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Behavioral Changes by Inhibition of Acetylcholinesterase Induced by Trizole (Propiconazole) Fungicide on Freshwater Fish *Clarias batrachus*

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Abstract: The fungicide PCZ (propiconazole) is widely used in agriculture for the production of vegetable crops. Because of its physical and chemical properties, the small concentration of PCZ in water bodies "habitat of aquatic flora and fauna" make it sever in conditions for survival. According to the obtained data from the present study, the central theory that environmentally relevant concentrations of PCZ affect acetylcholinesterase (AChE) activity in fish. The conclusion also holds that the theories such as metabolites of trizole convert the enzyme activities also. The LC values, (LC_{10}, LC_{50} and LC_{90}) were estimated on fish which was dose as well as time dependent. The exposure to sub-lethal concentration of PCZ *in vivo* assessment was studied after 24h and 72h during exposure with 80% and 40% of LC_{50} (1.11mg/l and 2.23mg/l respectively). Significant ($p<0.05$) inhibition was observed in AChE in nerve tissue of fish and behavioral changes also observed, very little changes in activity of AChE was observed in muscle, tissue of fish. The study shows that PCZ have potential to damage aquatic ecosystem. Therefore, we can say that this fungicide should be avoided in near water bodies.

Key words: PCZ (Propiconazole) · *In vivo* · Flora and Fauna · Acetylcholinesterase (AChE)

INTRODUCTION

For centuries, pesticides have been used in agriculture to enhance food production by eradicating unwanted insects and controlling disease vectors [1]. Unfortunately, the use and abuse of pesticides has surfaced that it not only lethal to targeted organisms but also sub-lethal to non-targeted organisms including Man also. Fishes serve as important bio-indicators for aquatic contamination. Indian fishery contributing about one third of total fish production of the country and share about 95% of total aquaculture production. Therefore, aquaculture as an enterprise has some innate advantages, such as high returns, high productivity, high feed conversion ratio, utilization of agriculture and animal wastes, high employment generation etc [2]. Recent evidence indicates that fish, which is the most important fauna, are quickly becoming scarce. One consequence of this scarcity is the increasing use of chemical pesticides in fields. Pesticides are among the most hazardous chemicals to men. Chemical pesticides with persistent molecules (long half-life periods) pose a threat to fish and also to the human population consuming the affected fish [3].

Fungicides are extensively used to protect agricultural crops against the damages caused by pests. Trizole containing fungicides was used as antifungal in agriculture for controlling pest and also for increasing food crops [4]. Propiconazole induced severe effects on hepatic nuclear receptor activation, hepatic hypertrophy, cytochrome P450s induction, cell proliferation, all-trans retinoic acid level and on serum cholesterol levels [5] in organisms. Propiconazole is a number of DMI (demethylation inhibitors) group with rapid acropetal systemicity. It acts on the pathogen inside the plant to stop disease development by interfering with sterol biosynthesis in fungal cell membrane. The foliar systemic fungicides propiconazole (PCZ), had chemical formula 1-(2-(2, 4-dichlorophenyl)-4- propyl-1, 3-dioxolan-2-ylmethyl)-1H-1,2,4-trizole. They have a shorter half-life and lower bioaccumulation but announced effects on the aquatic ecosystems may arise from spray drift or surface run-off [6]. They have been reported to undergo transformation of secondary metabolites in terrestrial

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mammals [5]. Series of study shows that the PCZ altered the metabolic pathways, cell signaling, cell growth pathways, cell cycle genes and other transcriptional factors [7].

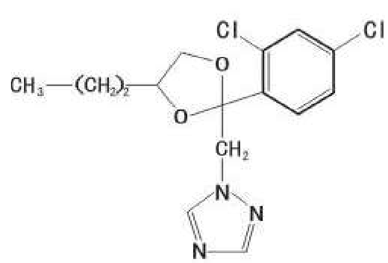
Effect of toxicants on enzymatic activity is one of the most important biochemical parameters, which affected under stress. When an organ is diseased due to the effect of a toxicant, enzyme activity appears to be increased or it may be inhibited due to the active site being either denatured or distorted. Acetylcholinesterase, or acetyl-hydrolase, is a serine protease that hydrolyses the neurotransmitter acetylcholine. AChE is found at mainly neuromuscular junctions and cholinergic brain synapse, where its activity serves to terminate synaptic transmission. It belongs to carboxylesterase family of enzymes. Inhibition in AChE may lead to muscular paralysis, bronchial constriction and death by asphyxiation in fish.

However, the toxic effects of PCZ on fishes have not been adequately researched. The aim of present study is to evaluate the toxicity and the effect of sub-lethal doses of PCZ to analyze the enzymatic responses is freshwater fish *Clarias batrachus*, is an important fish of Indian capture fishery.

MATERIALS AND METHODS

Chemical: Propiconazole (PCZ) (CAS No. 60207-90-1) has purchased from Syngenta Ltd. from India, a technical grade pesticide. Other chemicals such as Giemsa stain has purchased from local Indian market.

Chemical Structure of PCZ



Experimented Animals: The freshwater fish *Clarias batrachus* obtained from local hatchery (Chappy Hatchery). Fish had an average weight of 40.01±1.50g and average length 17.26±0.10cm for adult and for fingerlings, the average weight had 10.20±1.45g and average length 8±0.10cm. Fish were fed with commercial fish food and acclimatized under laboratory conditions for 2 weeks, containing de-chlorinated tap water (pH= 7.6, alkalinity 150 mg/l $CaCO_3$, DO= 7.03 mg/l, Temperature=22°C). The photoperiod used 12/12 dark/light. The aquaria used in study was made of glass had constant aeration. Physical dimensions of aquarium were 100×40×40 cm and a 120 L capacity.

Toxicity Test: Toxicity test has performed by the method of Singh and Agarwal, [8]. Five fishes kept in glass aquaria containing 10 L de-chlorinated tap water and this experiment replicated six times. Fish exposed for 24h to 96h to four different concentrations (1.7 mg/l, 2.0 mg/l, 2.3 mg/l and 2.5 mg/l for Propiconazole) of pesticides in laboratory. Control fish kept in similar conditions without any treatment. Each group of fish replicated three times. Mortality recorded after every 24h. Dead animals removed to prevent the decomposition of body in experimental aquaria. The effective doses (LC values, upper and lower confidence limits and slope value) calculated by probit log method of Russel *et al.* [9]. LC values calculated by POLO programmed. Product moment correlation co-efficient was applied in between exposure time and lethal concentration [10].

RESULT

Acute toxicity: After treatment, all the experimented fishes immediately settled down at the bottom of aquarium. Within 5-10 min, the breathing of fishes affected and they came to the water air interface for air breathing, the respiratory impairment, probably due to the effect of the fungicides on gills and general metabolisms. After 30-60 min, their swimming activity is also slow down. During exposure the loss of equilibrium, hypo and hyperactive activity and vertical position observed after 48h. Finally, their activity ceases and fishes died. LC values of PCZ for period ranging from 24-96h on fish *Clarias batrachus* presented in (Table 1). The toxicity in both the cases was time as well as dose dependent. There was a significant negative correlation between LC values and exposure periods. Thus with an increase in exposure period the values of LC of PCZ decreased.

Acetylcholinesterase Activity: After 24h exposures to 40% and 80% of 24 h LC_{50} of Propiconazole (Trizole), Acetylcholinesterase (AChE) activity was decreased to 68% and 44% in nervous tissues. After 72h, decrement in AChE activity reached up to 61% and 33% in nervous tissues at 40% and 80% of 24 h LC_{50}, respectively (Table 2 and Figure 1).

Table 1: Pisicidal activity of fungicide Propiconazole against fresh water fish *Clarias batrachus* at different time intervals

Exposure periods	Effective doses (mg/L)	Slope
	LC_{10} = 2.44 (2.14-2.55)	
24h	LC_{50} = 2.79 (2.68-3.01)	21.84±6.13
	LC_{90} = 3.19 (2.97-3.99)	
	LC_{10} = 2.16 (1.68-2.33)	
48h	LC_{50} = 2.59 (2.44-2.74)	16.51±4.84
	LC_{90} = 3.09 (2.87-3.98)	
	LC_{10} = 2.08 (1.64-2.24)	
72h	LC_{50} = 2.39 (2.22 -2.50)	20.71±5.74
	LC_{90} = 2.76 (2.63-3.14)	
	LC_{10} = 2.11 (1.75-2.24)	
96h	LC_{50} = 2.35 (2.19-2.44)	26.72±7.31
	LC_{90} = 2.63 (2.53-2.89)	

- Batches of fifteen fishes exposed to four different concentrations of the fungicides.
- Concentrations given are the final concentrations (v/v) in the aquarium water containing de-chlorinated tap water.
- Values given in parenthesis are lower and upper confidence limits of LC value.
- Negative correlation coefficient found between the product moment of LC values and exposure periods

Table 2: Acetylcholinesterase (AChE) activity (µM 'SH' hydrolyzed/min/mg tissue) in muscle and liver tissues of freshwater fish *Clarias batrachus* after 24h and 72h exposure to 40% and 80% of 24 h LC_{50} of Propiconazole (Trizole)

Pesticide	Tissue	Exposure Period	Control	40% of LC_{50}	80% of LC_{50}
Propiconazole (Trizole)	Nervous	24h	0.049±0.021(100)	0.033±0.014*#(68)	0.021±0.009*#(44)
		72h	0.049±0.021(100)	0.029±0.013*#(61)	0.016±0.007*#(33)
	Muscles	24h	0.087±0.016(100)	0.073±0.032*#(84)	0.069±0.030*#(80)
		72h	0.084±0.018(100)	0.068±0.030*#(82)	0.064±0.028*#(77)

- All these assay replicates six times and the values are mean ±SE of all the replicates.
- Values given in the parenthesis are percent values with control taken as 100%.
- *, Significant (P<0.05) when Student's 't' test was applied between treated and control groups.
- #, Significant (P<0.05) when Two-way ANOVA was applied between treated and control groups.

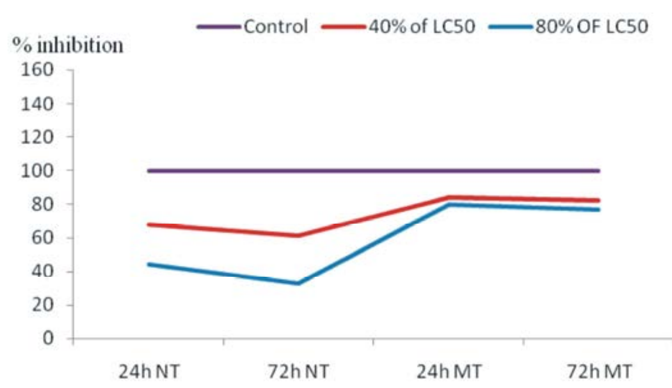


Fig. 1: Percent Acetylcholinesterase (AchE) activity in muscles and liver tissues of the freshwater fish *Clarias batrachus* after exposure to 40% and 80% of LC_{50} (24h) of Propiconazole (Trizole).

DISCUSSION

In tissues, propiconazole cleaved into propyl side chain and dioxolane ring structure. It metabolites into 2-4-dichlorophenyl, 1,2,4-trizole, trizole alanine ring, trizole acetic acid, trizole pyruvic acid and trizole lactic acid which was the metabolites of the PCZ. In which 1,2,4-trizole and trizole alanine are main metabolites of trizole containing fungicides. These metabolites may be conjugates with different metabolic pathways in body of fish and showed maximum effects.

In present study Acetylcholinesterase activity was decreased in nervous tissues of the fish *Clarias batrachus* after exposure to different sub-lethal doses of

PCZ. During neurotransmission, ACh is released from the nerve into the synaptic cleft and binds to ACh receptors on the post-synaptic membrane, relaying the signal from nerve. AChE, also located on the post-synaptic membrane, terminate the signal transmission by hydrolyzing ACh. The liberated choline is taken up again and ACh is synthesized by combining with acetyl-CoA through the action of choline acetyl-transferase. To receive another impulse, ACh must be released from the ACh receptor. This occurs when the concentration of ACh in the synaptic cleft is very low. Inhibition of AChE leads to accumulation of ACh in the synaptic cleft and results in impeded neurotransmission. AChE- inhibitors block the normal breakdown of the neurotransmitter, acetylcholine into acetic acid and choline. They do it by blocking the site where acetylcholine attaches the enzymes. In normal condition, ACh attaches to the serine hydroxyl group on AChE and PCZ may have high affinity to conjugate with serine and form enzyme-inhibitor complex. This prevents acetylcholine from interacting with cholinesterases enzyme and being break down. This leads to the buildup of excessive levels of neurotransmitter ACh at the neuromuscular junctions. Due to this nerves collapse in brain of fish that leads to behavioral changes. Behavior is subject of vast study and all aspects of behavior are not well established. It has been found that at every movement, animal showed a varied behavioral response, for its own survival and perhaps for evolutionary adaptation [11]. In the present study, PCZ shows significant behavioral changes in fish (hyperactive movement, hypo movement, vertical position and loss of equilibrium). Behavior provides a unique perspective linking the physiology and ecology of an organism and its environment [12]. Behavioral action is a sequence of quantifiable actions, which operated through the central and peripheral nervous systems [13] and the cumulative manifestation of genetic, biochemical and physiologic processes essential to life such as feeding, reproduction and predator avoidance. For the best meet of the challenge of surviving in a changing environment, behavior allows an organism to adjust to external and internal stimuli in order to adapted environmental variables. Since behavior is not a random process, but instead of it, is a highly structured and predictable sequence of activities designed to ensure maximal fitness and Survival of the individual. Alterations in fish behavior, particularly in non-migratory species, also provide important indices for ecosystem assessment. Hindrance in acetylcholineterase activity during fungicide exposure caused disruption in the learned behavior in fish that affect the survival of fish and ultimately lead mortality. Nagaraju *et al.* [14] analyzed the disruption in schoolings behavior in fish *Labeo rhotia*. Torre *et al*. [15] reported Cyprinus carpio and Cnesterodon decemmaculatus were highly sensitive to pollutant and showed reduced level of acetylcholinesterase.

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