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Short term toxicological effect of clofibric acid on *Labeo rohita* fingerlings (Hamilton, 1822): Behavioural and enzymatic responses

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Abstract
Clofibric acid (CA) is a hypolipidemic compound and an active derivative substance of clofibrate has been detected in the water bodies all over the world. For Swiss lakes and German rivers, the concentrations of CA in the surface water and from effluents of Sewage treatment plants are 0.551 µg/Land 1.6 µg/L, ground (4 µg/L), surface and drinking waters (0.07–0.27 µg/L) all over the world. Short term toxicological investigation (96 hours) on *Labeo rohita* fingerlings was studied at different nominal concentrations such as 1, 10 and 100 µg/L. In case of behavioural responses, opercular movement and mucus secretion both increased significantly. The disparity of behaviour in exposed fish became more pronounced with rising CA concentrations, showing a positive correlation with the concentration. Throughout the trial, there were no obvious signs of anomalous behaviour in the fish in the control group. In accordance with enzymatic responses, Glutamic Oxaloacetic Transaminase (GOT), Glutamic Pyruvic Transaminase (GPT) and Lactate Dehydrogenase (LDH) levels in all treatments were statistically significant (p <0.05).

Keywords: Clofibric acid, *Labeo rohita*, short term investigation, behavioural reponses, enzymatic responses

Introduction
Clofibric acid (CA) [IUPAC: 2-(4-Chlorophenoxy)-2-methylpropanoic acid] is a hypolipidemic compound and an active derivative substance of clofibrate has been detected in the water bodies all over the world (Koutsouba *et al.*, 2003) [13]. The overproduction causes aimless usage and careless disposal which increase the releasing rate into the aquatic environment as parent compounds, metabolites or conjugates (Guerra *et al.*, 2014) [9]. The excretion of pharmaceutical drug which are commonly detected like antibiotics, anti-inflammatory drugs, lipid-lowering agents and anticonvulsants (Fent *et al.*, 2006) [7] from manufacturing industry or any other source. The pharmaceutical compounds may occur in minimum concentrations (ng/l to µg/L) and its intervention in the biological system as well as aquatic ecosystem causes degradation of the water quality and affect the organisms and human health (Ramaswamy *et al.*, 2011) [20]. Clofibric acid is excreted by human being taking the cardiovascular drug Clofibrate (Heberer, 2002) [10] and it also act as an endocrine disrupter (Pfluger and Dietrich, 2001) [19]. Clofibric acid is detected (in the range of 0.8–2 µg/L) in treated waste water in the USA in 1976 (Hignite and Azarnoff, 1977) [11], ground (4 µg/L), surface and drinking waters (0.07–0.27 µg/L) all over the world (Tauxe-Wuersch *et al.*, 2005) [29]. Clofibric acid is non-biodegradable, highly mobile and very persistent in the environment, with a half-life of 21 years and a water residence time of 1–2 years (Richardson and Bowron, 1985; Buser and Muller, 1998) [22] [5]. Tixier *et al.*, 2003 [30] reported that the occurrences of Clofibric Acid in different water body i.e., the range of daily loads of Clofibric acid in WWTPs is 0.26-1.26 g/day, in river is 2 g/day, in lake 1 kg/ day. Exposure of fish to Clofibric acid might lead to changes in plasma lipoprotein and triglyceride concentrations. Significant alterations were observed in GOT, GPT and LDH enzyme activity in *Cyprinus carpio* exposed to Clofibric Acid (Saravanan *et al.*, 2011) [26]. The environmental detection level as well as occurrences and toxicity of various pharmaceuticals and its derivatives in aquatic body is now very important (Ramaswamy *et al*., 2011) [20] and their toxicological effect on the physiology of aquatic organisms specifically in freshwater fish are very limited (Saravanan *et al*., 2011) [26]. Hence, the present experiment is conducted to assess the toxicological impact of CA at different concentrations (1, 10 and 100 µg/L) on Indian Major Carp *Labeo rohita* which is most important cultured species with high growth rate and high economic value.

Materials and Methods

Experimental Chemical

Clofibric acid (a- (p-Chlorophenoxy) isobutyric acid, CAS No. 882-09-7) is purchased from Hind Biotech company. The Dimethyl Sulphoxideis used as a solvent in the present experiment and 0.2 mL/L used to prepare the stock solution at different concentrations (1, 10, and 100 µg/L) due to their low water solubility.

Procurement and acclimatization of experimental animal:

The advanced healthy and uniform sized with an average weight of 28.6 ± 1.4 gm (mean ± SD) and length 12.8 ± 1.2 cm (mean ± SD) *Labeo rohita* fingerlings were procured from Bengal Fish Hatchery of Sonarpur, Kolkata, was transported safely to wet laboratory (Dept of Aquatic Environment management) of the Faculty of Fishery sciences, through aluminium "Hundi'' during the morning hours (6 am – 9 am) to avoid stress. For acclimatization, the fishes were gently transferred in two FRP tanks (1000 L capacity, 950 L water volume) after 2% disinfection with KMnO4 treatment for three weeks. They were kept under constant temperature 16 °C ± 0.5 and 12:12 h of photoperiod with continuous aeration. During this period, fishes were fed with floating granules feed at the rate 3% of body weight and the tanks were subjected to 30% water exchange every alternative day and cleaning 2 times to avoid any kind of disease or infection and equal volume of water was restocked to sustain the water quality and water level.

Experimental design

Short-term exposure studies

Based on the nominal concentrations of CA (1, 10 and 100 µg/L) and control (0 µg/L)with triplicate, the short-term toxicity (96 hours)was carried out in accordance with EPA guidelines in each glass aquarium (75 L capacity and 60 L volume) and simultaneously, the guidelines of animal ethics of West Bengal University of Animal and Fishery Sciences, Kolkata, India were sternly followed during the present study period and 7 fishes (each of 28.6 ± 1.4g and length 12.8 ± 1.2 cm)were introduced and feeding was withheld during the bioassay experiment. The test water was replenished at the end of 24 h and freshly prepared solution was added to maintain the concentration of CA at a constant level. The mortality/survival of fish was recorded in every 24 h. No mortality occurred during the experiments. In the experimental period (96 hours), the physicochemical parameters (APHA, 2005) [3] were analysed two times are as follows: The ranges of temperature, dissolved oxygen (DO), pH, free carbon dioxide, total alkalinity were 16–18 ^{0}C, 5.7–6.0 mg /L, 7.3–7.6, 0.521–0.841 mg /L, and 159–178 mg /L, respectively during the short-term toxicity assay.

Collection of blood

Each fish was anaesthetized with clove oil (Apollo Pharmacy, India) @ 50 µl/L of water before taking blood. Blood was withdrawn from caudal vein using a medical syringe (23G), which was previously rinsed with 2.7% EDTA solution. Collected blood was then transferred immediately to EDTA test tubes (as anticoagulant) and was shaken well in order to prevent blood haemolysis and kept for 45 min followed by centrifugation in a cooling centrifuge (Thermo Fisher Scientific, USA) at 3500 rpm for 15 min at 4 ◦C. Some amount of blood was poured in eppendorf tubes without anticoagulant and kept for 2 h followed by centrifugation at 3500 rpm for 15 min at 4 ◦C to obtain the serum (Ali *et al*., 2018) [1].

Enzyme Assay

Plasma GOT and GPT activities were estimated by 2,4-DNPH method described by Reitmen and Franckel, 1957 [21] and LDH activity was measured following the methodology described by Kumar *et al.,* 2018 [14]. For plasma, the enzyme activity was expressed as International Unit (IU) per liter.

Statistical analysis

All values were analysed by one way analysis of variance (ANOVA) in a SPSS software version 22.0 (SPSS Inc. Chicago, USA), followed by a Duncan multiple range test (DMRT) test to determine the significant differences (P <0.05) among the concentrations on each parameter.

Results and Discussion

Behavioural Changes

The behavioural changes of *L. rohita,* short termly exposed to Clofibric Acid are observed and recorded time to time. It was evidenced that the exposed fish showed marked clinical signs of toxicity with varying degrees depending upon the concentration of the CA. The behavioural and morphological changes are recorded after adding the chemical in the experimental tanks. On the first day for every 30 minutes was noted and continued observation up to 12 hours, then followed by 24, 48 and 96 hours. During first few hours after adding the CA, the fish showed hyperactivity with erratic movements in aquarium. Mucus secretion and opercular movement was increased in very high level. In the fish of the control tank rate of the opercular movement was normal 76 ± 8 beats /min but in the treatment tank, the rate of opercular movement of fish was more than 114 ± 9beats /min. Rapid jerk movement of the fish was observed in the high concentration of the CA. Frequent surfacing also occurred in the all the treatments tanks except control tank. The behavioural irregularities, displayed by the exposed fish increased with increasing concentrations of CA thus, exhibiting a positive correlation with the concentration. No visible abnormal behaviour was observed in the control group of fish during the study. The details of behavioural changes are mentioned in Table 1.

Table 1: Behavioural changes in *Labeo rohita* during short term CA exposure

Behaviour	Concentration (µg /L)			
	C (0 µg/L)	T_1 (1 µg/L)	T_2 (10µg/L)	T_3 (100µg/L)
Jumping Tendency	-	-	++	+++
Opercular movement	-	-	+	+++
Mucus Secretion	-	+	++	+++
Erratic Swimming	-	-	+	+++
Loss of equilibrium	-	-	-	+
Rapid jerk movement	-	+	+	+++
Changes of body colour	-	-	-	++

None -, low +, moderate + +, high + + +, very high + + + +

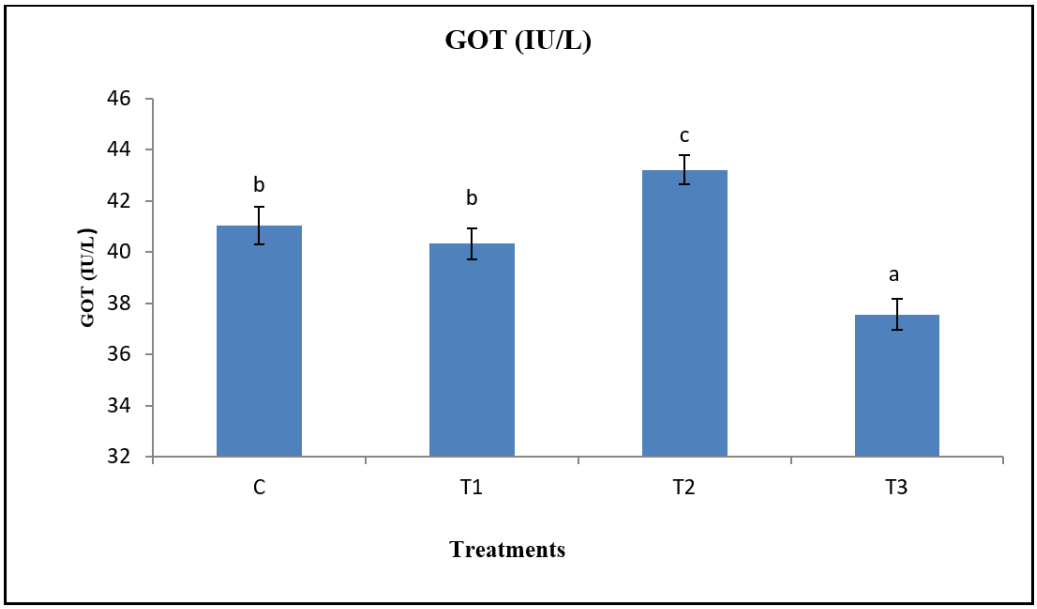
Several recent studies documented that pharmaceuticals and active neuro compounds have potential to change in the behaviours of aquatic species such as animal stay away from the centre of the experimental tank and prefer to stay corner of the tank, shoal coordination, feeding nature, dark and light preference (Gebauer *et al.*, 2011; Riehl *et al.*, 2011) [8, 23]. Respiratory distress, noticed in exposed fish, could be caused by mucous precipitation and neurological dysfunction of gill epithelia in response to the toxicant which resulted in high respiratory rate as reported by Banerjee (2007) [4]. High opercular ventilation has been reported by Omoregie *et al.*, 2009 [17] as an index of stress when *O. niloticus* comes in contact with an unfavourable environmental condition due to single super phosphate. Robinson, 2009 [24] concluded that the behavioural alterations of fish can provide important indices for ecosystem assessment and monitoring.

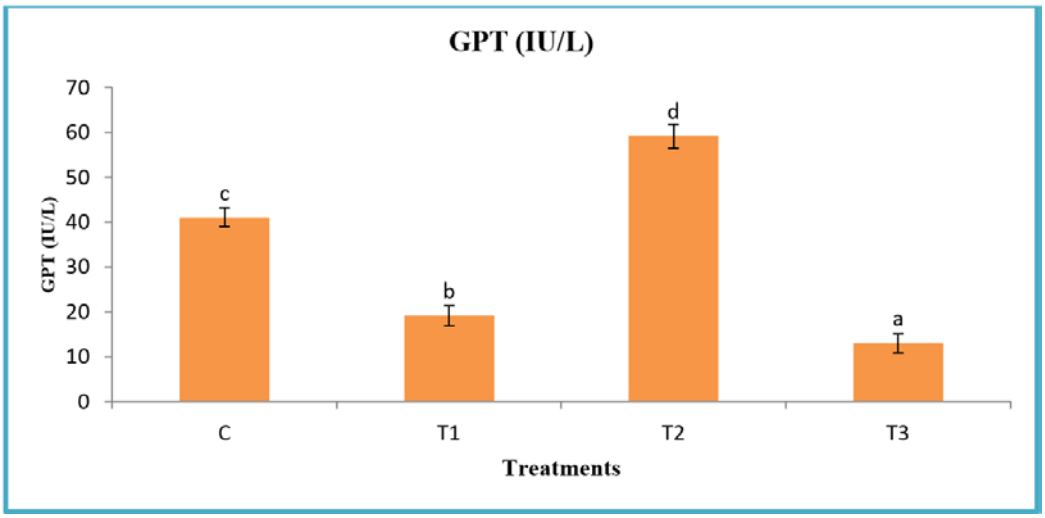
Enzyme assay

Glutamic Oxaloacetic Transaminase (GOT), Glutamic Pyruvic Transaminase (GPT) and Lactate Dehydrogenase (LDH) levels in all treatments were statistically significant ($p < 0.05$). But in case of GOT activity, no significant difference ($p > 0.05$) was observed between C and T_1. No significant alteration ($p > 0.05$) was observed between C and T_1 in case of LDH activity. Variations of enzyme activity with specific data are represented in Table 2.

Table 2: Enzymological (GOT, GPT and LDH) responses in a *L. rohita* after 96 h exposure to different concentrations of CA

Concentrations (µg/L)	Enzyme assay		
	GOT (IU/L)	GPT (IU/L)	LDH (IU/L)
C (0 µg/L)	41.04 ± 0.74^{b}	41.08 ± 2.09^{c}	588.67 ± 23.24^{a}
T_1 (1 µg/L)	40.32 ± 0.6^{b}	19.1 ± 2.23^{b}	618.45 ± 22.53^{a}
T_2 (10 µg/L)	43.22 ± 0.57^{c}	59.15 ± 2.66^{d}	926.78 ± 25.62^{b}
T_3 (100 µg/L)	37.57 ± 0.6^{a}	13.04 ± 2.09^{a}	1104.13 ± 33.55^{c}





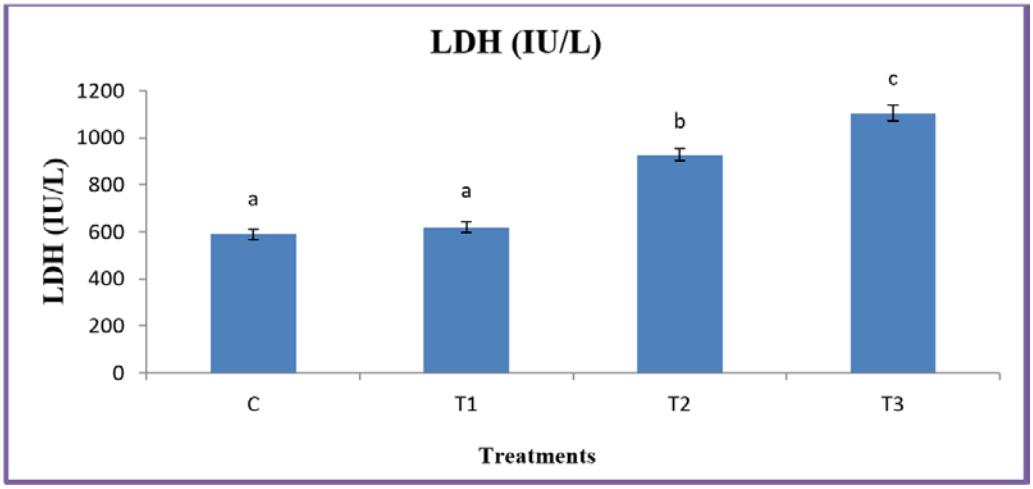


Fig 1: Mean value of different enzymological parameter of *L. rohita* after 96 h exposure to different concentrations of CA. Different numerical values designate significant difference ($p < 0.05$) between the different concentration and control within the 96 h. of exposure. Error bars indicate standard deviation (n = 3).

The GPT activity decreased in 1 and 100 µg/ l. The variations of GPT and GOT activity in blood plasma indicate organ dysfunction such a liver damage in aquatic organisms during stress function (Jung *et al.*, 2003) [12]. In the current investigation, short exposures led to a considerable rise in plasma GOT and GPT activity, which may be due to accumulation of drugs in tissues leading to tissue damage resulting in release of these enzymes into blood. It is commonly acknowledged that an increase in these enzyme activities in the extracellular fluid or plasma is a sensitive sign of even minimal cellular injury (Palanivelu *et al*., 2005) [18]. The increase in plasma GOT and GPT activities in *C. mrigala* exposed to the drug ibuprofen indicates the disorder in Kreb's cycle caused by the drug (Saravanan *et al.*, 2012) [25]. According to Sivakumari *et al*. (1997) [27], a rise in GOT and GPT activity in fish indicates a predominance of anaerobic carbohydrate metabolism, presumably to satisfy increasing energy needs under persistent and extended toxic stress. The increase in GOT and GPT activity may also be due to damage of the organs resulting in increased protein and CHO metabolism, and the changes in the histological structure of the hepatic and extra hepatic tissues may form another possible reasons (El-Sayed *et al.*, 2007) [6]. According to certain theories, stress elevates the transmination pathway generally (Li *et al*., 2011) [15], and the body tries to counteract harmful stress by speeding up metabolism. In contrast to the elevation of GOT and GPT activity, significant decrease in these enzymes has been reported in fishes exposed to various toxicants (Ambili *et al*., 2013) [2].

In the present study, also a significant decrease in GOT and GPT activity was noticed in *L. rohita* during short exposure of CA which indicate that detoxification mechanism may not be sufficiently effective to present the action of these drugs on the system or it indicates disturbance in the structure and integrity of cell organelles (Saravanan *et al.*, 2013) [2]. Lactase dehydrogenase (LDH) activity of *L. rohita* after CA exposure was also higher than control group. There was a significant increase ($P< 0.05$) in LDH activity with an increase of concentration of CA and a two fold increase was noticed in 100 µg/L. LDH is one of the important metabolic requirements for tissue and involve in energy production. After brief exposure to CA, *Gambusia holbrooki* mosquito fish showed increased LDH activity, which may have been induced by the CA's ability to trigger a stress response (Nunes *et al*., 2005) [16]. The structural damage to the cell membranes or the hepatic or cardiac tissues is indicated by the observed rise of LDH activity in fish treated with CA. LDH activity may also alter as a result of modifications in protein and carbohydrate metabolism (Szegletes *et al*., 1995) [28].

Conclusion

CA is toxic in acute exposure but it has broad chronic toxicity effect, and the toxicity increases with increasing chemical concentration as well as exposure time. These changes may have an immense capability to disrupt the servility of *Labeo rohita* in their aquatic atmosphere. Discharge of untreated sewage treatment water into the natural waters bodies should be taken into consideration, should treat the effluent water containing the pharmaceutical drug with modern, efficient technique process compare to the conventional approach. This effluent water can also alter the water quality parameter, which ultimately worst effects on the biodiversity of aquatic organisms and also the phytoplankton diversity.

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