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Determination of Acute Toxicity and the Effect of Sub-Lethal Concentration of Quinalphos on Protein and Lipid Peroxidation in *Oreochromis niloticus*

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Abstract

Quinalphos is an organophosphate used in agriculture that is considered to have potential hazardous effects on non-target organisms. The present study was designed to determine the median lethal concentration (LC_{50}) of quinalphos (an organophosphate pesticide) on the fish, *Oreochromis niloticus,* for 96 hours. The LC_{50} value of quinalphos on *Oreochromis niloticus* for 96 hours was determined as 3.65 µl/L. The study also focuses on the immediate effect of exposure to sub-lethal concentrations of quinalphos on protein and lipid peroxidation rate after 24 hours in brain and muscle tissues. Protein was found to be decreasing while lipid peroxidation rate increased in both tissues after 24 hours of exposure. By the estimation of antioxidant enzymes Superoxide Dismutase (SOD) and Catalase (CAT) activity, it was also identified that quinalphos is capable of impairing the antioxidant defense mechanism of the non-target organism and thereby increase the oxidative stress.

Keywords: Catalase, Lipid Peroxidation, Organophosphate Pesticide, Protein, Superoxide Dismutase

1. Introduction

Pesticides has become an inevitable chemical in the field of agriculture for the control of pests. But its uncontrolled usage has contributed to its spread all over the environment contributing to air, water and soil pollution, and also adverse effects on non-target organisms[1,2]. Instead of being a part of agricultural benefits, it has become the cause of many health-related issues in non-target organisms which includes humans as well[3]. Quinalphos is a widely used organophosphate pesticide, that can be toxic. Organophosphate pesticides are known to cause inhibition on cholinesterase enzyme activity[4]. Fishes come in direct contact with most of the aquatic pollutants within the surrounding water through gills[5] which made them most vulnerable to aquatic pollution.

Oreochromis niloticus is an economically important fish used in agriculture in many countries around the world. They are very adaptable and can withstand wide range of environmental changes like temperature, salinity *etc*. *Oreochromis* can intake natural feeds and formulated feeds as well which make them economically feasible in fish culture techniques[6]. They show variation in feeding behaviour by being a generalist as well as an opportunistic omnivore[7]. The food consumption may change with the size of the fish, habitat where it resides, maturity and environmental status[8].

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Pesticide toxicity can be tested in many ways. But usually, toxicity on humans is determined on the basis of effect on test organisms like rats or by means of organisms that enters human food chain like fishes. The toxicity level is primarily determined by acute toxicity tests[3]. Acute toxicity tests are usually considered as the primary step in the determination of toxicity of a substance. It provides information on the after effects of getting exposed to sub-lethal concentration of a drug or chemical for short term period. Application of median lethal concentration (LC_{50}) of drug would result in the death of 50% of the tested organisms[9]. Since alternation in the locomotor activity of fish can be an indication of effects on nervous system, swimming behaviour of fish is frequently assessed as a tool on toxicity investigations[10]. Similarly, assessment of protein content of an organism is a representation of physiological activities within a cell[11]. Lipid peroxidation is especially affected in acute pesticide toxicity as it is resulted after environmentally applicable pesticide exposure. There are many studies that support the statement that lipid peroxidation is increased in fish species undergone acute pesticide toxicity[12-14]. Pesticides are known to induce an imbalance between endogenous and external Reactive Oxygen Species (ROS), thereby disrupting the antioxidant defence[15,16]. When the equilibrium between generation of ROS and its neutralization by antioxidant enzymes get disrupted, it led to oxidative stress and tissue damage[17]. Oxidative stress can lead to protein damage by its degradation and modifications[18]. This results in the accumulation of end products of lipid peroxidation and protein degradation in cells. In general, oxidative stress along with lipid peroxidation and protein degradation can induce various pathological conditions such degenerative illness, inflammation and aging, eventually leading to apoptosis of cell[19]. Thus, protein analysis and lipid peroxidation rate are considered as an indication of level of toxicity at cellular level.

Even at a very low concentration, pesticides show effect on antioxidant and detoxifying enzymes that reduce oxidative stress[20]. Antioxidant enzymes are the first line of defence mechanism against oxidative stress. Superoxide Dismutase (SOD) and Catalase (CAT) are two important antioxidant enzymes that helps in the dismutation of free radical, superoxide anion, generated during metabolism. SOD converts two molecules of superoxide anion to hydrogen peroxide and a molecular oxygen, while CAT convert hydrogen peroxide to water and molecular oxygen[21]. Thus, any variation in the activity of SOD and CAT by quinalphos exposure can be considered as a severe damage to the antioxidant enzyme system.

2. Materials and Methods

The fish were collected from the near farms and were kept in the laboratory conditions and 73 similar sized fishes were selected for the acute toxicity test. They were allowed to acclimatize in the laboratory conditions for 14-21 days. The fish tanks were properly aerated and the fish were regularly fed during this period.

2.1 Experimental Animal

Oreochromis niloticus (Tilapia) with an average weight of 4.5 ± 1.5 g and 5.2 ± 1.2 cm in length were selected and kept in tanks filled with 10 L of water. The experiment was explained and presented before the doctoral committee and was approved by the chair.

2.2 Chemical

Quinalphos 25% EC (O, O- Diethyl O-(quinoxalin -2-yl) phosphorothioate) was purchased from a nearby retail shop. The chemical was kept in room temperature before the application on fish.

2.3 Method of Exposure

The pesticide was introduced to the fish, by dissolving it to the water in which they are kept. Dose selection was done by conducting pilot studies on the fish. The responses such as mortality and behavioural changes prior to the application of pesticides in various concentrations were observed for few months before the initiation of experiment.

2.4 Evaluation of Lethal Concentration (LC_{50})

The LC_{50} value was determined by probit analysis with 5% confident limit[22]. The acclimatized fish were transferred into six aquarium tanks. They were kept without feeding for 24 hours before the test was done. This avoids contamination of test water with excess fecal and food discharge after administration of toxin. Ten fish were placed in each aquarium tank filled with 10 L of water. Six different concentrations of quinalphos were added (0.5 µl/L, 1.75 µl/L, 3 µl/L, 4.25 µl/L, 5.5 µl/L, 6.25 µl/L) with a difference of 1.25 µl/L. These concentrations were fixed

after multiple trials and pilot study conducted before the experimentation. The fish were exposed to the pesticide for 96 hours. Observation was recorded on every 24 hours for 4 days. Fish mortality and variation in behaviours were observed and recorded. A set of fish were also kept as control batch. The observations were computed according to the method of calculation of probit analysis.

2.5 Estimation of Protein

The sub-lethal concentrations of quinalphos ($1/5^{th}$ and $1/10^{th}$ of LC_{50}) was used to determine the effect on protein and lipid peroxidation. Brain and muscle tissues were used for the analysis. Protein estimation was done using Lowry's method. The principle involved in this method is to determine the protein concentration by measuring the blue colour developed upon the reduction of phosphomolybdic phosphotungistic component in the Folin-Ciocalteau reagent by the amino acid tyrosine and tryptophan in the protein and reactivity of peptide nitrogen with copper [II] ions under alkaline conditions. For the analysis, 0.1ml of tissue homogenate was taken and made up the volume to 1 ml. 5 ml of alkaline copper solution was added to each tube including the blank (water), mixed well and incubated at 37˚C for 3 minutes. Then 0.5 ml of Folin-Ciocalteau reagent was added, mixed well and incubated at 37˚C for 30 minutes. A blue colour was developed and its absorbance was measured at 660 nm[23].

2.6 Determination of Lipid Peroxidation Rate

Lipid peroxidation rate was analysed by Malondialdehyde (MDA) assay[24]. MDA is the by-product of lipid peroxidation, can react with Thiobarbituric Acid (TBA) to generate MDA-TBA adduct. This adduct can be easily quantified spectrophotometrically. The tissue was homogenized in 0.1 M Tris – HCl buffer, pH 7.5 and allowed to stand for 5 minutes. 1ml of tissue homogenate was combined with 2 ml of TBA reagent and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. After cooling the flocculent, precipitate was removed by centrifugation at 1000 rpm for 10 minutes. Absorbance of the sample was read at 535 nm against the blank that contain no tissue homogenate.

2.7 Estimation of SOD Activity

Superoxide dismutase assay is used for the estimation of SOD[25]. The reduction of Nitro Blue Tetrazolium salt (NBT) by superoxide anion has been utilized as the basis of assays for superoxide dismutase, which exposes its presence by inhibiting the reduction of NBT. SOD assay is based on principle that NBT undergo reduction (which is a blue-coloured formazan) by superoxide radicals. It competes with enzyme SOD for superoxide anions. In the presence of SOD in reaction mixture, NBT will produce less amount of coloured complex than control. 50 µl of tissue samples were added to the reaction mixture containing 50 mM phosphate buffer (7.8), 45 µM methionine, 5.3 mM riboflavin, 0.1 M NBT and 84 µM potassium ferric cyanide. This anion can change NBT to formazan, but SOD inhibits this reaction. In the presence of SOD, NBT will produce less amount of coloured complex than control. Thus, inhibition of NBT is measured to estimate SOD. The tubes were then incubated at 25°C for 10 minutes and the absorbance was read on spectrophotometer at 600 nm.

2.8 Estimation of CAT Activity

Catalase was assayed according to the method of Takahara *et al*[26]. To 1.2 ml of 0.01 mM phosphate buffer (pH 7.0), 0.5 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of 0.2 mM hydrogen peroxide (H_2O_2) solution. Catalase convert H_2O_2 to water and oxygen. The decrease in absorbance was measured at 240 nm for every 30 seconds up to 3 minutes. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

2.9 Statistical Analysis

The median lethal concentration was calculated using MS-excel and the best fit line graph of concentration (Log C) on x-axis and probit value of mortality on y-axis was obtained. LC_{50} value for 96 hours was determined.

3. Result

Quinalphos is an organophosphate pesticide commonly used in agriculture. It is known to cause inhibition of cholinesterase enzyme[27]. In the present study, the fish were continuously observed and mortality was recorded in fixed time interval. It was observed that after 24 hours of application of pesticide, fish death was noted

Table 1. Probit analysis of median lethal concentration after 96 hours of observation

Con(µl/l)	Log (con)	Alive	Dead	Prop p	Corrected p	Probit
0		9	1	0.1		
0.5	–0.30103	9	1	0.1		
1.75	0.24	8	2	0.2	0.11	3.78
3	0.48	5	4	0.4	0.38	4.70
4.25	0.63	4	6	0.6	0.56	5.14
5.5	0.74	2	8	0.8	0.78	5.76
6.75	0.83	0	10			
Slope	3.86					
Intercept	2.83					
Test value	5.00					
Log c	0.56					
LC 50	3.65					

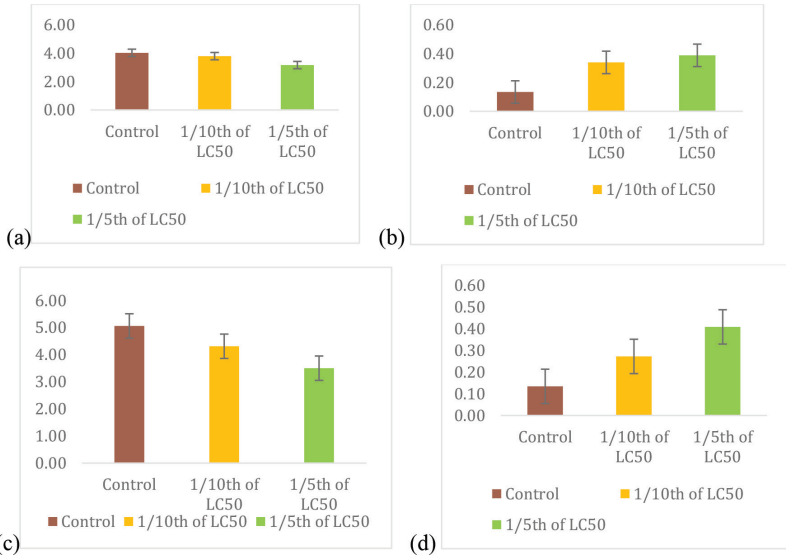


Figure 1. Graphs showing variation in protein concentration and lipid peroxidation rate in brain and muscle tissues after 24 hours of exposure to quinalphos (n = 9).
The units of variants at y- axis : protein concentration in mg and lipid peroxidation rate in *µg MDA units/mg of protein*
(a) Variation in protein concentration of brain tissues after exposure to quinalphos for 24 hours.
(b) Variation in lipid peroxidation rate of brain tissues after exposure to quinalphos for 24 hours.
(c) Variation in protein concentration of muscle tissues after exposure to quinalphos for 24 hours.
(d) Variation in lipid peroxidation rate of muscle tissues after exposure to quinalphos for 24 hours.

in 3 µl/L, 4.25 µl/L, 5.5 µl/L and 6.75 µl/L concentrations. Aquarium tanks treated with 1.75 µl/L of pesticide had fish death after 48 hours of the exposure while 0.5 µl/L of pesticide treated tank had fish death only after 72 hours of pesticide application. From the result obtained using probit analysis (Table 1), LC_{50} value of quinalphos on *Oreochromis niloticus* was determined for 96 hours.

After exposure to the pesticide, the fish showed disturbance. Fish showed active aggressive swimming soon after the application of pesticide indicating their

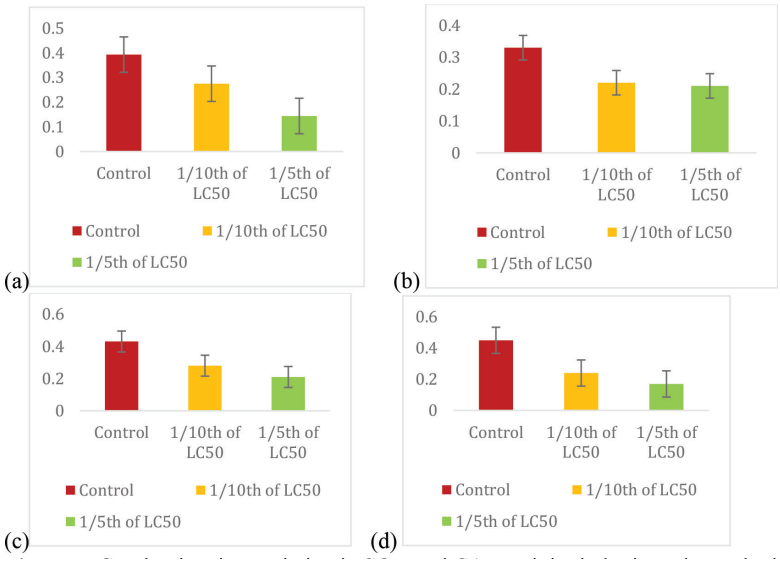


Figure 2. Graphs showing variation in SOD and CAT activity in brain and muscle tissues after 24 hours of exposure to quinalphos (n = 9).

The units of variants at y-axis : SOD activity in *enzyme units/mg of protein* and CAT activity in *enzyme units/mg of protein*.

(a) Variation in SOD activity of brain tissues after exposure to quinalphos for 24 hours.

(b) Variation in SOD activity of muscle tissues after exposure to quinalphos for 24 hours.

(c) Variation in CAT activity of brain tissues after exposure to quinalphos for 24 hours.

(d) Variation in CAT activity of muscle tissues after exposure to quinalphos for 24 hours.

response, to avoid the toxin. But after few minutes of exposure the swimming behaviour got restricted and they settle down at the bottom of the tanks. The pigmentation of scales got darker after the exposure in all the concentrations. They also showed quick opercular and fin movements which later become feeble. The opercular opening were wider and showed respiratory discomfort by often gulping of water. As the period of exposure increased, fish were found to settle down to bottom and become lethargic. They also showed loss of equilibrium. Fish in the control group were normal throughout the experiment.

Biochemical parameters such as protein and various stress related enzymes showed variation in activities upon exposure to quinalphos in sub-lethal concentrations[28,29]. Organophosphates can induce generation of ROS and lead to lipid peroxidation of cell membrane. In this study, protein concentration was found to be decreasing with increase in concentration of pesticide after 24 hours of exposure in both brain and muscle tissues. Meanwhile lipid peroxidation rate was increasing with increase in pesticide concentration (Figure 1). In the case of antioxidant enzymes, SOD and CAT activity showed decrease with increase in concentration of pesticide in both brain and muscle tissues of *Oreochromis niloticus* after 24 hour of exposure (Figure 2).

4. Discussion

In the present study, the fish were monitored for variation in physiology and behaviour. Application of pesticide showed abnormal behaviour in all fish despite the variation in concentration of chemical. Behavioural responses were used to assess the impact of a toxic substance in toxicology. This provides the physiological and behavioural changes upon exposure to the toxicant. Behaviour helps an organism to adjust and overcome the effects of internal and external stimuli[30]. Organophosphates are known for their ability to disrupt acetylcholinesterase activity which explains the alternation in swimming behaviour and lethargic nature of the fish[31,32].

The observations also suggest that very small concentration of the pesticides can have severe behavioural as well as physiological changes in fishes.

Death was recorded earlier when the concentration of pesticide was higher, which indicate the toxicity level of the pesticide. Fish death might have occurred due to any neurological defects and interruption in nerve function or may be due to respiratory failure[33]. Exposure to pesticide can cause serious alternations in biochemical parameters along with tissue and organ damages[34].

Proteins are the common biomolecular target of hazardous effect of pesticides. Exposure to quinalphos by *Channa punctatus* showed decreased protein content in liver, muscle, kidney, intestine, brain and gills[35]. The reduction in the protein content immediately after the exposure might be due to impairment in protein synthesis, damaged tissues and disturbance of cellular contents[36]. Under stressed conditions, proteins may act as a source of energy since the organism need to eliminate the toxicant that cause the stress[37]. Thus, it was concluded that exposure to sub-lethal doses of quinalphos can affect protein metabolism. Pesticides affect the protein by the generation and accumulation of protein carbonyls in animal tissues. Thus, various studies are being conducted to detect the biomarkers of protein damage[38,39]. Varieties of pesticides such as organophosphates, carbamates, organochlorines as well as pyrethroids have caused oxidative damages to protein[40,41].

Exposure to sub-lethal dosage of pesticide Roundup in *Prochilodus lineatus* for 96 h increased the MDA in the fish liver by three folds[12]. In *Catla catla,* following 96 h of exposure to acute and sublethal concentrations of methyl parathion caused increase in lipid peroxidation levels in gills and plasma. Significant elevation in lipid peroxidation may have contributed to imbalance in antioxidants and metabolic enzymes along with severe cellular and tissue damage[42]. Treatment of *Astyanax aeneus* fish with sublethal concentrations of ethoprophos also caused increase in lipid peroxidation rate[43]. Acute exposure of *Channa punctatus* to atrazine for 15 days resulted in elevation of lipid peroxidation in liver tissues[13]. Important fact is that, in all these findings it is consistently proved the ability of pesticide to cause oxidative damages on non- target aquatic organisms especially fish and eventually demonstrate human being as vulnerable. Exposure to acute concentrations of terbuthylazine resulted in significant increase in lipid peroxidation and De-Oxy Ribonucleic Acid (DNA) uncertainty in human blood samples[44].

Many organophosphate pesticides are known to cause impairment in antioxidant defence system[45-47]. Studies conducted on various organisms by organophosphate pesticide exposure showed various effects on different tissues. For example, a study conducted on *Oreochromis niloticus* showed that dimethoate, an organophosphate pesticide caused increased activity of SOD and LPO while decreased CAT activity in liver tissue. At the same time, LPO showed increased activity and antioxidant enzymes showed decreased activity in kidney tissues[48]. Exposure of quinalphos showed increased SOD and CAT activity in the liver tissues of *Cyprinus carpio*[28]. Sub-lethal concentration of chlorpyrifos caused decline in the activity of SOD and CAT in the liver, kidney and gill tissues of *Ctenopharyngodon idellus*[49]. Though antioxidant enzymes show diverse effects upon exposure to various organophosphate pesticide, here in this study, sub-lethal treatment of quinalphos resulted in the reduction of activity of both SOD and CAT enzymes of *Oreochromis niloticus*.

5. Conclusion

Pesticides have become an unavoidable chemical in the agriculture of most of the developing countries despite the hazardous effects it can cause on non-target organisms. Quinalphos is a widely used organophosphate pesticide that have effects on nervous system of an organism. Pesticides can have different levels of toxicity in organisms depend on age, sex, size and exposure time. Since, the usage of pesticides in the agriculture has become mandatory, the only way to avoid the consequence of toxicity is by minimizing its application. The above experiment demonstrates the lethal concentration of quinalphos that can cause severe effects on non- target organisms. By using a common cultivable fish as a test organism, the study represents the effects, pesticides can cause on vertebrates and also indicates a way of its entry to food chain. Generally, increase in lipid peroxidation could be related to uncontrolled ROS generation and impaired antioxidant system in fish. Both oxidative stress and ROS contribute to the initiation of apoptosis, results in the damage of protein and lipids, leading to cell death or inflammatory responses of cells. Thus, it is an indication that oxidative stress caused by pesticides in non-target organisms, could be the commencement of many severe health conditions including cancer. So, this type of studies could be considered as a warning to the health hazard, that may result, not just in aquatic organisms but also in other non-target organisms including humans.

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7. References

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