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Study of *In-Vivo* Effects Caused by Metabolites (1,2,4-Trizole Alanine) of Steroid-Inhibitor Fungicide on Aquatic Life (Fish)

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Abstract

The fungicide PCZ (propiconazole) is widely used in agriculture especially in Asian contries as India, China ect. for the production of vegetable crops. Because of its physical and chemical properties , the small concentration of PCZ in water bodies "habitate of aquatic flora and fauna" make it sever in conditions for survivle. On the basis of analysis of all data in present study, the central theory that environmentally relevant conentrations of PCZ affect biochemical parameters in fish. The conclusion also hold that the theories such as metabolites of trizole convert the enzyme activities also. The LC values (LC_{50}) estimate on different life stages of fish that was dose as well as time dependent. The exposure of sub-lethal concentration of PCZ *in vivo* assessment studied after 24 h and 72 h during exposure with 40% and 80% of LC_{50} (0.56 mg/l, 1.12 mg/l for fingerlings respectively) & (1.11 mg/l, 2.23 mg/l for adults respectively). Protein, Amino acids, Glycogen, Nucleic acids and enzyme succinic dehydrogenase decreased in liver and muscles, but lactic dehydrogenase levels, Protease, GOT and GPT increased in the both tissues. The study shows that PCZ have potential to damage aquatic ecosystem. Therefore, we can say that this fungicide should avoid in near water bodies.

Keywords: PCZ (Propiconazole); *In Vivo*; Flora and Fauna; Biochemical parameters

Introduction

Trizole containing fungicides are used as antifungal in agriculture for controling pest and also for increasing food crops [1,2]. 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 [3-5] in organisms.

Propiconazole is a number of DMI(demethylation inhiitors) 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 reported to undergo transformation of secondary metabolites in terrestrial mammals [7]. Series of study shows that the PCZ altered the metabolic pathways, cell signaling, cell growth pathways, cell cycle genes and other transcriptional factors [8,9]. Nevertheless, the toxic effects of PCZ on fishes have not adequately researched.

The aim of present study is to evaluate the toxicity and the effect of sub-lethal doses of PCZ to analyze the biochemical, physiological and enzymatic responses is fresh-water fish *Clarius batracus*, is an important fish of Indian capture fishery.

Materials and Methods

Chemical

Propiconazole have local name Tilt is a systemic fungicide purchased from Syngenta Ltd. from India, a technical grade pesticide.

Experimented animals

The fresh-water fish *Clarius batracus* (total average size 12-17 cm and average weight 35-50 g) for adult and for fingerlings (total average size 6-8 cm and average weight 9-12 g) brought from local fresh-water pond. They were stored in laboratory tank containing 100 liters of de-chlorinated tap water and acclimatized to the laboratory conditions for 72 h.

Experimental design

Toxicity test perform by the method of Singh and Agarwal [10]. Five fishes kept in glass aquaria containing 10 L de-chlorinated tap water. Fish exposed for 24 h to 96 h to four different concentrations of pesticides in laboratory. Control fish kept in similar conditions without any treatment. Each group of fish replicated three times. Mortality recorded after every 24 h. Dead animals removed to prevent the decomposition of body in experimental aquarium. The effective doses (LC values, upper and lower confidence limits, slope value, and heterogeneity) calculated by probit log method. Product moment co-relation co-efficient was applied in between exposure time and lethal concentration (Sokal and Rohef) [11].

Tissue preparation

Fishes exposed to 40% and 80% of 24 h LC_{50} doses (1.11 mg/l, 2.23 mg/l respectively). Experiment conducted from 24 h to 72 h. After completion of treatment the test, fishes were removed and washed with water, killed by severe blow on head, and operated their liver and muscles quickly dissected out in ice tray and used for biochemical and enzymatic analyses. Control fishes kept in similar condition without any treatment. Each experiment replicated at least 6 times and values expressed as mean ± SE of six replicates. Following parameters tested by different methods.

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Protein level estimated according to the method of Lowery et al. [12] using bovine serum albumin as standard. Estimation of total free amino acids made according to the method of Spices [13]. Estimation of DNA and RNA performed by method of Schneider [14] using diphenylamine and orcinol reagents respectively. Glycogen estimated by Anthrone method of Van Der Vies [15].

Lactic dehydrogenase, the method of Anon [16]. Succinic dehydrogenase, the method of Arrigoni and Singer [17]. Protease, method of Moore and Stein [18]. GOT and GPT, method of Reitman and Frankel [19]. Two ways ANOVA performed between control and tested group. The significant level was 0.05.

Results

Study shows, PCZ cause announce alteration in behavior and metabolic anomalies such as level of glycogen, protein, amino acids, nucleic acids and enzymes like Lactic dehydrogenase, Succinic dehydrogenase, Protease, GOT and GPT (Glutamic oxalic trasaminase and Glutamic pyruvate transaminase). The toxicity data is presented in Table 1 and biochemical and enzymatic values of 40% & 80% of LC_{50} (0.56 mg/l, 1.12 mg/l) presented in Tables 2 and 3.

After exposures of 40% of LC_{50}, (0.56 mg/l) the level of protein is depleted 85% in muscles tissue, 80% in liver at 24 h, 68% in muscle tissue, and 60% in liver at 72 h. While amino-acids level is increases 118% in muscles and 115% in liver at 24 h and 125% in muscles and 120% in liver at 72 h. Glycogen level become reduces 78% and 65% in muscle and liver respectively at 24 h, it progressively decreases 59% and 62% in muscle and liver respectively at 72 h. The trend of decrement also observed in DNA, RNA and level of SDH activity. It becomes 77%, 75%, 76%, 73%, 65% and 63% in muscles and liver respectively at 24 h and 62%, 60%, 65%, 62%, 66% and 58% in muscles and liver respectively at 72 h. The enzymetic level increase at 24 h and 72 h in both tissues. Protease becomes 115% and 130% in muscle and liver at 24 h and 125% and 138% in muscles and liver at 72 h. While Lactic dehydrogenase level is increases 112% in muscles and 113% in liver at 24 h and 116% in muscles and 120% in liver at 72 h. The transaminase reactivity also shows increase level in muscle and liver. GOT becomes 120% and 128% in muscle and liver at 24 h and 124% and 135% in muscles and liver at 72 h. GPT also increase 115% in muscles and 124% in liver at 24 h and 119% in muscles and 130% in liver at 72 h.

After exposures of 80% of LC_{50}, (1.12 mg/l) the trend is similar as level of protein is depleted 78% in muscles tissue and 71% in liver at 24 h and 62% in muscle tissue and 55% in liver at 72 h. While amino-acids level is increases 123% in muscles and 120% in liver at 24 h and 135% in muscles and 140% in liver at 72 h. Glycogen level become reduces 68% and 52% in muscle and liver respectively at 24 h, it progressively decreases55% and 49 % in muscle and liver respectively at 72 h. The trend of decrement also observed in DNA, RNA and level of SDH (Succinic dehydrogenase) activity. It becomes 70%, 68%, 72%, 66%, 60% and 57% in muscles and liver respectively at 24 h and 58%, 56%, 59%, 57%, 55% and 52% in muscles and liver respectively at 72 h. The enzymetic level increase at 24 h and 72 h in both tissues. Protease becomes 120% and 136% in muscle and liver at 24 h and 132% and 143% in muscles and liver at 72 h. While LDH (Lactic dehydrogenase) level is increases 118% in muscles and 120% in liver at 24 h and 125% in muscles and 130% in

Exposure periods	Effective doses (mg/L)	Slope	Effective doses (mg/L)	Slope
	Fingerlings		Adult Fish	
	LC_{10}=0.84 (0.26-1.06)		LC_{10}=2.44 (2.14-2.55)	
24 h	LC_{50} =1.40 (1.19-1.74)	5.75 ± 2.06	LC_{50}=2.79 (2.68-3.01)	21.84 ± 6.13
	LC_{90}=2.34 (1.83-2.69)		LC_{90}=3.19 (2.97-3.99)	
	LC_{10}=0.80 (0.32-1.01)		LC_{10}=2.16 (1.68-2.33)	
48 h	LC_{50}=1.28 (1.05-1.46)	6.35 ± 2.08	LC_{50}=2.59 (2.44-2.74)	16.51 ± 4.84
	LC_{90}=2.04 (1.69- 4.39)		LC_{90}=3.09 (2.87-3.98)	
	LC_{10}=0.72 (0.29-0.92)		LC_{10}=2.08 (1.64-2.24)	
72 h	LC_{50}=1.10 (0.81-1.24)	7.03 ± 2.17	LC_{50}=2.39 (2.22 -2.50)	20.71 ± 5.74
	LC_{90}=1.68 (1.46-2.62)		LC_{90}=2.76 (2.63-3.14)	
	LC_{10}= 0.71 (0.31-0.89)		LC_{10}= 2.11 (1.75-2.24)	
96 h	LC_{50}=0.99 (0.71-1.12)	8.77 ± 2.66	LC_{50}=2.35 (2.19-2.44)	26.72 ± 7.31
	LC_{90}=1.39 (1.24-1.82)		LC_{90}=2.63 (2.53-2.89)	

Batches of fifteen fishes were 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 is found between the product moment of LC values and exposure periods

Table 1: Pisicidal activity of fungicide propiconazole against different stages of fresh water fish *Clarius batracus* at different time intervals.

Con.	Protein		Amino-acids		Glycogen		DNA		RNA	
	M	L	M	L	M	L	M	L	M	L
Control	107.14 ± 0.20 (100)	94.20 ± 0.10 (100)	60.33 ± 0.16 (100)	68.22 ± 0.12 (100)	142.34 ± 0.2 (100)	182.20 ± 0.2 (100)	139.35 ± 0.09 (100)	129.21 ± 0.12 (100)	100.45 ± 0.10 (100)	93.20 ± 0.12 (100)
LC_{50} 40% 24 h*	91.06 ± 0.12 (85)	75.36 ± 0.10 (80)	71.18 ± 0.15 (118)	78.45 ± 0.10 (115)	111.0 ± 0.20 (78)	118.4 ± 0.15 (65)	107.3 ± 0.12 (77)	96.75 ± 0.14 (75)	76.34 ± 0.10 (76)	68.03 ± 0.09 (73)
LC_{50} 80% 24 h*	83.56 ± 0.10 (78)	66.88 ± 0.15 (71)	74.20 ± 0.16 (123)	81.86 ± 0.20 (120)	96.79 ± 0.13 (68)	100.21 ± 0.10 (55)	97.54 ± 0.08 (70)	87.86 ± 0.17 (68)	72.32 ± 0.07 (72)	61.51 ± 0.08 (66)
LC_{50} 40% 72 h*	72.85 ± 0.15 (68)	56.52 ± 0.12 (60)	75.41 ± 0.20 (125)	81.86 ± 0.22 (120)	83.98 ± 0.16 (59)	112.96 ± 0.15 (62)	86.39 ± 0.10 (62)	77.52 ± 0.13 (60)	65.29 ± 0.08 (65)	61.51 ± 0.10 (66)
LC_{50} 80% 72 h*	66.42 ± 0.13 (62)	51.81 ± 0.14 (55)	81.44 ± 0.229 (135)	95.50 ± 0.15 (140)	74.01 ± 0.10 (52)	89.27 ± 0.18 (49)	80.82 ± 0.07 (58)	72.35 ± 0.10 (56)	59.26 ± 0.12 (59)	53.12 ± 0.13 (57)

Table 2: changes in total protein (µg/mg), total free amino acids (µg/mg), glycogen (mg/g), nucleic acids (µg/mg) level and activity of protease (tyrosine/mg protein/h), LDH (pyruvate reduced/min/mg protein), SDH (µmoles dye/min/mg protein), GOT (µmoles pyruvate/mg protein/h) and GPT (µmoles pyruvate/mg protein/h) in different tissues of fresh water fish *clarius batracus* exposure to **40% & 80% of LC_{50}** of PCZ at different time intervals.

Con.	Protease		LDH		SDH		GOT		GPT	
	M	L	M	L	M	L	M	L	M	L
Control	0.97 ± 0.011 (100)	1.03 ± 0.015 (100)	4.30 ± 0.16 (100)	7.35 ± 0.12 (100)	1.98 ± 0.02 (100)	2.01 ± 0.05(100)	3.9 ± 0.09 (100)	2.58 ± 0.07 (100)	1.45 ± 0.01 (100)	1.87 ± 0.06 (100)
40% of LC_{50} 24h*	1.11 ± 0.10 (115)	1.33 ± 0.12 (130)	4.81 ± 0.13 (112)	8.30 ± 0.10 (113)	1.28 ± 0.20 (65)	1.26 ± 0.15 (63)	4.6 ± 0.12 (120)	3.30 ± 0.14 (128)	1.66 ± 0.10 (115)	2.31 ± 0.09 (124)
80% of LC_{50} 24h*	1.16 ± 0.09 (120)	1.40 ± 0.15 (136)	5.07 ± 0.16 (118)	8.82 ± 0.20 (120)	1.18 ± 0.13 (60)	1.14 ± 0.10 (57)	4.9 ± 0.08 (127)	3.61 ± 0.17 (140)	1.74 ± 0.07 (120)	2.39 ± 0.08 (128)
40% of LC_{50} 72h*	1.21 ± 0.11 (125)	1.42 ± 0.10 (138)	4.98 ± 0.20 (116)	8.82 ± 0.22 (120)	1.30 ± 0.16 (66)	1.16 ± 0.15 (58)	4.8 ± 0.10 (124)	3.48 ± 0.13 (135)	1.72 ± 0.08 (119)	2.43 ± 0.10 (130)
80% of LC_{50} 72h*	1.28 ± 0.07 (132)	1.47 ± 0.13 (143)	5.37 ± 0.18 (125)	9.55 ± 0.15 (130)	1.08 ± 0.10 (55)	1.04 ± 0.18 (52)	5.1 ± 0.07 (133)	3.74 ± 0.10 (145)	1.78 ± 0.12 (123)	2.52 ± 0.13 (135)

Values given in parenthesis were percent change in parameters, *($p<0.05$) significant shows in all parameters

LDH=Lactic dehydrogenase,
SHD=Succinic dehydrogenase,
GOT=Glutamic oxalic transferase, GPT=Glutamic pyruvate transferase

Table 3: Changes in activity of protease (tyrosine/mg protein/h), LDH (pyruvate reduced/min/mg protein), SDH (µmoles dye/min/mg protein), GOT (µmoles pyruvate/mg protein/h) and GPT (µmoles pyruvate/mg protein/h) in different tissues of fresh water fish clarius batracus exposure to **40% & 80% of LC_{50}** of PCZ at different time intervals.

liver at 72 h. The transaminase reactivity also shows increase level in muscle and liver. GOT (Glutamic oxalic transaminase) becomes 127% and 140% in muscle and liver at 24 h and 133% and 145% in muscles and liver at 72 h. GPT (Glutamic pyruvate transaminase) also increase 120% in muscles and 128% in liver at 24 h and 123% in muscles and 135% in liver at 72 h.

Discussion

In tissues, propiconazole cleaved into propyl side chain and dioxolane ring structure. In liver and muscles tissues it metabolites into 2-4-dichlorophenyl ,1,2,4-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 alanine is main metabolite of trizole containing fungicides. These metabolites may be conjugates with different metabolic pathways in body of fish and showed maximum effects.

Alanine played most important role in glucose-alanine cycle between tissues and liver. The metabolite 1,2,4-trizole alanine after degradation may be entered into glucose-alanine cycle and induced the collection of amino groups in the form of glutamate by inducing transaminase reaction. This tend to increase in transaminase enzymes such as GOT (Glutamic oxalic transaminase) and GPT (Glutamic pyruvic transaminase) in tissues. In present investigation increase level of GOT and GPT have seen. However, some pesticides caused increased transaminase activity (GPT and GOT) levels in liver and muscle tissues. Begum [20] found the activity levels of GPT and GOT increased in liver and muscle tissues of *Clarias batrachus* during exposed to carbofuran. Murugesan et al. [21] also found that *Sarotherodon mossambicus*, when exposed to sublethal and lethal concentrations of carbaryl, showed adaptive elevation in the activity levels of GOT and GPT enzymes, particularly in liver and muscle.

Glutamate can transferred its amino groups to pyruvate by the action of alanine aminotransferase, which leads the glycolysis pathways in liver and muscles tissues. The metabolite 1,2,4-trizole alanine form N-(1H-1,2,4 trizole-3-yl)-glucopyranosylamine by the reaction of glucose1-PO_4 and affect the synthesis of oligosaccharides and polysaccharides. Present study showed the reduced value of glycogen in both tissues. Propiconazole treatment altered carbohydrate metabolism with the levels of a number of oligosaccharides reduced in a dose-response manner such as maltohexaose, maltopentaose, maltotetraose, maltotriose and maltose levels. Increase level of blood glucose concentration caused stress condition in fish respond to acute toxic effects of fungicide. Increase concentration of pesticides considered as reliable indicator of environmental stress [22]. Cicik and Engin [23] observed serum glucose increment in fish under stress condition. In present study, Hyperglycemia indicated disruption in carbohydrate metabolism by elevated breakdown of liver glycogen. Cypermethrin-induced hyperglycemia has been recorded in *L. rohita* [24] and in *S. schlegeli* [25].Stress is the condition of high energy demanding process and that energy used to cope with stress metabolically [26].The stress hormone cortisol has responsible to increase glucose production by glycolysis pathway in fish [27]. Borges et al. reported that cypermethrin-induced hyperglycema in *R. quelen* and associated with the increase in cortisol level in stress condition [28].

Stress condition shows behavior changes in organisms. Fish are ideal indicators for behavioral assays of various stressors and toxic chemicals exposure due to their constant, direct contact with the aquatic environment [29]. Behavior provide a unique perspective linking the physiology and ecology of an organism and its environment [30]. Behavioral action is in a sequence of quantifiable actions that operated through the central and peripheral nervous systems [31] 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 with external and internal stimuli in order to adapted environmental variables. Selective evolutionary processes have conserved stable behavioral patterns in concert with morphologic and physiologic adaptations [30]. 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. Fish are able to uptake and retain different toxicants dissolved in water via active or passive processes. Sub-lethal concentrations of pesticides in aquatic environments cause structural and functional changes in aquatic organisms and this is more common than mortality [32]. Behavioral modification is one of the most sensitive indicators of environmental stress and many affect survival [33]. Alterations in fish behavior, particularly in non-migratory species, can also provide important indices for ecosystem assessment. In the present study, PCZ shows significant behavioral changes in fish (hyperactive movement, hypo movement, vertical position and loss of equilibrium). Toxicity data clearly indicate that the fingerlings are more susceptible than adult fish due to the dependence of age and body size. During stress conditions, the glycogen reserves depleted to meet energy demand [34,35]. The freshwater fish, *Clarias batracus*, has reported to exhibit significant reduction in the level of glycogen Saha et al. studied effects of cypermethrin on various biochemical parameters [36].

PCZ in tissues degrade into different types of amino acids based metabolites so it incorporate with several polypeptide chains and altered its fate. The quantity of protein may also be affected due to impaired incorporation of amino acids in the polypeptide chains [37].

The protein is the alternative source of energy. Reduction in level of protein in experimental fish under pesticide influence is indicates hepatic insufficiency and probably malnutrition. Protein reduction observes in the present study due to high-energy demand in TCA cycle. The decrease level is also associated with the increase level of protease enzyme in tissues. Decrease in protein content under toxicity stress has already being reported [38]. The decrease in total protein level and increase in free amino acids level in both tissue and liver suggest the high protein hydrolytic activity due to elevation of protease activity [39]. Increase in free amino acids level was the result of breakdown of protein for energy requirements and impaired incorporation of amino acids in protein synthesis and decline in nucleic acids level [40]. In the present study increase, level of amino acids and protease has observed. Prasanth et al. [41] Observed significant elevation in the levels of free amino acids and protease activity in the Indian major carp (*Cirrhinus mrigala*) in response to cypermethrin. Gregor and John 1995 in serum protein level also observed reduced level.

1H-1,2,4-trizole bearing 4-position of 1,3-dioxolane moiety and an aryloxymethyl group and also nitrogen containing hetero-aromatic ring. Metabolites of trizole produce many N_2-containing substances that might be effect on enzymes activity and affect nucleic acids. Reduction in the DNA content is due to impairments of nucleic acids metabolism and the degradation of cells resulting in the tissues. Furthermore, inhibition of DNA synthesis, thus, might affect both protein as well as amino acid levels by decreasing the level of RNA in protein synthesis machinery. The regulatory roles of nucleic acid metabolism as observed in the different animals when treated with the different pesticides [24]. Das and Mukhrejee also reported the reduced level of DNA and SDH activity in brain and liver and increase level of LDH activity in both tissues [24]. Similar results have found in this study. In this study the level of LDH, was significantly increases under the effect of PCZ and depletion occurred in level of SDH and nucleic acids. PCZ has ability to modify the effect of several enzymes. These enzymes are blood soluble enzyme and best indicator of stress conditions [42]. The activity of these two enzymes might be governed by stress hormon cortisol that is the main hormone responsible for stress condition (Iwama et al.) [27]. LDH may indicate changes and hypofunction of liver under the toxicants effects on the hepatocytes are in the form of tissue damage in which cellular enzymes released from the cells into the blood serum. Increase level of LDH shown by the [24]. In the present study, the activity of SDH reduces. It is due to the mitochondrial disruption. SDH activity indicated anoxic hypoxic conditions when the fish exposed to toxicant and it was possibly, leading to decrease in the activities of oxidative enzymes and an increase in the glycolic enzymes reported by Dubale and Awasthi [43]. Reddy et al. reported decreased SDH activity in different tissues of food fish *Clarias batrachus* exposed to chlorpyrifos [44]. The decrease in SDH activity has reported in the fresh water crab, *Spiralothelphusa hydrodroma* treated with the pesticides, cypermethrin by Sreenivasan et al. [45].

Conclusion

Our studied showed that the fungicide (PCZ) which is widely used in vegetable crop fields have potential to damage aquatic fauna. It is highly toxic for fingerlings and adults. It caused severe biochemical and enzymatic alteration in fish. Therefore, we should avoid running off that fungicide in near water bodies.

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