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Changes in Anti-Oxidative Enzyme and Hematological Parameters of Rohu (*Labeo Rohita*) Fingerlings Exposed To Ivermectin

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ABSTRACT

Argulosis is the sever problem in carp culture and its severity turns to be panic in case of unregulated treatment with ivermectin, when the post treatment mortality of the fish or subsequent slow growth becomes common. Therefore, present study was aimed to study the effect of acute ivermectin exposure (96 hr) of different concentration on antioxidative enzymes (catalase, superoxide dismutase, glutathione S-transferase), acetyl choline enzyme activity and hematological parameters like platelets, white blood cell (WBC), red blood cell (RBC), haemoglobin content, haematocrit (HCT), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and Nitro-blue tetrazolium (NBT) of a tropical freshwater fish, rohu (*Labeo rohita*) fingerlings (5.5 g). The estimated LC₅₀ value of 7.89 µg/L was found at 96 h of exposure by probit analysis. Activities of anti-oxidative enzymes, immune hematological profile and neurotransmitter activity were significantly altered ($P < 0.05$) in a dose dependent manner. The early gross sign like sluggishness and dark body were, followed by disorientation and imbalance in swimming, finally mortality during acute toxicity.

Keyword: Ivermectin, Argulus, Rohu, Hematological parameters, Anti-oxidative enzymes

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INTRODUCTION

Carp dominate in aquaculture among the 541 number of species recorded in FAO aquaculture production statistics. Argulosis is a severe problem in tropical carp culture and causes huge loss to farmers. *Argulus* attachment scare becomes an open wound and site of secondary infection, leading to Argulosis outbreaks and high mortalities. *Argulus* infestation is the major problem encountered by the carp farmers. It has compelled the farmers to use various pesticides and anti-parasitic drugs. Among the various chemicals and drugs, Ivermectin is the most frequently used by the farmers.

Ivermectin belongs to a family of avermectins, one of the most recently developed antiparasitic agents isolated from the actinomycete, *Streptomyces avermitilis*. Ivermectin (22,23-dihydroavermectin B1a/22,23-dihydroavermectin B1b) is a broad-spectrum anti-parasitic drug, which has been proposed as an alternative treatment to Organophosphorous, Nuvan and Endosulfan pesticides that have been used for the control of *Argulus* (fish lice) earlier in carp aquaculture. Ivermectin may be administered either orally, intra-subcutaneously or in feed formulation as medicated feed. But most of the farmers dump it in water bodies with occasional use through feed.

Ivermectin is a derivative of avermectin B1 and the known anti- parasitic effects caused by paralysis of the pharyngeal muscles and paralysis of somatic muscles¹. The paralyzing effects on the pharyngeal muscles are associated with the interaction of avermectins with glutamate-gated chloride (GluCl) channel receptors. The physiological role of GluCl in the pharynx is to mediate the action of glutamate released from pharyngeal motor neurons. Exogenous glutamate inhibits pharyngeal pumping, which is mimicked by Ivermectin². Paralysis of somatic muscles, on the other hand, is associated with gamma-aminobutyric acid (GABA)-gated chloride channel receptors¹. As the toxicities in fish are concerned the *Argulus* infested fish get more prone to it due to the poor health status and open wound leading osmotic imbalance.

It is well established that pesticide and drugs alter hematological and enzyme profile, however, the infestation may also be associated with haematological and enzymological alteration as well as inflammatory changes³.The exposure of fish to toxic compounds results in the production of reactive oxygen species (ROS)^{4, 5} and ROS may overwhelm cellular defenses and damage proteins, mostly the membranes proteins, lipids, and DNA⁶. The oxidative damage concurrent to haematological and enzymological alteration may be the root cause of slow growth and mortality on farm during *Argulosis* treatment by ivermectin^{7,8}.

ROS generated in tissues are effectively scavenged by antioxidant defense systems. The aquatic organisms like fish comprise specific antioxidant enzymes, such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GSH-Px; EC 1.11.1.9) and glutathione reductase (GR; EC 1.8.1.7). There are no such information's available pertaining to antioxidative status, neurotransmission and haemato-immunomodulatory status of *Labeo rohita* exposed to Ivermectin. Level of drugs used for treatment of Argulosis in the carp culture system is high and varies from practice to practice, one of the recent comparative study has been done for efficacy of two avermectins, Doramectin and Ivermectin against *Argulus siamensis* infestation in Indian major carp by Hemaprashant et al.⁹. He suggested, the toxicity and pharmacokinetics study of Doramectin and Ivermectin in carps beside environmental, before recommending the safe use of these drugs to control of parasitic infections. The toxic effect of ivermectin at higher dose appears as poor feed intake, black coloration, ataxia and even death of the fish. Hence, use of this drug should be done based on systematic assessment of toxicity, lethal concentration and physiological changes at sub lethal doses. Until there was no systematic study basically focused on determining the acute toxicity and the potential mortality that occurs when using a higher dose of Ivermectin to *Labeo rohita* in water immersion. Thus, the aim of this study was to determine the effects of Ivermectin on selected haemato immunological parameters, acetyl choline enzyme activity and oxidative stress in *Labeo rohita*.

MATERIAL AND METHOD

Experimental animals

In the present experiment, two hundred and forty fishes were randomly distributed in 8 distinct experimental groups with 10 fishes in each group. Fishes of uniform size with initial weight ranging from 5.35 g to 5.75 g were stocked in each plastic tub of 100 L capacity. The experiment for study of LC₅₀, was designed after the range finding test. *Labeo rohita* fingerlings used for the experiment were obtained from the Array Farm Goregaon, Mumbai, India. The fingerlings were maintained in the rectangular, plastic 100 L experimental tubs and acclimatized for 15 days prior to the experiment.

Experimental conditions

Round the clock aeration was provided to all the experimental tubs and water temperature was recorded to be in the range of 26.4–28.8 °C. The animals were fed ad libitum with experimental diet (30% crude protein) at 3% of their body weight till 48 hr. before the start of the experiment. Water quality parameters viz. dissolved oxygen and temperature (dissolved oxygen and

temperature meter, Merck, Germany), pH (digital pH meter, LABINDIA, Mumbai), free carbon dioxide (titrimetric method, ¹⁰; total hardness (carbonate hardness test kit, Merck, Germany), ammonia (at 635 nm by phenate method ¹⁰), nitrite and nitrate (543 nm wave length ¹⁰) were recorded during the experimental period. Technical grade (97 %) ivermectin (Malti Enterprises India) was applied in water at different concentration after dissolving in ethanol to dissolve in water.

Determination of Lethal Concentration 50 (LC₅₀)

A static non-renewable acute toxicity bioassay was conducted according to standard ¹⁰ for determining LC₅₀ of Ivermectin exposure to *Labeo rohita* fingerlings (average weight of 5.5 g). Initially, a range finding test was conducted to ascertain the range to be followed in the definitive test. In this trial, the animals were exposed to a range of concentration in logarithmic scale such 20, 40, 60, 80 and 100 µg/L. Static nonrenewable bioassay was conducted in triplicates for each concentration of Ivermectin. No water exchange was done and the fishes were not fed during the period of experiment. Dead fishes were removed from each tank immediately and mortality was recorded at, 24, 48, 72 and 96 hrs. The maximum ethanol volume which was used for dissolving the ivermectin was added to the control group. The range of LC₅₀ for *Labeo rohita* fingerling under above mentioned conditions was ascertained to lie below 20 µg/L, since all the fishes died within the 18 hrs. Hence, for the definitive test, Ivermectin at 2.5, 5, 7.5, 10, 12.5, 15 and 17.5 µg/l in triplicates were used for 10 fishes kept in each tub. The data obtained from the experiment was processed by probit analysis using a computer programme, Basic LC₅₀ version 1.1 ¹¹.

Preparation of tissue homogenate

The organs of the fishes from the different groups were dissected carefully, weighed and kept on ice. The iced tissues were then homogenized with chilled sucrose solution (0.25 M) in a glass tube using Teflon coated mechanical tissue homogenizer (MICCRA D-9, Digitronic, Germany) to get 5 % homogenate and centrifuged at 5000 rpm for 20 min at 4 °C. The supernatant was separated and stored at - 20 °C for further analysis.

Biochemical assays

Antioxidant enzymes

Catalase

Catalase activity was estimated according to the method of Takahara et al. ¹². To 2.45 ml of phosphate buffer (50mM, pH 7.0), 50 µl of the tissue homogenate was added and the reaction was started by the addition of 1.0 ml of H₂O₂ solution, the decrease in absorbance was measured at 240 nm at 30 sec. intervals for 2 min. The enzyme blank was run simultaneously with 1.0 ml of

distilled water instead of hydrogen peroxide. The enzyme activity was expressed as n moles of H₂O₂ decomposed per min per mg protein

Superoxide dismutase

The SOD activity was estimated by the method of Misra & Fridovich¹³. The assay is based on the oxidation of epinephrine-adrenochrome transition by the enzyme. The reaction mixture consisted of 50 µl of sample, 1.5 ml phosphate buffer and 0.5 ml epinephrine. The solution were mixed well and immediately read the change in optical density at 480 nm for 2 min in a Shimadzu- UV spectrophotometer. One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

Glutathione-s-Transferase (GST)

Glutathione-S-transferase (GST; EC 2.5.1.18) activity was measured by spectrophotometric method of Habing *et al.*¹⁴, using S-2, 4-dinitrophenyl glutathione (CDNB) as substrate. The method was based on the principle of formation of adduct of CDNB, S-2, 4-dinitrophenyl glutathione was monitored by measuring the increase in absorbance at 340 nm against blank. The GST activity was expressed as nmol/min/mg protein.

Acetylcholine Esterase (AChE)

The enzyme (E.C.3.1.1.7) was assayed by the method of Hestrin¹⁵. Acetylcholine esterase assay system comprised of 1.0 ml of M/15 phosphate buffer (pH 7.2), 1 ml acetyl choline (0.004M, pH 4.0) substrate buffer mixture (1:9 dilution), and 0.2 ml of homogenate and incubated for 30 min at 37°C. Alkaline hydroxylamine (2.0 ml) was added to terminate the reaction. The solution was mixed thoroughly and 1 ml of HCl (2:1) was added followed by thorough mixing. Enzyme solution was then added to the control tubes. The color was developed by addition of 1 ml of FeCl₃ (10%) and OD was recorded at 540 nm after thorough mixing. In this assay, mixing the solution in every step is very essential to avoid trapping of air bubbles.

Collection of blood

Each fish was anesthetized with clove oil at 50µl of clove oil per liter of water before taking blood from fish. Blood was withdrawn from *vena caudalis* using a medical syringe which was previously rinsed with 2.7% EDTA solution. Blood collected was then transferred immediately to test tube containing thin layer of EDTA powder (as an anticoagulant) and shaken well in order to prevent hemolysis of blood.

The blood was kept at low temperature and analyzed within 6 h after collection. Hematocrit value (Hct %) was determined after centrifugation at 15,000 rpm for 3 min in a table centrifuge. Hemoglobin contents (Hb) were determined using cyanomethemoglobin method with Drabkin's

solution. Red Blood Cell count (RBC), white blood cell count (WBC) and differential WBC counts were performed as described in Schaperclaus¹⁶.

Total leucocytes count (WBC), Total erythrocyte count (RBC) and platelets

20 µl of blood was mixed with 3980 µl of WBC, RBC and platelets diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. Care was taken that here were no air bubbles trapped. The numbers of cells were counted in four big squares under high power (40x) magnification of light microscope and expressed as the number per cubic ml.

Haemoglobin content

The haemoglobin level of blood was analyzed following the cyanmethemoglobin method using Drabkins Fluid (Qualigens). Blood (20 µl) was mixed with 5 ml of Drabkin's working solution. The absorbance was measured using a spectrophotometer at 540 nm. The final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens Diagnostics). The hemoglobin concentration was then calculated as described in diagnostic kit.

Derived hematological parameters

The derived haematological profile of the mean corpuscular volume (MCV; fl), mean corpuscular haemoglobin (MCH; pg) and mean corpuscular haemoglobin concentration (MCHC; %) were calculated according to the equation suggested by Haney *et al.*, (1992).

$$\text{MCV (fl)} = \text{HCT (\%)} * 10/\text{RBC (million/mm}^3\text{)}$$

$$\text{MCH (pg)} = \text{Hgb (gm/dl)} * 10/\text{RBC (million/mm}^3\text{)}$$

$$\text{MCHC (\%)} = \text{Hgb (gm/dl)} * 100/\text{HCT (\%)}$$

Nitroblue tetrazolium (NBT) assay

Nitroblue tetrazolium assay was done by the method of Secombe¹⁷ as modified by Stasiack and Bauman¹⁸. Fifty microliters of blood was placed into the wells of 'U' bottom microliter plates and incubated at 37⁰C for 1 h to facilitate adhesion of cells. Then the supernatant was removed and the loaded wells were washed three times in PBS. After washing, 50 microliters of 0.2% NBT was added and plate was incubated for further 1 hr. The cells were then fixed with 100% methanol for 2-3 min and again washed thrice with 30% methanol. The plates were then air dried. Sixty microliters 2N potassium hydroxide and 70 microliters dimethyl sulphoxide were added into each well to dissolve the formazon blue precipitate formed. The OD of the blue colored solution was then read in ELISA reader at 540 nm.

Statistical analysis

The data were statistically analyzed using statistical package of SPSS version 16, in which data

were subjected to one-way ANOVA and Duncan's multiple range tests was used to determine the significant differences between the means at 5 % level of significance.

RESULTS AND DISCUSSION

Physio-chemical parameters of water

All the physico-chemical parameters of water were recorded and average values of all the treatments were found within the optimum range. Recorded dissolved oxygen concentration of all the experimental tubs were within the range of 6.2 to 8.4 mg L⁻¹ and the water temperature ranged from 28.4 °C to 32.8 °C during the experimental period. The pH values were recorded within the range of 7.2 to 8.4 and free carbon dioxide in water was found to be negligible (1.4 to 3.0 mg L⁻¹) and the carbonate hardness was found to be 236 – 245 mg L⁻¹. The total ammonia content of all the experimental tubs were recorded before water exchange. It was found to be in the range of 0.14 to 0.19 mg L⁻¹. The nitrite – N and nitrate-N content were found to be in the range of 0.003 to 0.005 mgL⁻¹ and 0.04 to 0.06 mg L⁻¹, respectively.

Acute toxicity and Lethal Concentration (LC₅₀)

The recorded values of LC₅₀ at 24, 48, 72 and 96 hrs were 18.87, 10.55, 8.92 and 7.9 µg /L, respectively (table. 1). The safe value of Ivermectin was found to be 1.01 µg /L with Sensitivity-value (S- value) of 1.77 µg /L for rohu fingerlings. The cumulative percentage mortality recorded at 24, 48, 72 and 96 hrs are given in figure 1.

Table 1: LC₅₀ values of ivermectin for *L. rohita* fingerlings (5.4 gm) at different hours for a period of 96 h (n =30 each concentration).

Period of Exposure (h)	LC 50 (µg/L)	95 % confidence interval		S- Value (µg/L)	Safe Level (µg/L)
		Lower Limit(µg/L)	Upper Limit(µg/L)		
24	18.87	14.52	32.12		
48	10.55	8.63	13.40	1.77	1.01
72	8.920	7.29	8.41		
96	7.896	6.38	9.55		

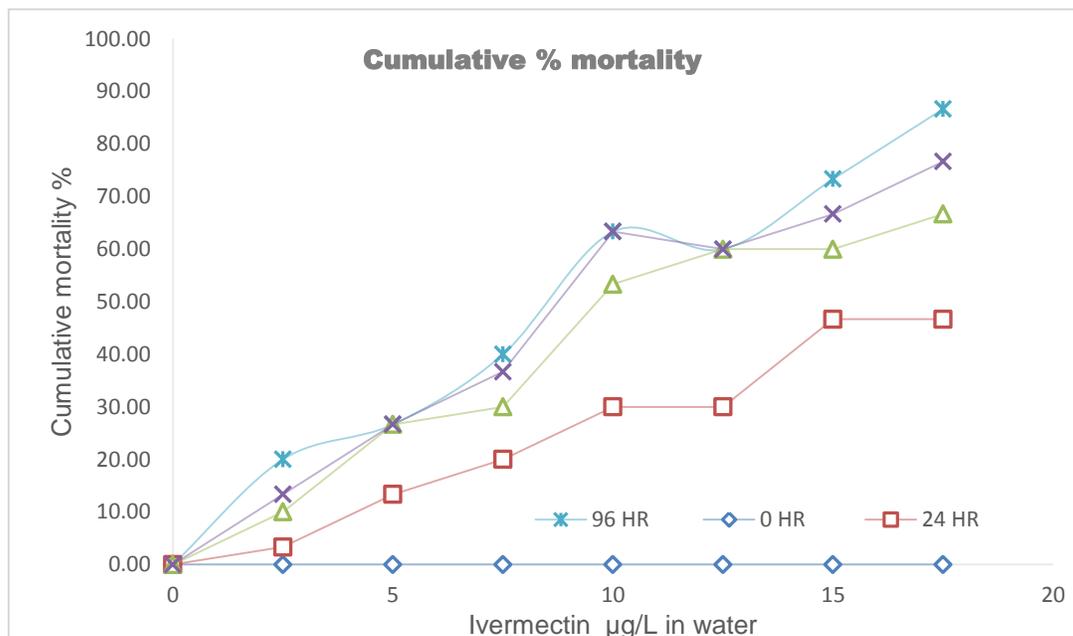


Figure 1: Percentage mortality of *Labeo rohita* fingerlings exposed to different concentration of ivermectin in water for a period of 96 h (n =30 each concentration)

Enzymes of oxidative damage

Exposure of Ivermectin had significant effect ($p < 0.05$) on both gill and liver catalase activity compared to the control (unexposed) group. The activities were substantially high in the highest concentration group (17.5 µg/L) and least in the control group both in liver and gill. Exposure of Ivermectin had no significant effect ($p > 0.05$) on SOD activities in gill and liver compared to the control group. GST activities of the liver and brain were significantly increased ($p < 0.05$) with the level of exposure at different concentration. (Table 2 & Figure 2)

Table 2: Activity of Catalase and SOD enzymes in liver and muscle tissue of *Labeo rohita* fingerlings exposed to graded level of ivermectin concentration in water for a period of 96 h.

Concentrations	Catalase		SOD	
	Gill	Liver	Gill	Liver
0 µg/kg	13.19 ^a ±1.18	19.60 ^a ±1.42	80.07 ^b ±3.86	55.43±4.12
2.5 µg/kg	53.09±5.76 ^b	71.10±7.51 ^{bcd}	69.24±2.13 ^a	52.96±4.03
5 µg/kg	55.18 ^b ±3.57	65.32 ^{bc} ±5.89	66.9 ^a ±3.87	55.57±4.61
7.5 µg/kg	56.45 ^b ±2.20	55.54 ^b ±4.55	75.09 ^{ab} ±2.09	60.10±4.92
10.0	69.90 ^{bc} ±8.01	79.45 ^{cd} ±9.20	75.96 ^{ab} ±4.48	58.04±5.91
12.5	70.58 ^{bc} ±5.72	87.74 ^{de} ±6.65	81.68 ^b ±2.97	60.35±4.84
15.0	84.95 ^{cd} ±9.55	103.56 ^{ef} ±6.46	71.55 ^{ab} ±2.34	57.89±4.38
17.5	95.93 ^d ±3.83	120.57 ^f ±6.85	72.50 ^{ab} ±3.69	52.05±2.42
P value	0.005	0.001	0.052	0.851

SOD: Super oxide dismutase: Units/mg protein) (n = 6)

Different superscripts in the same column signify statistical differences ($p < 0.05$) (mean ± S.E)

Catalase: Units/mg protein) (n = 6)

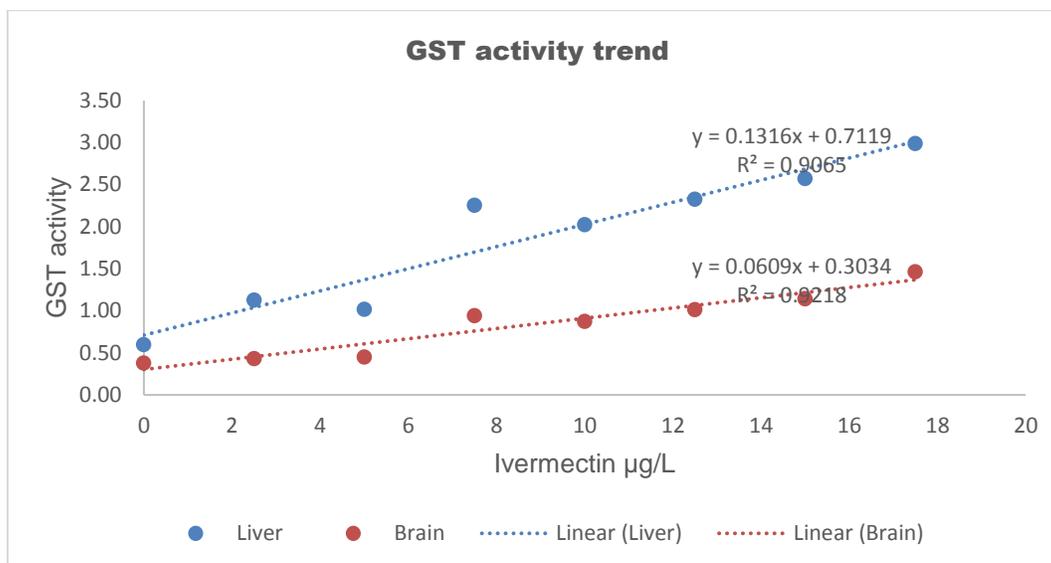


Figure 2: Trend of GST enzymes activity in liver and brain tissue of *Labeo rohita* fingerlings exposed to grade level of ivermectin concentration in water for a period of 96 h.

Different superscripts in the same column signify statistical differences ($p < 0.05$) (mean \pm S.E.) (n = 6), GST: Glutathione S- transferase Units/mg protein.

Acetylcholine esterase (Ache)

Exposure of ivermectin had significant dose dependent effect ($p < 0.05$) on brain Ache activity, which increased with the increasing dose of ivermectin in water, compared to control group. The values were substantially the highest in the highest concentration exposed group (17.5 $\mu\text{g/L}$) of in brain (9.27 ± 0.66^d), and least activity in control group. (Figure 3)

Table 3: Different blood parameters of *Labeo rohita* fingerlings exposed to different level of ivermectin concentration in water for a period of 96 h.

Conc.	WBC	RBC	Hb	Platelets	HCT	MCV	MCH	MCHC
Control	216.00±10.39 ^{ab}	1.36±0.03 ^{ab}	5.59±0.20 ^{bcd}	186.67±6.36 ^c	10.10±0.55 ^a	73.63±1.30 ^a	40.00±1.15 ^{abc}	57.53±1.78 ^e
2.5 µg/L	195.53±13.03 ^a	1.25±0.06 ^a	4.53±0.19 ^{ab}	213.00±31.43 ^c	13.27±0.98 ^{ab}	116.20±2.46 ^b	36.73±2.87 ^{ab}	34.67±2.23 ^c
5.0 µg/L	248.20±8.70 ^c	2.32±0.10 ^d	8.37±0.38 ^e	54.33±3.76 ^a	30.53±2.77 ^c	136.30±4.95 ^c	38.00±1.53 ^{ab}	26.60±0.98 ^a
7.5 µg/L	210.93±7.69 ^{ab}	1.31±0.10 ^{ab}	6.30±0.31 ^d	97.00±7.55 ^b	15.40±1.70 ^{abc}	111.17±3.55 ^b	46.2±1.21 ^d	41.80±1.59 ^d
10.0 µg/L	226.30±934 ^{bc}	1.30±0.08 ^{ab}	6.10±0.44 ^{cd}	67.67±5.24 ^{ab}	21.41±0.60 ^{cd}	160.00±3.06 ^d	42.10±1.46 ^{bc}	27.67±2.52 ^{ab}
12.5 µg/L	210.40±6.04 ^{ab}	1.70±0.08 ^c	6.53±0.44 ^d	59.33±2.40 ^{ab}	24.73±3.89 ^{de}	136.23±3.02 ^c	34.03±2.98 ^a	26.30±1.40 ^a
15.0 µg/L	196.10±5.27 ^a	1.11±0.10 ^a	3.90±0.36 ^a	50.33±4.10 ^a	10.77±0.79 ^a	113.03±5.37 ^b	38.33±2.18 ^{ab}	35.47±1.31 ^c
17.5 µg/L	198.10±3.46 ^a	1.53±0.06 ^{bc}	5.13±0.35 ^{bc}	38.67±2.91 ^a	19.03±1.85 ^{bcd}	118.8±7.80 ^b	35.87±1.83 ^{ab}	32.87±2.94 ^{bc}
P-value	0.002	0.002	0.001	0.001	0.002	0.001	0.002	0.001

Values in the same column with different superscript differ significantly (P<0.05);

Platelets, WBC; 10^3 cells/ mm^3 , RBC, million cells/ mm^3 , Haemoglobin, gm/100 ml of blood; HCT as percentage, MCHC. g/dl; MCV, fl , or 10^{-15}L ; MCH, fmol/cell.

Data expressed as Mean \pm SE, n = 3

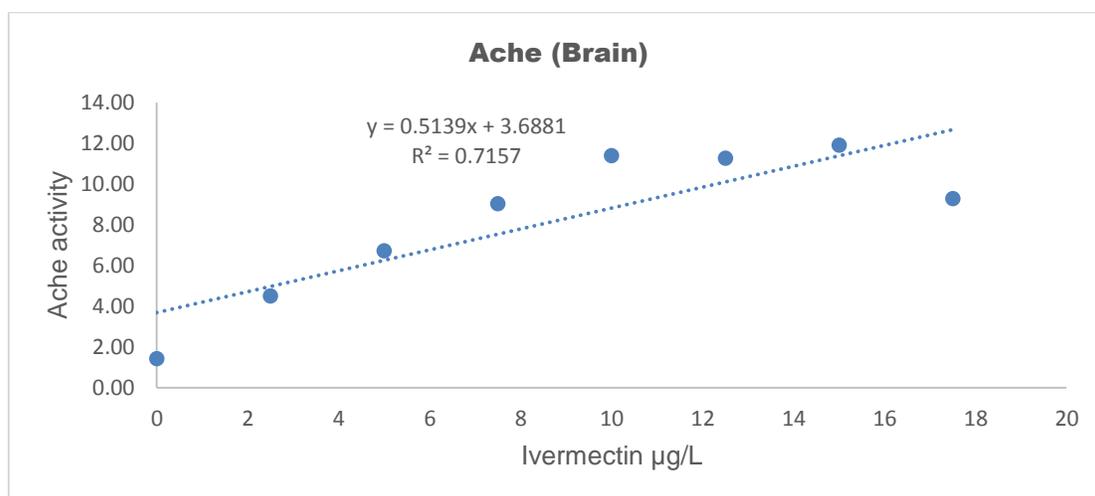


Figure 3: Trend of Ache enzyme Activity in brain tissue of *Labeo rohita* fingerlings exposed to grade level of ivermectin concentration in water for a period of 96 h.

Mean values bearing different superscripts under each column vary significantly ($P < 0.05$)

Acetyl choline esterase (AChE) specific activities expressed as nano moles of Acetyl choline released/min/mg protein at 37°C

Hematological parameters

The WBC count ($\times 10^5$ cells/ mm^3) and RBC count (10^6 cells/ mm^3) of the different treatments were significantly affected by the Ivermectin exposure in comparison to the control. The second level of ivermectin exposure ($5.0 \mu\text{g/L}$) showed significantly ($p < 0.01$) higher WBC and RBC count than the higher level of exposure and control group. The Hemoglobin content (gm/dl) of the fishes exposed to different concentration of the Ivermectin did not show any dose dependent variation, while the highest content was found to be in concentration $5.0 \mu\text{g/L}$, among all the group of fishes and the trend decreased with further increase in Ivermectin concentration. Similarly HCT of all exposed groups were found to be significantly ($p < 0.01$) higher than the control group and the highest value was found to be in concentration $5.0 \mu\text{g/L}$ and the least in control group among all the treatments (Table 3).

The platelets count ($\times 10^3$ cells/ mm^3) of the fishes exposed to different concentration of the Ivermectin had showed significantly dose dependent decrease. The highest count was found to be in control group ($0.0 \mu\text{g/L}$) and the least in the highest concentration ($17.5 \mu\text{g/L}$) exposed group among all the group of fishes. It bears a clear negative correlation and dose dependent decrease with increasing concentration (Table 3 and Figure 4)

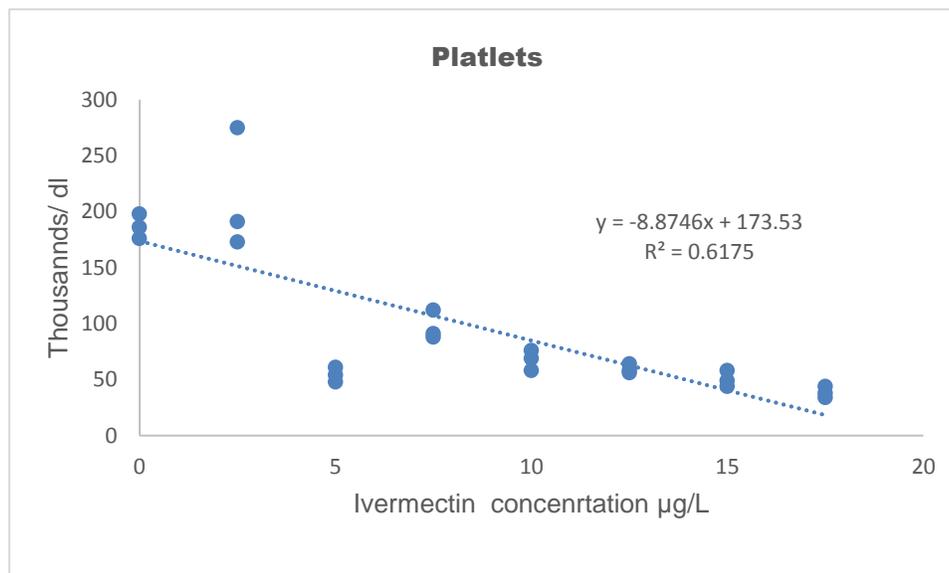


Figure 4: Trend of blood platelets count of *Labeo rohita* fingerlings exposed to different level of ivermectin concentration in water for a period of 96 h. (n = 3 for each concentration)

Mean corpuscular volume (fL, or 10^{-15} L), of fishes exposed to different concentration of the Ivermectin showed significantly the dose dependent effect. The all exposed groups were found to be significantly ($p < 0.01$) more than the control group and the highest value was found to be in concentration 10.0 $\mu\text{g/L}$, further increase in Ivermectin concentration lead decrease in MCV. However, there was no dose dependent impact of the Ivermectin exposure on the MCH value of the fishes and the highest value was found to be in exposure concentration 7.50 $\mu\text{g/L}$ and least in exposure concentration group 12.5 $\mu\text{g/L}$ among all the group of fishes. The Mean corpuscular hemoglobin concentration of the different treatments of Ivermectin lead significant decrease in the value compared to the control group. The highest value was found to be in control group and the least in exposed concentration 12.5 $\mu\text{g/L}$ group among all the groups (Table 3).

Nitroblue tetrazolium (NBT) assay

Respiratory burst activity (NBT reduction) of neutrophils of *Labeo rohita* due to acute exposure of different Ivermectin levels showed significant increase compared to the control group. Though ivermectin exposed group showed higher NBT values compared to the control, there was no variation ($P > 0.05$) among the exposed groups (Figure 5).

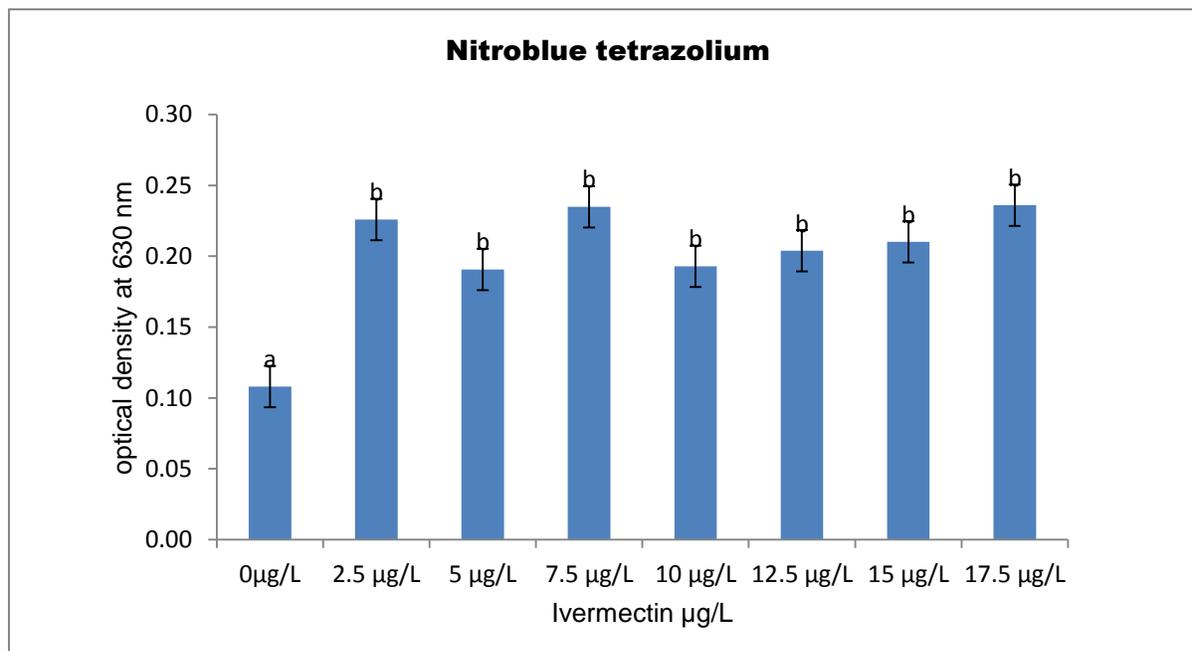


Figure 5: Respiratory burst activity of *Labeo rohita* fingerlings exposed to different ivermectin concentrations as experimental groups at the end of 96 hours

Values in the different bar with different superscript differ significantly ($P < 0.05$);

NBT: Nitroblue tetrazolium, units: optical density at 630 nm, Data expressed as Mean \pm SE, n = 6

DISCUSSION

Physiochemical parameters of water

All the physico-chemical parameters of water were recorded to be within the optimal range of *L. rohita*¹⁹. Therefore, the chance of interference of water quality parameter in response to treatment was negligible. It was further confirmed by no mortality of *L. rohita* fingerlings in the control group.

Acute toxicity and Lethal Concentration (LC₅₀)

In the present study, LC₅₀ of the Ivermectin to *L. rohita* fingerlings at 96 h was found to be 7.9 µg/L. It has been found by earlier studies that the difference between treatment dosage and toxic effects of Ivermectin in Atlantic salmon is very²⁰. A commonly recommended treatment dose of Ivermectin is 0.05 mg/kg, given twice weekly in feed, with no treatments in winter. However, the 96 h LD₅₀ was determined to be 0.5 mg/kg²⁰ with a 96 h LC₅₀ set at 17 µg/L²¹. But toxicity response depends upon weight, age and species. This reflects the varying degree of toxicity in different species of fishes. Symptoms of Ivermectin neurotoxicity in Atlantic salmon appear like loss of appetite, dark skin coloration, lethargy and erratic swimming behavior²⁰. These were also noticed in the present study in rohu fingerlings besides a yellow coloration of pelvic portion which

was prominent at end of experiment in most of the fishes. This can be attributed due to the elimination of the Ivermectin through intestinal secretion and potential damage of intestinal mucosa. The routinely used dose on farm in carp culture is more than 0.5 mg/Kg body weight and immersion doses are mostly unregulated as even the rough estimate of tank volume and dosing is not practiced. The higher sensitivity to Ivermectin in some fishes like rohu can be attributed to their poor evolutionary adaptation, the poor blood brain barrier ability to restrict the permeation of drug in brain. While in fishes the lower LC₅₀ dose compared to LD₅₀ may be due to the pumping of the oxygenated blood from gill to brain directly and paralysis of bronchial muscle leading to ataxia and death. Further, the high brain concentrations of Ivermectin found in Atlantic salmon, *Salmon salar*, and gilthead sea bream after oral and intraperitoneal administration, respectively^{22, 23} point towards a less selective barrier in fish as compared to mammals.

Enzymes of Oxidative Damage

Under normal physiological condition animal cell produce reactive oxygen species (ROS) such as H₂O₂ that may damage most cellular components leading to cell death. Living organisms are protected from ROS by several defense mechanisms, including antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, etc. Catalase (CAT) activity plays important role in antioxidative defense of the cell by reducing H₂O₂. When the rate of ROS generation exceeds that of their removal, oxidative stress occurs. In the present study, exposure of Ivermectin had significant effect (p<0.05) on both gill and liver catalase activity. Highest activity was recorded in the highest concentration (17.5 µg/L) exposure in liver and gill, whereas the unexposed group (control) had the least activity. In a similar study done in rabbits infested with *Psoroptes cuniculi*, doramectin caused an increase in CAT activity²⁴. In their study, the increased activity of CAT was found in the liver, kidney, heart of rabbit exposed to Ivermectin at 0.02 mg/kg. Similarly, in the present study there was dose dependent increase in CAT activity due to Ivermectin exposure. This indicates excessive, H₂O₂, reactive oxygen species (ROS) and reactive nitrogen species (NOS) free radical production due to Ivermectin toxicity^{25, 26}.

Phagocytosis in fish is the result of phagocytic cells, which include mononuclear phagocytes (monocytes and macrophages) and neutrophils. Phagocytes produce large quantities of superoxide anion (O₂⁻) upon stimulation with a variety of agents and pathogen. In the present study SOD activity was not significantly different from the control group. While the values were found to decrease in Ivermectin exposed group compared to their unexposed counterpart.

In the present study, Ivermectin had significant effect (P<0.05) on both liver and brain GST activity compared to the control group. The highest activity was recorded in the highest

concentration exposed (17.5 µg/L) in both liver and brain, whereas the control group had the least activity. The enzyme activity bears strong correlation ($r^2= 0.906$ in liver and $r^2= 0.921$ in brain) with Ivermectin level, indicating the potentiality of the enzyme activity as biochemical marker for the Ivermectin induced stress and toxicity. Several authors have reported, GSTs are a family of enzymes that play a significant role in detoxification of xenobiotics such as insecticides²⁷. Increased activity of delta and epsilon class GSTs had been linked to resistance to organophosphates, DDT and pyrethroids²⁸. GSTs have also been associated with another macrocyclic lactone resistance in mites, with elevated GST activity observed in abamectin-resistant *Tetranychus urticae*²⁹. Additionally, *Caenorhabditis elegans* isolates selected for Ivermectin resistance *in vitro* show increased transcription of GSTs and glutathione conjugate Multidrug related protein (MRP) transporters, together with reduced intracellular glutathione, suggesting its ivermectin induced acceleration of drug conjugation and removal³⁰.

Acetylcholine esterase (Ache)

Acetylcholine is a neurotransmitter found widely distributed in nervous tissue, stored and released from the synaptic vesicles. Acetylcholine is removed by acetylcholine esterase. This hydrolytic degradation ensures that the signal does not over stimulate the post-synaptic membrane. In the present study, exposure of Ivermectin had significant effect ($P<0.05$) on brain Ache activity compared to the control group. The highest value was recorded in the highest concentration (17.5 µg/L) exposed group in brain, whereas the, control group had the least activity. This is attributed due to the neural blocking by GABA. The inhibitory neurotransmitters in brain and the compensatory Ache activity rises to maintain the homeostasis. This possible adaptation becomes viable through alternative channel "activator" sites. This is a subject of further study in particular species *Labeo rohita*. But the strong dose dependent activity of acetylcholine esterase (Fig.3) indicates the possible function of alternate channel activator.

As explained by Hyaland et al⁸, in the Alternative channel "Activator" site neuronal nAChR function may also be enhanced via ligand binding sites distinct from those at which ACh or (-)-nicotine interact. These sites are thought to be present at the level of the α -subunit and are not subject to the same desensitization mechanisms described for (-)-nicotine. Compounds that interact with this novel site to increase neuronal nAChR mediated ion conductance have been termed "channel activators." The cholinesterase inhibitors physostigmine and galanthamine, (+)-2-methylpiperidine, and the antihelminthic agent, Ivermectin, are examples of compounds that act as channel activators at this site which is distinct from the (-)-nicotine site. Ivermectin (30 µM) has been shown to enhance ACh-evoked current in chick or human nAChRs. Even they found the

concomitant increase in apparent affinity and cooperatively of the ACh, dose-response curve also suggests that Ivermectin acts as a positive allosteric effector of this sub type.

Haemato - immunological Parameters

In the present study, exposure to different level of Ivermectin concentration did not cause any dose dependent effect on WBC, RBC count and hemoglobin content (gm/dl) in comparison to the control. The lower level of Ivermectin exposure showed significantly ($P < 0.01$) higher WBC, RBC count and hemoglobin content (gm/dl) than the higher Ivermectin level exposure and control group. It was evident that the lower level of Ivermectin exposure enhanced the hemoglobin content and RBC counts, whereas opposite trend was recorded with further increase in Ivermectin level. This could be the body own mechanism to compensate the oxygen deprivation caused due to branchial paralysis and slow opercular pumping. But the same mechanism fails to compensate at the higher toxicity level. Reduced hemoglobin content and elevation in WBC and RBC count was observed in long term toxicity study by Sarma (2003), which is in agreement with Katharios *et al.*²³. The platelets count ($\times 10^3$ cells /mm³) of the different exposure to different concentration of the Ivermectin showed dose dependent effect. The highest number was found in unexposed group and the least in highest concentration exposure (17.5 $\mu\text{g/L}$) among all the group of fishes (Figure 4.). This could be due to vitamin K deficiency triggered by the Ivermectin. Similar results were reported with the drugs like adbenzazole and Ivermectin³¹.

Hematocrit value and mean corpuscular volume were not affected ($P > 0.05$) with exposure to Ivermectin, the exposed groups were found to have significantly ($P < 0.01$) increased values than the control group. The highest value was found in lower concentration exposure and the least in control group. There was no significant impact of the Ivermectin exposure on the MCH value of the fishes. Similarly mean corpuscular hemoglobin concentration (MCHC) of the different treatments exposure to different concentration of the ivermectin showed significant decrease in the value compared to the control group while there was no dose dependent significant variation in value among the exposed groups. The highest value was found in unexposed group and the least in exposed concentration of 12.5 $\mu\text{g/L}$. A decrease in MCV may be attributed to shrinking of the erythrocytes, resulting in a microcytic anemia. The erythrocytes shrink due to insufficient synthesis of haemoglobin^{32,33}. The fluctuations in MCH and MCHC values of exposed group indicate that the concentration of haemoglobin in the RBCs was much high in the exposed fish compared with control. The MCHC is a good indicator of RBC swelling³⁴. The fluctuation of MCH and MCHC in different treated groups is function of the haemoglobin and RBC variation.

In the respiratory burst activity study, nitroblue tetrazolium (NBT) reduction of neutrophils in *Labeo rohita* experimental groups exposed to Ivermectin, significantly raised ($P < 0.01$) the NBT value than the unexposed group. The highest level of NBT value was found in exposed group (17.5 $\mu\text{g/L}$) and the lowest blood NBT value in the unexposed group. It is contrast to the finding by Siwicki *et al.*³⁵ where, exposure to chemotherapeutics caused significant inhibition in the activity of NBT in the blood. In present study the there is significant increase ($P < 0.01$) but not in dose dependent manner, which may be due to stress, leading ROS production or by activation of free radicals²⁵. In a similar study on the oxidative burst of eosinophilic granulocytes, the result revealed a dual, dose-dependent modulatory in vitro effect of the investigated anthelmintic drugs on the respiratory burst of eosinophilic effector cells indicating that these compounds may modulate host defense in vivo. Inhibitory effects on the generation of toxic oxygen intermediates were demonstrated for ivermectin at concentrations higher than 200 ng/ml (0.5 micro M). An increased production of the reactive oxygen metabolites was demonstrated at low doses of ivermectin (20-40 ng/ml; corresponding to 0.02-0.04 micro M).³⁶

CONCLUSION

In conclusion the ivermectin could be toxic in bath treatment when used at high concentration in water solution. Haematological, serum biochemical, antioxidant and enzymological parameters showed a shift of homeostasis during the exposure of ivermectin and reflected significant increase in the level of antioxidant and stress enzyme at concentration of ivermectin exposure below determined LC_{50} at 96 h, while the homeostatic mechanism seems to fail at higher concentration indicating adverse impact on function of vital tissues and physiology of rohu fingerlings. Moreover, the effect of modulation of haemato-immune system of the fish also shows that higher doses of exposure of ivermectin lead harsh and toxic impact.

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