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Assessment of the effect of sub-lethal acute toxicity of Emamectin benzoate in *Labeo rohita* using multiple biomarker approach

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ABSTRACT

Emamectin benzoate (EMB) is a potent neurotoxin agent, widely used for ectoparasites control in aquaculture, but their detailed toxicological implications in *Labeo rohita* are unknown. Thus, this study was conceptualized to determine the LC_{50} and to investigate the effects of two sub-lethal concentrations 1/50th of 96 h LC_{50} (1.82 μgL^{-1}) and 1/10thof 96 h LC_{50} (9.1 μgL^{-1}) on hemato-immunological and biochemical responses in *L. rohita* (mean weight 25.54 ± 2.3 g and length 10.35 ± 2.4 cm) for a period of 24 h, 48 h, and 72 h. LC_{50} of EMB were 163 μgL^{-1}, 112 μgL^{-1}, 99 μgL^{-1} and 91 μgL^{-1} at 24 h, 48 h, 72 h, and 96 h respectively. The safe limit at 96 h LC_{50} of EMB was 2.30 μgL^{-1}. In EMB treated fish, red blood cells, white blood cells, hemoglobin, and hematocrit counts were reduced ($p < 0.05$) significantly. Superoxide dismutase (SOD) activity in the liver and kidney declined ($p < 0.05$) at 72 h while in gill and muscle the activity increased significantly. Glutathione-s-transferase (GST) activity in the liver, gill, and kidney increased ($p < 0.05$) while muscle decreased significantly. Catalase (CAT) activity in liver, gill, and muscle decreased while in kidney increases. Glutamic-oxaloacetic acid transaminase (GOT) activity and Glutamate pyruvate transaminase (GPT) activity were increased in liver, kidney, and muscle tissue. The change in serum triglycerides, serum protein level was noticed. The level of cortisol, heat shock protein 70 (HSP70), and HSP90 increased ($p < 0.05$) while the immunological responses like immunoglobulin M (IgM) and complement 3(C3) activity decreased ($p < 0.05$) in EMB exposed fish. Thus, EMB exposure at two sub-lethal concentrations in *L. rohita* induces several hemato-immuno, and biochemical alterations in blood, serum, and different organs. The overall result of the present study indicated that EMB is toxic to fish even for a short-term exposure and low doses, and therefore utmost caution should be taken to prevent their drainage into water bodies.

1. Introduction

Pesticides accumulation and persistence in the aquatic environment is an alarming issue that may create new selective pressures, thus affecting the behavior, physiology, and ultimately fitness of aquatic life [1–5]. Fishes are quite sensitive to the presence of toxic chemicals that can be used as bio-indicators in various aquatic ecosystems. The intensive and indiscriminate use of pesticides in agriculture leads to environmental concern which ultimately leads to aquatic pollution. In the aquatic environment, pesticide/xenobiotic pollution is a severe ecological concern because of its bioaccumulation nature in non-target aquatic organisms especially fish or fish-eating organisms [6].

EMB is a potent neurotoxic agent which enters the aquatic environment directly from the waste feed, feces from aquaculture, and agricultural runoff and remains in sediments for a considerable period having a half-life time over 120 days [2]. EMB has two active homologs B_{1a}; ($C_{49}H_{75}NO_{13}C_7H_6O_2$, 90 %) and B_{1b} ($C_{48}H_{73}NO_{13}C_7H_6O_2$, 10 %) and it is a novel, broad-spectrum, semi-synthetic, and anti-parasitic drug that belongs to the avermectin family isolated from soil-dwelling actinomycetes (*Streptomyces avermitilis*). EMB acts as acaricide, bactericide, and insecticide used to control different pests in crops [7]. Additionally EMB was employed for parasites control in aquaculture like; *Argulus sp.* in koi carp and goldfish [8]; *Argulus coregoni* in rainbow trout (*Oncorhynchus mykiss*) [9]; sea lice (*Lepeophtheirus salmonis*) in Atlantic salmon

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[10–12]; *Salmincolacaliforniensis* in rainbow trout [13,14]; sea louse (Caligusminimus) in Asian sea bass (Lates calcarifer) [15]; *Anguillicoloides crassus* in American eels (*Anguilla rostrata*) [16]. Acute or chronic exposure to pesticides even at low concentrations alters the physiological and biochemical parameters in fish [17–20]. The toxic effect of EMB was noticed in many aquatic organisms like *Oreochromis niloticus* [21,22]; rainbow trout [23]; *L. rohita* [20] and benthic species [24]; *Clarias gariepinus* [1]; Asian seabass [15]; American lobsters [25]; *Danio rerio* [26]; zebrafish embryos [27]; Atlantic salmon [28]; planktonic marine copepods [29].

Fish act as an excellent bio-indicator and model organism for the toxicological and safety assessment of chemicals [30]. The Indian major carp *L. rohita* was selected for the present toxicological study of EMB. *L. rohita* has great consumer preference among all the six species of carp and contributed more than 60 % to the total carp production in India, 3% to major fish species produced globally, and15 % to global freshwater aquaculture production among the Asian countries [20,31]. It is easily available and quickly adapts to laboratory conditions.

Blood biomarkers (RBC, WBC, HGB); stress biomarkers (SOD, CAT, GST, cortisol, HSP70, HSP90), and immunological responses like IgM and C3 are the validated method to study the impact of acute and chronic toxicity of agrochemicals in fish [1,4,32,33]. Fish blood acts as a carrier medium for distributing metabolites, toxicants, and chemicals to different parts of the body [4,33]. Upon pesticide exposure, free radicals such as O_2^-, H_2O_2, OH- are produced inside the body which leads to tissue damage. The antioxidant defense mechanism of fish includes enzymatic and non-enzymatic components such as SOD and CAT which act on H_2O_2 and O_2^-respectively. At the same time, GST scavenges H_2O_2 and lipid hydroperoxides. However, GOT and GPT can be used to indicate the tissue damage under stress conditions [1,32] which ultimately impaired the immunoglobulin level, protein synthesis and,/or cellular apoptosis [4,32–34].

The detailed literature on the toxic implication of EMB on hemato-biochemical perturbations, oxidative stress, and immunological responses in *L. rohita* is scanty. Thus, the present investigation aims to determine the LC_{50} (24 h, 48 h, 72 h, 96 h) and to study the effect of sub-lethal acute exposure of EMB.

2. Material and methods

2.1. Test chemical

EMB (CAS Number 155569-91-8, purity >98 %, Sigma-Aldrich)stock solution (200 mg L^{-1}) was prepared in 50 mL Milli Q water by using 0.5 mL of Tween-20 surfactant, and different concentrations of 1.82, 9.1, 10, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 500 and 1000 μgL^{-1} were prepared from stock solution.

2.2. Experimental animals

The fingerlings of *L. rohita* (25.54 ± 2.3 g and 10.35 ± 2.4 cm) were obtained from ICAR-CIFRI fish farm, Balagarh, West Bengal, India and maintained in the wet laboratory with proper care. As a prophylactic measure, the fish were treated with 1 % salt solution for 5 min, followed by dip treatment with 50 mg L^{-1} potassium permanganate, then transferred carefully to the circular 500 L FRP (fiber reinforced plastic) tanks, and acclimatized for 15 days before starting the experiment with the provision of continuous aeration. Fish was fed twice daily with commercial Growfin™feed (crude protein 32 %, crude fat 5 %, crude fiber 3 %, and moisture content 11.5 %). The fecal matter and unused feed were siphoned off every day.

2.3. Experimental design

Organization for Economic Cooperation and Development (OECD) guidelines [35] were followed to design the experimental setup and permission was taken from Institutional Animal Ethics Committee, ICAR-Central Inland Fisheries Research Institute, Kolkata, India (IAEC/2021/04) for the toxicological study. To determine the $LC_{50,}$ 12 fishes were randomly distributed in each glass aquarium having 50 L water. Fish were exposed with different concentrations of EMB such as 50, 60, 70, 80, 90, 100, 125, 150, 175, 200 μgL^{-1} for a period of 96 h in triplicate. Fish were kept without EMB exposure treated as control. These concentrations were chosen for the range finding test by exposing the fish with low (10 μgL^{-1}) to high (1000 μgL^{-1}) concentrations of EMB. Fish mortality was recorded at 24 h, 48 h, 72 h, and 96 h. Probit analysis was used for the calculation of LC_{50}. The safe limit was calculated as per the method of Hart et al. [36].

Based on the obtained value of 96 h LC_{50}, two concentrations *i.e.* 1.82 μgL^{-1} (1/50th LC_{50} 96 h) and 9.1 μgL^{-1} (1/10th LC_{50} 96 h) were selected for sub-lethal acute toxicity test. No pesticides were added in control group. Twelve fish were randomly distributed in each glass tank containing 50 L water. The experiment was carried out in triplicate. Water quality parameters were measured daily and 10 % water was exchanged daily with the test solution. Fish samples were collected on 24 h, 48 h, and 72 h of exposure by scarifying two fish from each replicate. No mortality was observed during the sub-lethal test. Blood was collected from the caudal vein using a 2 mL disposable syringe. Fishes were anesthetized with clove oil (0.05 ml L^{-1} of water) before collection of blood. The collected blood was stored in EDTA vials for the complete blood cell count, and 1.5 mL eppendorf tube for serum collection. Blood was left for 30−60 min at 4 °C for coagulation and centrifuged at 5000 rpm for 10 min, supernatant was collected and stored at −20 °C for further analysis. The liver, gill, kidney, and muscle tissue were dissected out and washed in phosphate buffer saline (PBS, pH 7.4) solution. For biochemical assays, 0.2 g tissue was homogenized in 2 mL PBS solution (0.1 M, pH 7.4) using QIAGEN® TissueLyser II (QIAGEN, Hilden, Germany), followed by centrifugation at 15,000 rpm for 15 min at 4 °C. The supernatant was collected and stored at −20 °C for further analysis.

2.4. Hematological analysis

Hematological parameters like erythrocytes (RBC-red blood cells), leucocyte (WBC-white blood cells), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (HGB), hematocrit (HCT), platelets (PLT) were analyzed by using Sysmex XP-100 differential cell counter (Sysmex Corporation, Kobe, Japan) as per the manufacturer's instructions.

2.5. Antioxidant and metabolic enzymes

The activity of antioxidant enzymes like catalase, CAT [37,38]; superoxide dismutase, SOD [38,39]; glutathione-s-transferase, GST [40,41] in liver, gill, kidney, and muscle were analyzed using standard protocols. The glutamic-oxaloacetic acid transaminase, (GOT) activity and Glutamate pyruvate transaminase (GPT) activity were measured in the liver, kidney, and muscle as per standard procedure [42]. The change in absorbance (OD) was measured using ELISA-cum-Spectro reader (BioTekEpoch™2 microplate reader). Total protein concentration in liver, gill, kidney, and muscle was estimated at 750 nm using bovine serum albumin as a standard, according to Lowry et al. [43].

2.6. Serum biochemical parameters

Serum triglyceride, serum albumin, and serum protein level were determined using a Serum Biochemical Analyzer (Transasia-Erba® EM–200, USA). The serum globulin was calculated as total serum protein-serum albumin. The albumin globulin ratio was calculated as, = serum albumin/serum globulin.

2.7. Stress hormones and immunological responses

Cortisol, immunoglobulin–M (IgM), and complement3 (C3) were quantified in serum; heat shock protein 70 (HSP 70), heat shock protein 90 (HSP 90) were quantified in gill. These assays were performed by using a commercially available ELISA-based kit (BT BioAssay, Shanghai, China) as per the manufacturer's instruction and the final OD was taken at 450 nm using ELISA cum Spectro reader (BioTek Epoch M2 Take-3 plate reader).

2.8. Water quality parameters

Water quality parameters (pH, temperature, conductivity, total dissolved solids, and dissolved oxygen) were analyzed by a Multiparameter water quality probe (YSI ProDSS®) while the hardness was analyzed by titration method [44].

2.9. Statistical analysis

Probit analysis was used to calculate LC_{50}. The significant difference ($p < 0.05$) between the treated and control group was analyzed by one-way analysis of variance (ANOVA), followed by Tukey test using SPSS (IBM version 20) software. Results are expressed as mean ± standard deviation (SD).

3. Result

3.1. Lethal concentration (LC_{50}) and safe level

In the present study, LC_{50} values at 24 h, 48 h, 72 h and 96 h of EMB were 163 μgL^{-1}, 112 μgL^{-1}, 99 $\mu gL^{-1,}$ and 91 μgL^{-1} respectively. No mortality was observed in control group. The result showed a decreasing trend of lethal pesticide concentration with increasing time of exposure (Table 1). The estimated safe level of EMB was 2.30 μgL^{-1} (Table 1).

3.2. Symptomatological observation

EMB exposed fishes at lethal concentrations expressed numerous behavioral changes including slow and erratic movement, lethargy, surfacing, loss of balance, body flattened, exhibited respiratory distress with the opening of the mouth and expanded buccal cavity. The first symptom appeared after 20−30 min at a higher lethal dose (500–1000 μgL^{-1}), while 24−30 hours at a lower lethal dose (125–200 μgL^{-1}). Before death, fish showed erratic movement and slowly settled at the bottom with elevated opercular movement @ 75–80 times/minute. The EMB exposed fish showed darkening on the dorsal side of the body surface during the lethal toxicity test. Noticeable symptomatological behavior has not been observed during the sub-lethal toxicity test.

3.3. Hematological parameters

Hematological alteration in fish was studied in *L. rohita* after exposure to EMB, and presented in Table 2. WBC count varies significantly ($p < 0.05$) at higher concentrations of EMB after 48 h and at both concentrations after 72 h in comparison to control fish. WBC count declined up to 6 % and 17 %, in case of 48 h and 72 h EMB exposure respectively, with the lowest recorded in 9.1 μgL^{-1} concentration, at 72 h exposure ($165.45 \pm 1.41 \times 10^3$ μL^{-1}). HGB and HCT activity was gradually decreased with increment in the EMB exposure, and significantly ($p < 0.05$) decreased at 9.1 μgL^{-1} concentration at 24 h, and both the concentration (1.82 μgL^{-1} and 9.1 μgL^{-1}) onward after 48 h of EMB exposure. The decrease in HGB and HCT ranged up to 34 % and 50 % respectively, with the increase in concentration, and exposure duration. RBC levels were reduced significantly ($p < 0.05$) at 1.82 μgL^{-1} (42 %), and 9.1 μgL^{-1} (44 %) in compared to control. PLT counts were significantly ($p < 0.05$) elevated at 9.1 μgL^{-1} of EMB, at 72 h of exposure. The highest PLT count was recorded in 9.1 μgL^{-1} concentration, at 72 h exposure ($42 \pm 2.83 \times 10^3$ μL^{-1}). In the case of MCV and MCHC, the only significant change was observed in 9.1 μgL^{-1} concentration of EMB at 48 h exposure. The MCH were varied non-significantly between EMB exposed fish and control fish.

3.4. Antioxidant and metabolic enzymes

In the liver (Fig. 1A) and kidney (Fig. 1C) a significant ($p < 0.05$) reduction in SOD activity was observed in EMB exposure fish groups at 72 h in comparison to control fish. SOD activity in gill was increased significantly ($p < 0.05$) over the concentrations (Fig. 1B). SOD activity was significantly ($p < 0.05$) higher in fish muscle at higher concentration of EMB after 48 h (1.55 ± 0.08 U mg protein^{-1}) and both the concentrations of EMB at 72 h (1.44 ± 0.03 and 1.58 ± 0.02 U mg protein^{-1}) (Fig. 1D) in comparison to control. The CAT activity in the liver decreased significantly after 24 h at both concentrations (Fig. 2A), while in gill it decreases at the higher concentration at 24 h and both the concentration of EMB after 48 h and 72 h (Fig. 2B). In the muscle, CAT activity decreased significantly at higher dose after 72 h (Fig. 2D). In contrast, CAT activity in the kidney increased ($p < 0.05$) at 72 h of the higher dose of EMB (Fig. 2C) when compared to control.

The GST activity was gradually increased due to an increase in EMB concentration as well as the duration of its exposure in comparison to control fish. In the liver, a significant upsurge was observed at 48 h at high dose, and in both the concentration of EMB at 72 h of exposure (Fig. 3A). In gill tissue, a significant increase in GST activity was noticed at the higher concentrations at 24 h, and both the concentration after 48 h (Fig. 3B). Kidney tissue showed significant elevation in GST activity as compared to control with the highest value at higher concentration exposure of 72 h (0.153 ± 0.005 U mg protein^{-1}) (Fig. 3C), on the

Table 1
Cumulative mortality of *L. rohita* exposed to various concentrations of EMB.

Concentration (μgL^{-1})	No. of fish taken	Cumulative mortality of fish at different durations (hours)				Survival (%)	Mortality (%)
		24 h	48 h	72 h	96 h		
Control	12	0	0	0	0	100	0
50	12	0	0	0	0	100	0
60	12	0	0	0	0	100	0
70	12	0	0	0	0	100	0
80	12	0	0	1	2	83.89	16.11
90	12	0	2	6	8	33.34	66.66
100	12	0	2	4	7	41.67	58.33
125	12	4	8	11	12	0	100
150	12	4	12	12	12	0	100
175	12	10	12	12	12	0	100
200	12	12	12	12	12	0	100
LC_{50} (μgL^{-1})		163	112	99	91		
Safe limit 96 h LC_{50}		2.30 μgL^{-1}				*Hart et al. (1948)	

* C = 48 h LC_{50} × 0.03/S, where C = Presumable harmless concentration and S = 24 h LC_{50}/48 h LC_{50}.

Table 2
Change in hematological parameters of *L. rohita* exposed to two sub-lethal concentrations of EMB.

Parameters		24 h	48 h	72 h
WBC ($\times 10^3$ μL^{-1})	Control	198.8 ± 1.84^a	199.05 ± 2.62^a	200.54 ± 0.95^a
	1.82 μgL^{-1}	205 ± 0.64^a	193.1 ± 0.57^{ab}	167.5 ± 1.91^b
	9.1 μgL^{-1}	210 ± 7.07^a	187 ± 1.41^b	165.45 ± 1.41^b
RBC ($\times 10^6$ μL^{-1})	Control	2.40 ± 0.02^a	2.35 ± 0.02^a	2.40 ± 0.014^a
	1.82 μgL^{-1}	1.92 ± 0.03^b	1.84 ± 0.03^b	1.39 ± 0.16^b
	9.1 μgL^{-1}	1.72 ± 0.02^c	1.35 ± 0.04^c	1.33 ± 0.01^b
HGB (gdL^{-1})	Control	9.55 ± 0.07^a	9.35 ± 0.64^a	9.05 ± 0.78^a
	1.82 μgL^{-1}	8.7 ± 0.71^a	6.7 ± 0.71^b	6.195 ± 0.29^b
	9.1 μgL^{-1}	6.8 ± 0.14^b	6.35 ± 0.21^b	5.89 ± 0.31^b
HCT (%)	Control	33.47 ± 1.32^a	31.69 ± 1.63^a	32.325 ± 2.06^a
	1.82 μgL^{-1}	28.52 ± 2.90^a	26.875 ± 0.13^b	22.825 ± 4.31^{ab}
	9.1 μgL^{-1}	17.825 ± 1.31^b	15.725 ± 1.10^c	16.125 ± 1.80^b
MCV (fL)	Control	139.73 ± 4.25^a	132.35 ± 7.96^a	135.79 ± 7.84^a
	1.82 μgL^{-1}	148.60 ± 17.29^a	146.08 ± 2.98^a	166.98 ± 49.67^a
	9.1 μgL^{-1}	103.89 ± 6.34^a	116.84 ± 5.08^b	121.32 ± 14.85^a
MCH (pg)	Control	39.87 ± 0.05^a	39.05 ± 3.00^a	38.01 ± 3.00^a
	1.82 μgL^{-1}	45.29 ± 3.01^a	36.44 ± 4.40^a	44.96 ± 7.11^a
	9.1 μgL^{-1}	39.64 ± 0.33^a	47.24 ± 2.81^a	44.27 ± 1.86^a
MCHC (gdL^{-1})	Control	28.55 ± 0.91^a	29.49 ± 0.49^b	27.98 ± 0.63^a
	1.82 μgL^{-1}	30.79 ± 5.61^a	24.92 ± 2.51^b	27.51 ± 3.92^a
	9.1 μgL^{-1}	38.22 ± 2.01^a	40.53 ± 4.17^a	36.87 ± 6.05^a
PLT ($\times 10^3$ μL^{-1})	Control	23.25 ± 1.06^a	24.5 ± 2.12^a	24.055 ± 1.34^b
	1.82 μgL^{-1}	24 ± 1.41^a	25.5 ± 2.12^a	36 ± 1.41^a
	9.1 μgL^{-1}	25.5 ± 0.71^a	29.5 ± 0.71^a	42 ± 2.83^a

Values are in mean±SD, different superscript letters showing significantly difference ($p<0.05$) among the treatments.

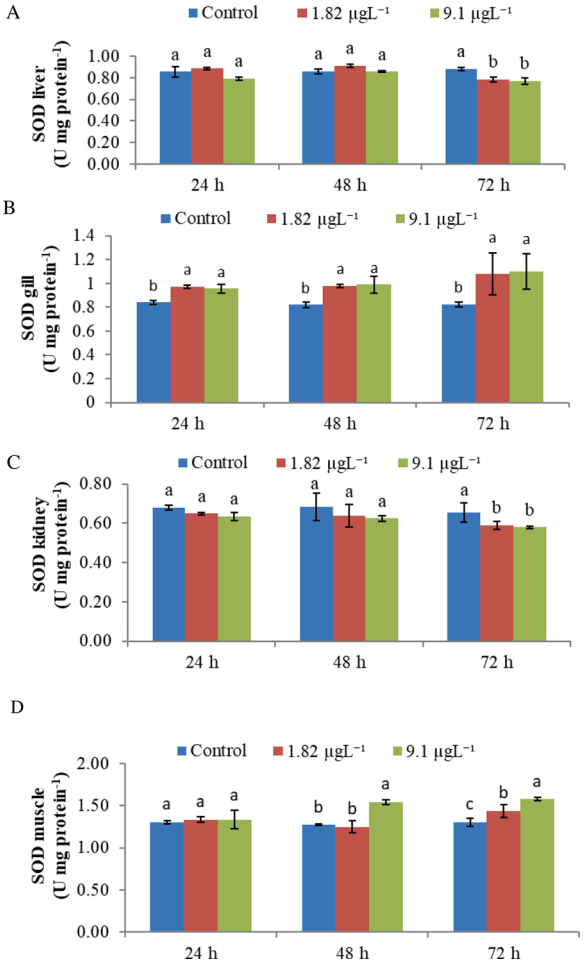

Fig. 1. Change in SOD activity in liver (1A), gill (1B), kidney (1C), and muscle (1D) of *L. rohita* exposed to two sub-lethal concentrations of EMB.
Data showing different letters are significantly different ($p < 0.05$).

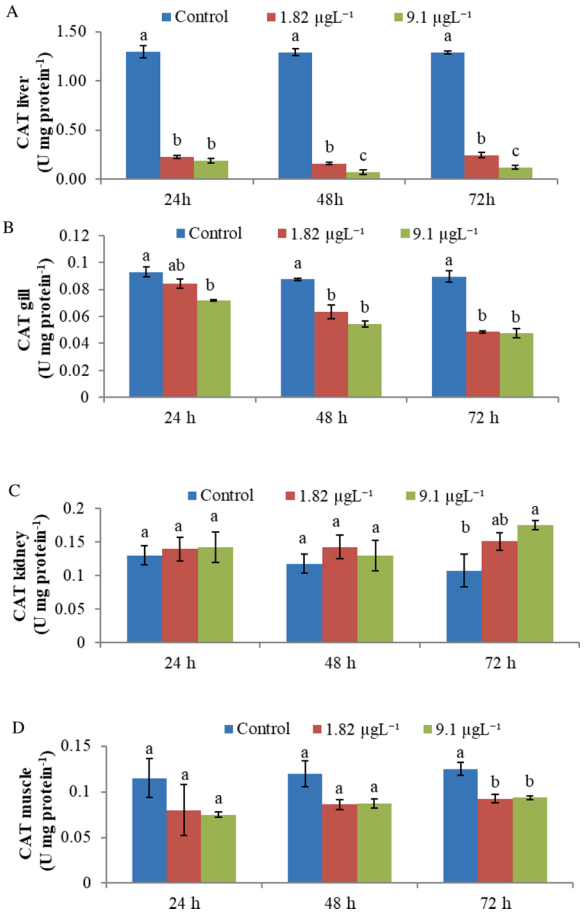

Fig. 2. Change in catalase (CAT) activity in liver (2A), gill (2B), kidney (2C), and muscle (2D) of *L. rohita* exposed to two sub-lethal concentrations of EMB.
Data showing different letters are significantly different ($p < 0.05$).

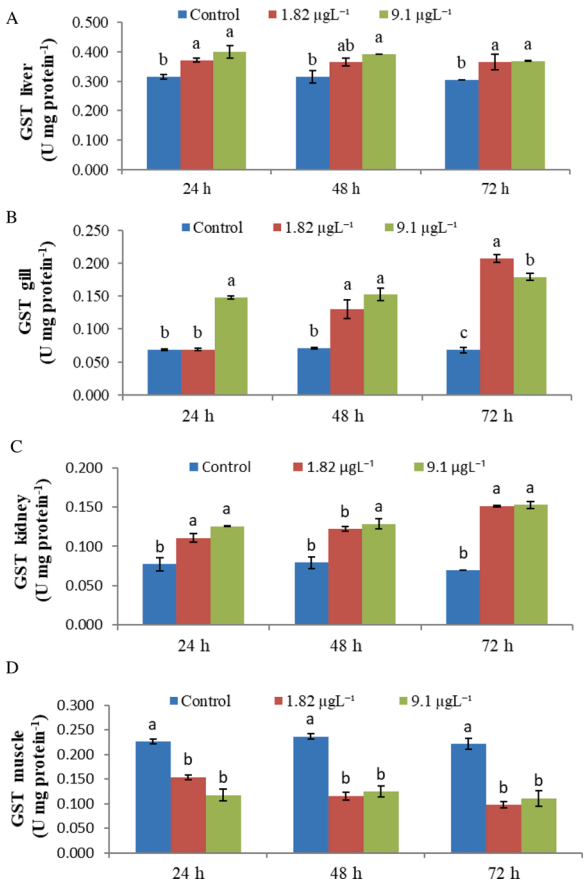

Fig. 3. Change in glutathione-s-transferase (GST) activity in liver (3A), gill (3B), kidney (3C), and muscle (3D) of *L. rohita* exposed to two sub-lethal concentrations of EMB.
Data showing different letters are significantly different ($p < 0.05$).

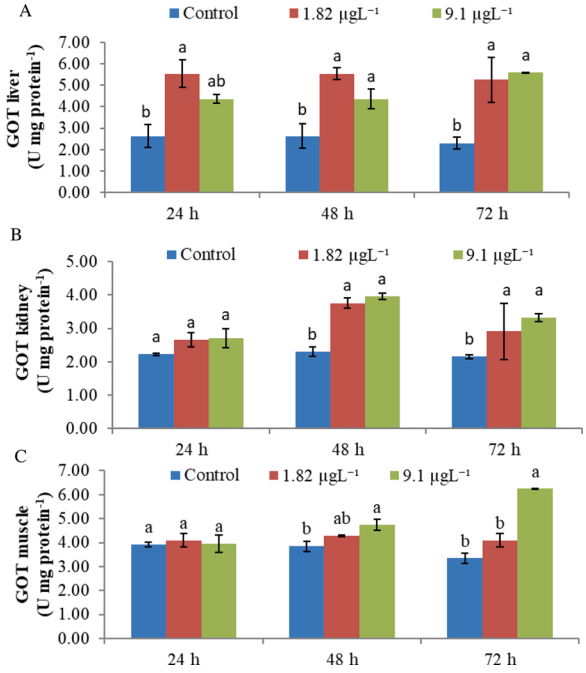

Fig. 4. Change in glutamic-oxaloacetic acid transaminase (GOT) activity in liver (4A), kidney (4B), and muscle (4C) of *L. rohita* exposed to two sub-lethal concentrations of EMB.
Data showing different letters are significantly different ($p < 0.05$).

contrary, GST activity in muscle tissue was significantly ($p < 0.05$) reduced up to 55 % as compared to control (Fig. 3D).

Figs. 4 and 5 show the effects of EMB on the GOT and GPT activity in the liver, kidney, and muscle tissue of *L. rohita*. The GOT activity in the liver was significantly ($p < 0.05$) elevated in both the concentration of EMB (Fig. 4A) in comparison to control fish. In the kidney tissues, significant enhancement of GOT was noticed after 48 h at both the concentration of EMB (Fig. 4B) however, in muscle, GOT activity was increased after 48 h at higher concentration only. The GPT activity in the liver was significantly ($p < 0.05$) elevated at both the concentration of EMB at 48 h and 72 h at higher concentrations (Fig. 5A). In kidney and muscle tissues, significant enhancement of GPT was noticed only at 72 h of EMB exposure in comparison to control the group.

3.5. Serum biochemical parameters (triglycerides, albumin, globulin, and protein)

Serum biochemical parameters like triglycerides, protein, albumin, and globulin, exposed to EMB at both the sub-lethal concentration are depicted in Table 3. EMB exposed fish at both concentrations showed significantly ($p < 0.05$) higher triglycerides levels. At higher EMB concentration, serum protein activity was increased significantly ($p < 0.05$) at 72 h (3.67 ± 0.10 gdL^{-1}). Albumin concentration was significantly higher at higher doses of EMB during the exposure period. Though the globulin, and albumin, and globulin ratio showed an increasing trend, however, values were found non-significant.

3.6. Stress hormones

The level of stress hormones and steroids like cortisol, HSP70, and HSP90 in EMB exposed fish are depicted in Fig. 6. Serum cortisol level

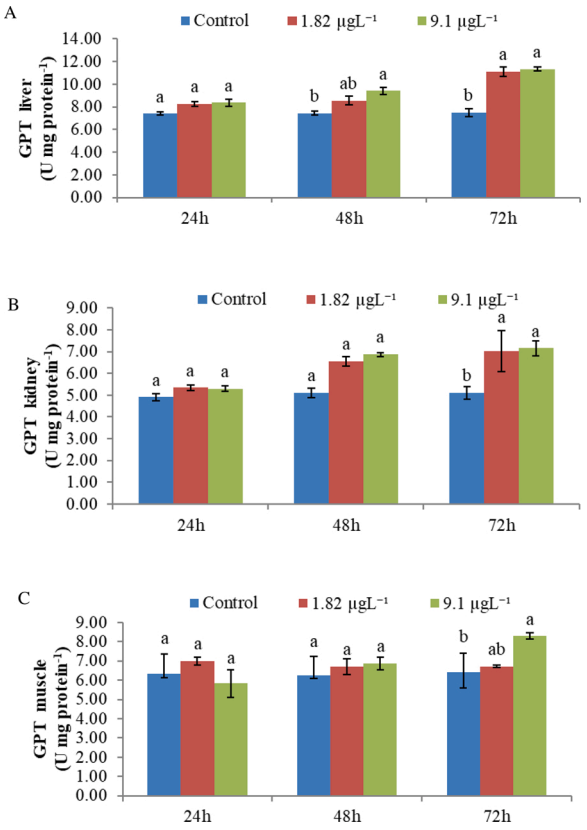

Fig. 5. Change in Glutamate pyruvate transaminase (GPT) activity in liver (5A), kidney (5B), and muscle (5C) of *L. rohita* exposed to two sub-lethal concentrations of EMB.
Data showing different letters are significantly different ($p < 0.05$).

Table 3
Change in level of serum triglyceride, albumin, globulin, and protein of *L. rohita* exposed to two sub-lethal concentrations of EMB.

Parameters	Treatments	24 h	48 h	72 h
Triglyceride (mgdL^{-1})	Control	25.95 ± 0.78^{a}	26.75 ± 1.06^{a}	25 ± 0.71^{a}
	1.82 μgL^{-1}	47.22 ± 1.44^{b}	51.31 ± 1.56^{b}	59.65 ± 1.91^{b}
	9.1 μgL^{-1}	60.97 ± 0.81^{c}	50.40 ± 3.97^{b}	60.57 ± 0.95^{b}
Albumin (gdL^{-1})	Control	0.57 ± 0.02^{a}	0.59 ± 0.01^{a}	0.58 ± 0.01^{a}
	1.82 μgL^{-1}	0.60 ± 0.02^{a}	0.69 ± 0.01^{a}	0.62 ± 0.01^{a}
	9.1 μgL^{-1}	0.69 ± 0.01^{b}	0.66 ± 0.01^{b}	0.94 ± 0.06^{b}
Globulin (gdL^{-1})	Control	2.13 ± 0.11^{a}	2.16 ± 0.06^{a}	2.18 ± 0.03^{a}
	1.82 μgL^{-1}	2.26 ± 0.23^{a}	2.38 ± 0.06^{a}	2.21 ± 0.28^{a}
	9.1 μgL^{-1}	2.27 ± 0.21^{a}	2.30 ± 0.48^{a}	2.73 ± 0.04^{a}
Albumin: Globulin	Control	0.266 ± 0.003^{a}	0.27 ± 0.11^{a}	0.26 ± 0.001^{a}
	1.82 μgL^{-1}	0.265 ± 0.03^{a}	0.29 ± 0.01^{a}	0.28 ± 0.04^{a}
	9.1 μgL^{-1}	0.3 ± 0.03^{a}	0.29 ± 0.06^{a}	0.34 ± 0.01^{a}
Protein (gdL^{-1})	Control	2.69 ± 0.13^{a}	2.74 ± 0.06^{a}	2.76 ± 0.04^{a}
	1.82 μgL^{-1}	2.85 ± 0.21^{a}	3.07 ± 0.05^{a}	2.83 ± 0.27^{a}
	9.1 μgL^{-1}	2.96 ± 0.20^{a}	2.97 ± 0.47^{a}	3.67 ± 0.10^{b}

Values are in mean±SD, different superscript letters showing significantly difference ($p<0.05$) among the treatments.

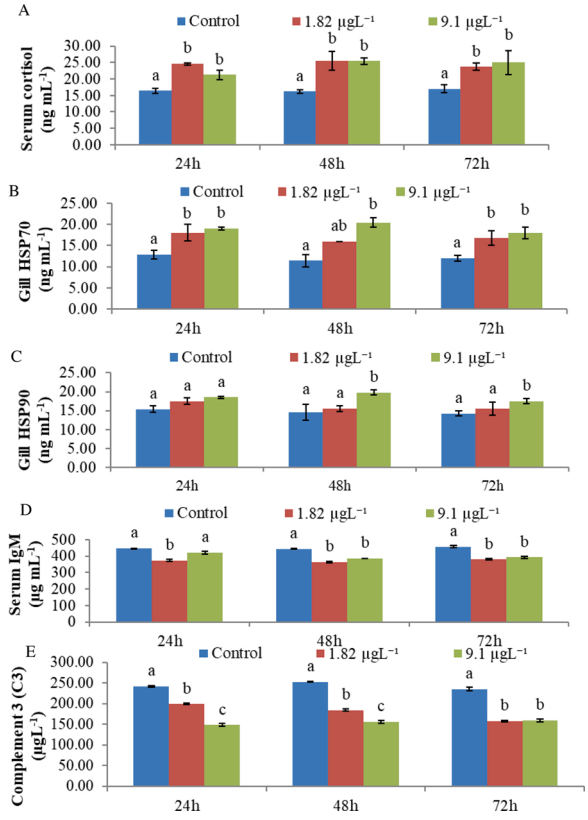

Fig. 6. Change in serum cortisol (6A), gill HSP70 (6B), gill HSP90 (6C), serum IgM (6D) and serum C3 (6E) activity in *L. rohita* exposed to two sub-lethal concentrations of EMB.
Data showing different letters are significantly different ($p < 0.05$).

increased significantly ($p < 0.05$) in EMB exposed fish (Fig. 6A) in comparison to control fish. Gill tissue showed significantly higher HSP70 levels in the EMB exposed fish group (Fig. 6B) however, HSP90 content was found higher ($p < 0.05$) only after 48 h at higher concentration of EMB (Fig. 6C).

3.7. Immunological responses

The concentration of serum IgM declined after 48 h when fish were exposed with both the concentration of EMB (Fig. 6D). The serum complement (C3) levels were found to be decreased ($p < 0.05$) in EMB treated fish groups as compared to control in 24 h, 48 h, and 72 h (Fig. 6E).

3.8. Water quality parameters

In the present study the range of water quality parameters were as follows; temperature 28–30 °C, pH 7.71–8.28, dissolved oxygen 5.54–7.21 mg L^{-1}, conductivity 438–555 μScm^{-1}, TDS 257–328 mgL^{-1}, hardness 142–152 mgL^{-1}. No significant changes in water quality parameters were noticed in EMB treated groups and control fish.

4. Discussion

The present study was to evaluate the short-term effects of EMB pesticide on hemato-immuno, serum biochemical, and stress hormone alterations in *L. rohita*. The calculated LC_{50} (96 h) of EMB was 91 μgL^{-1} which was found to be highly toxic. The present LC_{50} was comparatively lower than the value reported in *Oncorhynchus mykiss* (rainbow trout) 176 μgL^{-1}, *Cyprinus carpio* 180 μgL^{-1}, *Pimephales promelas* (Fathead minnow) 384 μgL^{-1}, *Lepomis macrochirus* (Bluegill Sunfish) 184 μgL^{-1} [2]. The toxic effects of pesticides to the fish and other aquatic organism is influenced by several factors such as concentration, duration of exposure, bio-accumulation factor, species, age, size, sex of the organism, water quality parameters, type of formulation of pesticide, and its absorption, accumulation, metabolism, excretion, and degradation [4, 45].

Pesticides exposed to fish at lethal concentrations of EMB expressed abnormal behaviour like lethargy, rapid opercular movement, loss of balance, excess mucus secretion, and darkening on the body surface. However, no visible symptom was observed in the fish exposed to EMB during the sub-lethal toxicity test for a period of 72 h. In an earlier study, erratic and irregular swimming movements, loss of balance, sudden overexcitability were observed at 0.093 mg L^{-1} of malathion in rainbow trout [46]; rapid swimming, higher opercular movements were noticed in cypermethrin treated *L. Rohita* [47]; erratic behaviour with sluggish swimming patterns, loss of balance and elevation of opercular respiration in *C. carpio* exposed to glyphosate [48]. EMB and abamectin induced hypoactivity in *Danio rerio*, Zebrafish [26,27].

Hematological alteration due to xenobiotic exposure is a valuable indicator of physiological status in fish [48–50]. *L. rohita* exposed to sub-lethal concentration (1.82 μgL^{-1} and 9.1 μgL^{-1}) of EMB caused a significant decline in the levels of RBC, HGB, HCT, and MCV levels in comparison to control fish. The red blood cell destruction and inhibition of erythropoiesis are the probable reasons for the reduction of RBC. It has been proved that disorders in the hemopoietic process accelerated the disintegration and shrinkage of RBC and erythrocytes lysis which can further lead to the reduction of RBC, HGB, and HCT content [48]. The present study is in agreement with the results of Gholami-seyedkolai et al. [48], where HGB, RBC, HCT, and WBC are reduced in *C. carpio* while exposed to glyphosate (Roundup®). In a similar line, *O. niloticus* exposed to chlorpyrifos caused a significant reduction in the value of RBC, HGB, MCH, MCV [18,49]. WBCs play a key role in regulating the immunological function in organisms however, organisms exposed to pesticides inhibit their maturation process [51]. The decrease in WBC count ($p < 0.05$) was noticed in EMB exposed *L. rohita* at higher concentrations suggesting that the immune system probably has been compromised [50]. A similar result was also reported in common carp exposed to glyphosate (Roundup®) [48], and in *L. rohita* exposed to fenvalerate [50]. In contrast, no significant changes in WBC and RBC activity in *O. niloticus* after 96 h exposure of EMB [22].

SOD and CAT are antioxidant enzymes that play an important role in maintaining reactive oxygen species (ROS) balance as they scavenge the excess superoxide generated in the body and may act as the first line of defense mechanism against ROS [52]. SOD converts superoxide radicals (O_2^-) into H_2O_2 which is further converted into H_2O and O_2 by catalase

enzymes. If SOD activity is not regulated, this may lead to cell dysfunction [1]. In the present study, a significant decline in SOD activity was observed in the liver and kidney at 72 h of EMB exposure as compared to the control. This decreased activity of SOD might be due to the utilization of enzymes in the liberation of O_2 to H_2O from superoxide thereby compromising cell membrane integrity [1,52]. In agreement with our finding, Firat and Tutus [22], reported a significant decrease in SOD activity in the liver of *O. niloticus* after 48 h and 96 h exposure to EMB; in the liver and kidney due to imidacloprid exposure in female rats [52,53], in the liver of *L. rohita* exposed with profenofos [54]. In contrast, in the present study, the gill, and muscle showed an elevation in SOD activity which may be because, gill and muscle play a major role in the xenobiotics metabolism and gill functions of defense mechanism, which counteracts the oxidative stress induced by EMB pesticides thus maintain cellular membrane integrity [1]. It was also observed that fenvalerate exposed zebrafish [55] and *L. rohita* [50] showed a decline in SOD activity in gill tissue as compared to the control. Concentration and time duration-dependent increase in SOD activity due to sub-lethal exposure to ivermectin has been observed in *Clarias gariepinus* [1]. In present findings, CAT activity in the liver, gill, and muscle decreased, however, in the kidney its activity increased in compared to the control. This reduction of CAT activity was probably due to excess production of ROS or peroxidative damage to the liver tissue. The findings of Firat and Tutus [22], corroborate to present findings as CAT activity decreased after 96 h of EMB exposure in liver tissue in case of *O. niloticus*, in the liver of *L. rohita* exposed with profenofos [54], and in liver and gill tissue of *Danio rerio* [55]. However, Prusty et al. [50] noticed that gill CAT activity remains unaffected due to fenvalerate exposure to *L. rohita* though the liver SOD activity declined.

GST is an important detoxifying enzyme, which converts xenobiotics/pesticides to non-toxic metabolites by conjugation with GSH as part of phase II of xenobiotic biotransformation [54,56,57]. In the present finding, increased GST activity in the gill, liver, and kidney was observed elevated after exposure to EMB. This indicated that GST was induced either by GSH conjugation or by the detoxification of hydroperoxides [54]. Our result was in parallel with the finding of Nataraj et al. [54], in the liver of *L. rohita* exposed to profenofos; in the liver of *Catla catla* exposed to cypermethrin [58], in the hepatopancreas of *Astacuslepto dactylus* exposed to azoxystrobin [59] and in the gill, liver, kidney, brain, heart, and muscle tissue of *L. rohita* exposed to endosulfan and chlorpyrifos [60].

Pesticide exposure produces oxidative stress which leads to tissue dysfunction and enhances the production of GOT and GPT. These enzymes play an important role in carbohydrate and protein metabolism, thus acting as an indicator for cell rupture and tissue damage/dysfunctions under pesticide exposure [1,49]. In the present study, significantly increased activity of GOT and GPT was found in the liver, kidney, and muscle of *L. rohita* upon EMB exposure in comparison to the control fish. The probable reason for the increased level of GOT and GPT was the damage of hepatic cells or liver cirrhosis due to pesticide exposure [1]. The present result is in line with Firat and Tutus [22], indicating a significant increase in GOT and GPT activity in serum after 96 h exposure of EMB in *O. niloticus*. GOT, and GPT activity was significantly elevated in *Cirrhinus mrigala* on exposure to chlorfenapyr, acetamiprid, and dimethoate on 10th, 20^{th,} and 30th days [19]. Emamectin benzoate exposure in rainbow trout also increases the activity of GOT, and GPT [23].

EMB exposed fish showed higher triglycerides activity as compared to the control; this might be due to free radical-induced oxidative stress which leads to hepatopathy. Similar results were obtained in *L. rohita*, exposed to fenvalerate [50]. The increased levels of serum albumin, globulin, and protein in EMB treated fish were associated with innate response in fish. Similar to our result, enhanced levels of serum protein and albumin were noticed in rainbow trout exposed to EMB for 21 days [23]. Prusty et al. [50] found the decreased activity of albumin, globulin, and protein in serum of *L. rohita*, exposed to fenvalerate, and *C. gariepinus* to sub-lethal exposure of ivermectin [1].

The major stress hormone, cortisol plays an important role to maintain blood glucose levels by increasing gluconeogenesis [19,22]. During stress, its concentration rises rapidly in the blood. Our current finding demonstrated a significant upsurge of serum cortisol levels in EMB treated fish which might be able to cope with the increased demand for energy during EMB induced stress. Our result is in agreement with Firat and Tutus [22], indicating a significant elevation in serum cortisol activity in *O. niloticus* after 96 h exposure with EMB. Decreased level of serum cortisol was observed in *Pseudetroplus maculatus* exposed to chlorpyrifos at sub-lethal exposure for a period of 15 and 30 days [61]; in Nile tilapia exposed with bifenthrin [18]; in C. mrigala exposed to dimethoate, chlorfenapyr, and acetamiprid [19]. The decrease in cortisol level might be due to variation in species, pesticides, and duration of exposure.

IgM is a primary immunoglobulin as well as a major antibody and plays an important role in mediating immune response in fish. A marked reduction in serum IgM levels found in *L. rohita* exposed to EMB indicates the inhibition of innate immune responses. Reduction of IgM activity respondent to tissue damage, cellular apoptosis, and suppressed B lymphocyte function. Reduction of IgM level in this study agreed with the effect of bifenthrin on Nile tilapia [18]; Ronstar® on *Clarias gariepinus* [33]; deltamethrin on *Gobiocypris rarus,* Chinese rare minnow [62].

Complement 3 (C3) is the key component in the innate immune system, plays a crucial role to protect the tissue against xenobiotic exposure or stress [32]. In the current study, C3 activity in serum of *L. rohita* exposed to EMB was significantly ($p < 0.05$) decreased as compared to control, indicating that EMB may disturb the innate immune system of *L. rohita* by changing C3 content. The present findings corroborate with the earlier studies in *Gobiocypris rarus* [62] and *Channa argus* [32] exposed to deltamethrin.

Heat shock proteins (HSPs), induced by pesticide exposure can be used as a biomarker [63]. Induced HSP activity works as molecular chaperons, thus protecting the cell during stress by repairing lipids and proteins. In the present study, HSP70 /HSP90 showed a significant elevation ($p < 0.05$) as compared to control fish, this was probably due to protective effects of the gill tissue against the structural defects of protein under EMB induced stress. The present findings are in agreement with earlier results of *O. niloticus* exposed to fipronil [64]; in rainbow trout exposed with deltamethrin [65]. It was noticed that the HSP70 level in the brain and liver did not change significantly after 48 h but its activity increased after 14 days due to thiamethoxam exposure in *O. niloticus* [63].

5. Conclusion

This study revealed that sub-lethal acute exposure of emamectin benzoate (1/50th of 96 h LC_{50}1.82μgL^{-1} and 1/10th of 96 h LC_{50}9.1 μgL^{-1}) to *L. rohita* causes significant alteration in hematological parameters, oxidative enzyme, stress hormone, biochemical and immunological (IgM, C3) responses. However, only concentration-dependent significant alteration was observed. Reduction in RBC, HGB, and HCT level was due to the acceleration in process of erythrocytes lysis and shrinkage of RBC induced by EMB on the erythropoietic tissue. The sign of cellular damage seen in the liver, gill, and kidney might compromise the detoxification of the chemicals. Elevated SOD, GOT, GPT, GST, HSP70, HSP90, and serum cortisol activity might be due to the functioning of the defense mechanism, which counteracts the oxidative stress induced by EMB pesticides. Reduction in IgM and C3levels indicating inhibition of innate immune response. The multiple biomarkers studied during the experiment may be applied as sensitive tests to evaluate the health status and physiological responses of *L. rohita* exposed to pesticide EMB.

Data availability

Raw data and other supplementary material are available at the following repository: osf.io/7zpcj.

No data was used for the research described in the article.

Data will be made available on request.

Conflict of Interest

The authors declare no conflict of interest.

Authors contribution

Vikas Kumar: Conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing-original draft, review and editing, visualization, supervision, Software; **Himanshu Sekhar Swain**: Conceptualization, methodology, investigation, resources, data curation, writing-original draft, review, and editing; **Basanta Kumar Das**: Conceptualization, methodology, investigation, resources, data curation, editing, visualization, supervision, funding acquisition, project administration; **Sankhajit Roy:** Conceptualization, methodology, investigation, resources, data curation, editing, visualization, supervision, project administration; **Aurobinda Upadhyay:** Formal analysis, investigation, data curation, review and editing, Software; **Mitesh H. Ramteke:** Formal analysis, review, and editing; **Ramen Kumar Kole**: Methodology, validation, data curation, review, and editing; **Hemanta Banerjee**: Methodology, validation, data curation, review, and editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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