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Acute and sublethal toxicity of chlorpyrifos on developmental stages of *Dattaphrynus melanostictus*

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ABSTRACT

Organophosphate (OP) compounds are commonly used as pesticides in agriculture, in homes, offices and gardens, parasiticides in veterinary medicine, and also employed as chemical warfare agents (CWA). However, extensive application of these OPs within agricultural proximity leads to the deleterious effects on inhabiting fauna. This study was aimed to find out acute toxicity and elucidates the sub-lethal effect of commercially formulated organophosphate chlorpyrifos (CPF) on *Duttaphrynus melanostictus* tadpoles. In this study, the 96 h LC_{50} value found to be 5.9 mg/L. Tadpoles exposed to three sub-lethal concentrations (0.7, 1.1, and 1.9 mg/L) of CPF showed a significant ($p < 0.05$) alterations in enzymatic antioxidants like catalase, superoxide dismutase glutathione peroxidase and non-enzymatic malondialdehyde, further the anticholinesterase potential of CPF was evident in concentration-dependent trend at five days of exposure tenure. It could be therefore emphasized that the CPF poses a potential threat to *D. melanostictus* tadpoles under the selected concentrations and hence it is advised that care should be taken when the toxicant is used and disposed under aquatic proximity. The investigation further serves as the preliminary data in the due course of regulatory surveillance and could be of a greater help in monitoring water with suspected CPF contamination.

INTRODUCTION

Large-scale anthropogenic activities have been associated with the drastic decline of amphibian populations globally (Jones *et al.,* 2009). According to International Union for Conservation of Nature (IUCN) report, 32.5% of total amphibian species have declined in terms of their number, which is far more critical than for birds and mammals (Quaranta *et al.*, 2009). Amphibians, unlike other animals, constitute a unique group among many ecosystems due to their active and multiple roles as, prey, predators, and herbivores (Touchon and Wojdak, 2014). Their contribution to tropic dynamics makes them one of the crucial features in determining the survival ability of other organisms through the food chain (Arribas *et al.*, 2014). Hence their existence at certain population ratio could be accountable for other species continuity as well.

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Even though the loss of habitat is considered to be the primary reason for the amphibian decline (Collins and Storfer, 2003), the role of pesticide contamination in freshwater habitats often questions its contribution in survival rate and reproduction of anurans (Knapp *et al.*, 2007). In addition to this, amphibians complete their larval development in an aquatic medium like ponds and lakes to which the pesticidal effluents from agricultural runoffs often find their way (Hayes *et al.*, 2003; Grayson *et al.*, 2011). The rich permeability of skin and egg which often get absorbed, persist and bioaccumulate further explains the vulnerability of tadpoles to environmental xenobiotics (Brühl *et al.*, 2013). A number of factors like morphological deformities compromised reproducing ability, immune-suppression, and reduction in growth and development have indicated the potential risk of pesticide contamination against anurans (Johnson *et al.*, 2007; Groner and Relyea, 2011). The use of integrated biomarker approaches for studying the inter-cascading changes in physiology and biochemistry has become an advanced strategy for reporting the overall health of tadpoles under the toxicological point of view (Boone and Semlitsch, 2001). Amphibian susceptibility to insecticides has been very well acknowledged in the past (Brühl

et al., 2013) and proven to be critical enough for compromising growth and development (Hartman *et al.*, 2014).

Currently, many pesticides are extensively used in agricultural operations. These pesticides act on the various physiological process and general development stages of non targeted organisms (David and Kartheek, 2014). Organophosphate (OP) compounds are commonly used as pesticides in agriculture, industry and in homes, offices, and gardens. These compounds are also used as parasiticides in veterinary medicine. In addition, some OPs are also employed as chemical warfare agents (CWA), while others are used as flame retardants and plasticizers (Ramesh *et al.*, 2018). Synthetic organophosphates chlorpyrifos is a pharmaceutical product, widely used to control various pests of agriculture and veterinary for public health protection. (Singh and Walker, 2006). According to a recent report, children exposed to chlorpyrifos while in the womb have an increased risk of delays in mental and motor development at age 3 and an increased occurrence attention-deficit hyperactivity disorder (Rauh *et al.*, 2006). There are some reports shows toxic potentials of OP pesticides on tadpoles (Geng *et al.*, 2005; Li *et al.*, 2010). The chemical contamination and its impact on non-targeted animals is an important aspect of the present study.

The reactive oxygen species (ROS) formed by pesticide exposure causes oxidative stress (Hernández *et al.*, 2013) and also known to pose catastrophic potentials against neurotransmission in tadpoles (Peltzer *et al.*, 2013). However, literature support on evaluations of CPF toxicity in tadpoles of *Duttaphrynus melanostictus* is found to be limited. However, *D. Melanostictus* is found to be fairly resistant in comparison to other species of tadpoles (David and Kartheek, 2015). Its susceptibility against organophosphates cannot be overlooked as these are often found to influence the antioxidant enzyme status and neurobiochemical activity ultimately determining survival rate among anurans. Therefore, this study aims to inspect the toxic potentials of CPF by determining the acute and sub-lethal toxicity of commercially formulated CPF on *D. melanostictus* tadpoles.

MATERIALS AND METHODS

Toxicant selected and preparation of test solution

Commercial grade Chlorpyrifos of 20% EC (CPF) was procured from the Dharwad local market (Karnataka, India). The stock solution was prepared by dissolving 1.0 gram of CPF in 100 ml of double distilled water. The requisite test concentrations were freshly prepared by diluting the stock solution prior to the initiation of toxicity studies.

Procurement and maintenance of tadpoles

Five hundred and fifty tadpoles of *Duttaphrynus melanostictus* (Gosner stage 20) were collected from uncontaminated ponds located in Karnatak University, Dharwad city (Karnataka, India) and were transported to the laboratory with care. The tadpoles were acclimatized to laboratory conditions for the duration of 7 days (one week). Everyday aquaria were aerated for 2 h, the temperature was maintained at 22 ± 2°C. The tadpoles were fed with green algae and boiled spinach during acclimatization, as well as during the 5 days of sub-acute toxicity test, but they were not fed during the 96 h of median lethal toxicity test.

Acute toxicity test

A static renewal assay test was employed as a method of exposure for each acute toxicity test. A range finding test was performed prior in order to find the upper and lower limits of acute toxicity value of CPF against tadpoles of *D. melanostictus*. This step was carried out in order to minimize the animal killing. The experimental tadpoles (10 each) were transferred to the aquaria consisting of concentrations ranging from 5000-10000 µg/L of commercial grade CPF in eleven different groups. Among these, one set, however, served as a control group consisting of tadpoles, which were being placed in dechlorinated tap water without any traces of CPF (n = 10). Each concentration, 3 parallel groups were made in this study. Mortality rate was observed at every 24 h intervals during the 96 h of the exposure period, the number of dead animals was recorded and the same were separated from the aquaria. The toxicity test results for the tadpoles are expressed as the median lethal concentration at 96 h, with 95% confidence limits (Table 2).

Exposure to sub-lethal concentrations

For sub-lethal exposure studies, four different groups of tadpoles were made in this experiment, first group keep without any traces of CPF in a dechlorinated water served as control (C), second (E1), third (E2) and fourth (E3) groups are intoxicated with CPF concentrations of 0.0, 0.7, 1.1 and 1.9 mg/L respectively for duration of 5 days (120 h). Each group consisted of 10 tadpoles (n = 10) and were maintained in triplicates. Tadpoles were transferred to glass aquaria only after thorough inspection and identification of its stage (Stage 27) according to Gosner (1960). Each glass aquaria consisted of 10 liters of water which was dechlorinated. All the experiments were carried out at 24 ± 2°C. Besides, temperature, pH, and dissolved oxygen levels were monitored daily (Table 1). The mean values of the individual group were taken into account for the present study.

Antioxidant assay

Catalase activity

Catalase (CAT) activity was determined by Luck (1963). Briefly, the assay mixture consisted of 3 ml of H_2O_2 (2 mmol), phosphate buffer 0.067 M (pH 7.0) and 10% of 0.05 ml of supernatant of whole tadpole homogenate and the changes in absorbance were recorded at 240 nm using spectrophotometer further, the activity of CAT was expressed as U/mg protein.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity measurement was done by Kakkar *et al.* (1984). The reaction mixture contains sodium pyrophosphate buffer 0.052 mM (pH 8.4), phenazine methosulphate 0.186 mM, nitroblue tetrazolium chloride 0.030 mM and NADH 0.780 mM in 50 µl of supernatant of tadpole homogenate. The enzyme concentration required to inhibit 50% of the chromagen formed in one unit at 560 nm was used for SOD activity prediction and is denoted in terms of U/mg protein.

Table 1: Showing values for quality assessment of water used in the present investigation.

Parameter	Values obtained
Temperature	24 ± 2°C
pH	7.1 ± 0.3
Dissolved oxygen	6.1 ± 0.4 mg/l
Total hardness	37.3 ± 3.1 mg as $CaCO_3$/l
Salinity	Nil
Specific gravity	1.003
Calcium	21.31 ± 0.27 mg/l
Phosphate	0.9 ± 0.04 mg/l
Magnesium	0.85 ± 0.3 mg/l

Table 2: Acute toxicity and 95% confidence limits of chlorpyrifos against *D. melanostictus* tadpoles.

Toxicant	96h LC_{50} (mg/L)	R^2 Linear Value	Probit	95% Confidence limits	
				Upper limit	Lower limit
Chlorpyrifos	5.9	0.797	0.512	6.192	5.532

Glutathione peroxidase activity

The Glutathione peroxidase (GPx) activity determined by Paglia and Valentine (1967). The reaction medium was composed of potassium phosphate buffer (171 mmol), sodium azide (4.28 mmol), EDTA (2.14 mmol), reduced glutathione (6 mmol), NADPH (0.9 mmol), and glutathione reductase (2 µmol). The reaction took place at 22°C ± 1, starting with the addition of H_2O_2 0.72 mmol. The absorbance of the samples was measured at 340 nm using a spectrophotometer and activity was expressed as mU/mg protein (mU = Catalyses of 1 nmoles of NADPH/min)

Lipid peroxidation

Determination of lipid peroxidation (LPO) level was achieved by the method of Buege and Aust (1978). The 2.0 ml of reaction mixture contains 10% trichloroacetic, 0.67% thiobarbituric acid and 200 µl of whole tadpole homogenate. The reaction mixture was incubated for 15 min at 90 ± 2°C. After cooling, it was centrifuged at 7500× g for 8 min, and optical density was measured at 530 nm by a spectrophotometer. LPO levels were expressed as nmol MDA/mg protein.

Acetylcholinesterase Activity

The rate of AChE activity was measured by Ellman *et al.* (1961). 3.0 ml of reaction mixture containing 0.05 M Tris–HCl buffer (pH 8.0), 0.34 mM DTNB, 1 mM acetylthiocholine and tadpole tissue homogenate in a suitable amount. The formation of thiocholine–DTNB complex at room temperature (25°C) was measured by photometrically at 412 nm. The AChE activity was expressed as nmole thiocholine (product) formed/min/mg protein.

Statistical analysis

All the data were expressed as the mean ± standard error of the mean (SEM). Analysis of Variance (ANOVA) test performed to analyze the differences between the groups and p-values less than 0.05 were considered statistically significant.

Ethical Statement

All the experiments were conducted in accordance with IAEC and Committee for the Purpose of Control and Supervision of Experiments on Animals (Registration number 639/02/a/CPCSEA).

RESULTS AND DISCUSSION

The *in-vivo* studies on endpoint evaluation for determining acute toxicity in addition to sub-lethal investigations is of principal importance under regulatory systems in order to understand the toxicity exerted by the selected chemical and additionally suggest the receptiveness and vulnerability of the exposed organism against it (David and Kartheek, 2014; David and Kartheek, 2015; David and Kartheek, 2016). Current investigation showed that there is no mortality among the *D. melanostictus* tadpoles under the control group. However, the 96 h LC_{50} value of CPF against the experimental tadpoles was identified to be 5.9 mg/L. The results are represented in Table 2 and their respective figures are provided in Figures 1–2. Changes in the reception of toxicity are known to vary for different organisms and are found to be mainly dependent on nature of the compound exposed to it. CPF is an organophosphate (OP) is known to demonstrate high toxicity against the aquatic forms including fish and amphibians (Giddings *et al.*, 2014).

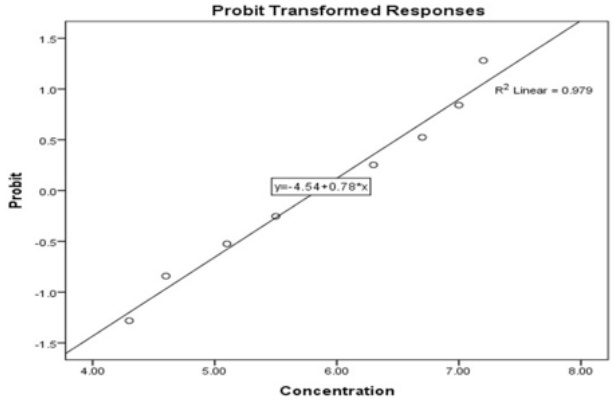

Fig. 1: Showing concentration versus probit for tadpoles of *D. melanostictus* exposed to commercially formulated chlorpyrifos.

Reports on the acute toxicity of other organophosphates, pyrethroids, and organochlorine compounds have been discussed so far. Nonetheless, the same for CPF against anurans are not presented so far to the best of our knowledge. Previously, the investigations on OPs toxicity against *D. melanostictus* tadpoles have been presented by David and Kartheek (2015), which suggests the comparatively lesser degree of toxicity at 7.5 mg/l by malathion. In yet another report, by David *et al.* (2012), the impact of a synthetic pyrethroid has been reported in which the recorded LC_{50} was found to be 3.34 µg/l. Correlating the outcome from the above, it could be ascertained that the CPF was found to be more toxic as compared to malathion. However, the same has been found to be lesser toxic as compared to the synthetic pyrethroid cypermethrin. According to Relyea (2004), *Rana sylvatica, Rana pipiens, Rana clamitans, Rana catesbeiana, Bufo americanus, and Hyla versicolor* were found to be moderately responding to the malathion residues with LC_{50} value ranges being located

between 1.25 and 5.9 mg/L. Exploration of this report elucidates the resistance ability of American toad *Bufo americanus* against the combined stress of predator and malathion residues with that of the *D. melanostictus* against CPF alone. Even though the higher resistance ability of the toad is apparent as compared with the *D. melanostictus*, it is highly implicit that the other factors influencing the toxicity within the aquatic systems in addition to the stages of the tadpoles exposed be evaluated for the precise understanding.

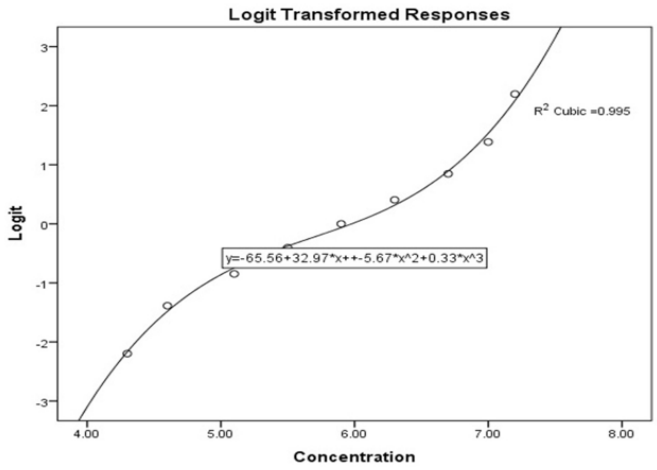

Fig. 2: Showing concentration versus logit for tadpoles of *D. melanostictus* exposed to commercially formulated chlorpyrifos.

Sub-lethal investigations have been yet another pointer under aquatic toxicology and have been in use for decades. The results from the present investigation suggested the toxic insult due to CPF intoxication evidenced by variations in all the antioxidant enzyme activities in all groups of exposed tadpoles. Changes in catalase activity under E1 were noticed that an elevation of +18.43%, followed by a decline of -28.49% and -60.41% in E2 and E3 respectively when compared with control (Figure 3). The activity of SOD was shown that continuous downfall in its activity. The percent decline in SOD activity was -17.13%, -39.60% and -73.73% in E1, E2, and E3 respectively as compared to C (Figure 4). The activity of GPx in the tadpoles also shows significant differences in the experimental groups (Figure 5). The percent change in expression patterns of GPx was found to be elevated in E1 (+28.27%) followed by diminish in E2 (-12.48%) and E3 (-45.85%) when compared with C.

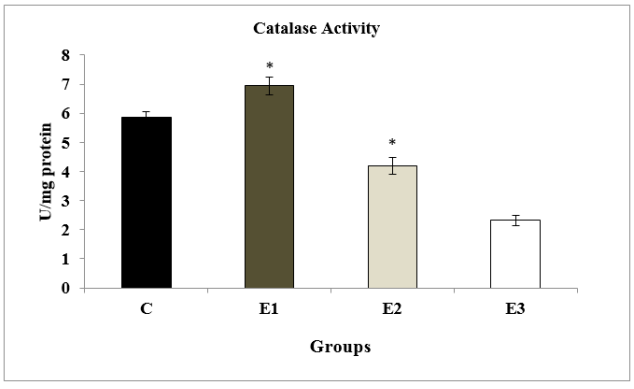

Fig. 3: Changes in catalase activity of *D. melanostictus* following exposure to commercially formulated chlorpyrifos.

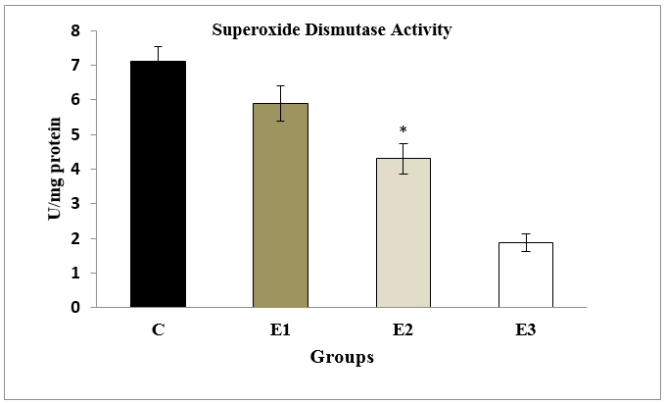

Fig. 4: Changes in superoxide dismutase activity of *D. melanostictus* following exposure to commercially formulated chlorpyrifos.

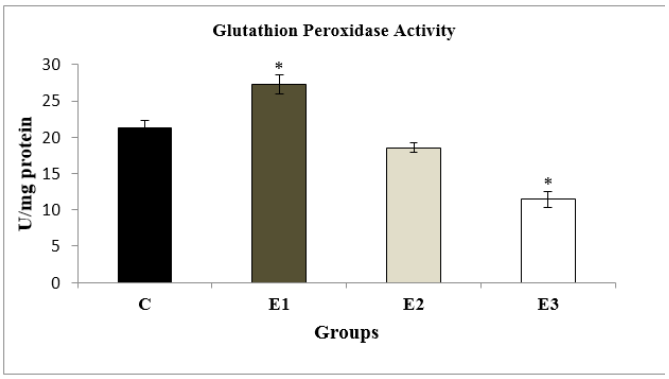

Fig. 5: Changes in glutathione peroxidase activity of *D. melanostictus* following exposure to commercially formulated chlorpyrifos.

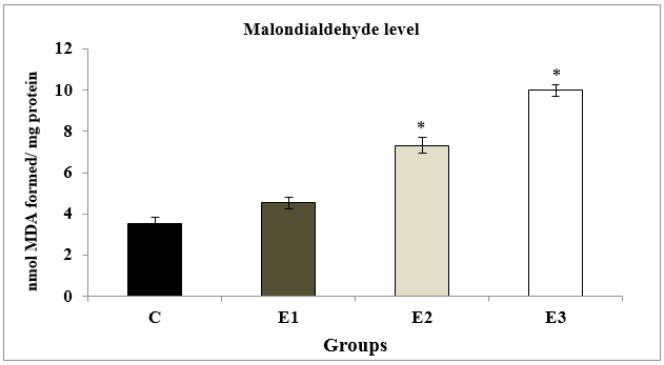

Fig. 6: Changes in malondialdehyde levels of *D. melanostictus* following exposure to commercially formulated chlorpyrifos.

The lipid peroxidation was found to elevate under the influence of CPF and the variations of the same have been presented in Figure 6. When compared to control changes in LPO were noticed that an elevation of +27.68% in E1, 106.8% in E2 and 181.92% in E3. A significant difference in E2 and E3 was noticed in exposed tadpoles, unlike E1. Upon correlation, the results indicated the damage to be highest in tadpoles belonging to E3 at 5 days of the exposure period.

Aquatic pollution due to indiscriminate use of pesticides has been acknowledged to be one of an important anthropogenic

source of amphibian survival and is known to affect a large fraction of the aquatic ecosystems around the globe. Amphibian species are vulnerable to contaminants because of the greater permeability of the skin and gills during the larval stage (Azzam *et al.*, 2003). Oxidative metabolism is a regular phenomenon in the cell environment (Hagedorn *et al.*, 2012) and its controlled limits are governed by biochemical makeup in different aquatic organisms including fish (Zhang *et al.*, 2012) and amphibians (Antonio-Garcia *et al.*, 2008). The biochemical makeup constitutes a number of sections, of which, antioxidant enzymes form an imperative part (Hagedorn *et al.*, 2012). The antioxidant pool further comprises a cluster of free oxyradical scavenging enzymes, *viz* CAT, SOD, and GPx which are known to be the defense enzymes against oxidative stress triggered by an arbitrary metabolic process *in vivo* (Ojha *et al.*, 2011). Thus, the over and under expressions are known to play a crucial role in the maintenance of health status of an organism (Poljsak *et al.*, 2013).

The present investigation reveals the interference of CPF in the oxidative metabolism and thus initiating the cascade changes by inducement of oxidative stress in *D. melanostictus* tadpoles. Metabolic breakdown of intoxicated xenobiotics can lead to ROS production at the considerable amount, which is sufficient to compromise the health status of an organism (David *et al.*, 2008). The significant variation in activity of catalase enzyme witnessed in the present study, these results coincide with studies of David *et al.* (2012), who reported the decline in catalase enzyme activity in *D. melastictus* under the influence of a synthetic pyrethroid, cypermethrin. In the present study, elevation of CAT at E1 and depletion at E2 and E3 are in line up with Costa *et al.* (2003) who suggested the discrepancies in CAT activity of bullfrog under the influence of Round up®. Variation in catalase activity in the present study did not follow uniformity and was inconsistent with CPF concentration. This may be due to some other extraneous factors which perhaps may not be captured within the boundary of the present study. However, it could be emphasized that the biochemical catalytic and repair are the two simultaneous yet individual processes and this could have accounted for uneven patterns of catalase expression.

The SOD activity in tadpoles was found to decline under the presence of CPF. Ojha *et al.* (2011) suggested an active role of OPs modulates the activity of SOD in a significant pattern. The intracellular environment could be the space for the generation of superoxide anion which may be the consequence of foreign chemical component participation (Stead and Park, 2000). This generally initiates the active role of SOD upon which it swiftly terminates the harmful radical under the action of body defense mechanism. The constant interaction between elevated levels of superoxide radicals and inadequate magnitude of its counterpart SOD might have resulted in an incessant enzymatic decline in the present investigations.

The GPx activity is known to be a crucial indicator in determining the levels of oxidative stress (Blokhina *et al.*, 2003). The elevated activity of GPx at E1 and its subsequent reduction at E2 and E3 may be due to its active role during the countering of the free oxyradicals generated during CPF detoxification. The other reason may be perhaps due to the GPx sharing of its substrate H_2O_2 with CAT. Since the affinity of CAT towards H_2O_2 is much lesser than GPx (Baud *et al.*, 2004). The uniform and irregular trend in decline of SOD and CAT respectively, in the tadpoles exposed to CPF, begs the need for an alternative mechanism to reimburse enzymatic status which was seen as a response to a compensatory act of GPx (Wang *et al.*, 2012). This mechanism further convinces the marked increase in GPx activity at E1, during which there was an insignificant change of CAT and significant change in SOD activity. These statements are supported by studies of Santos *et al.* (2015), who witnessed the changes in GPx activity in *Phyllomedusa inhering* exposed to polluted water. This phenomenon may perhaps be adequate to influence the metabolic synthesis of GPx activity under varying concentrations of CPF.

MDA, being one of the major end products of lipid peroxidation (Hodgson, 2004) and is also thought to be a cause in fabricating the arrest of cell function through elevating the condition of oxidative stress (Lushchak, 2011). Increased LPO was observed in the present investigation which can be implicated in CPF interaction with cellular metabolism. Sharma and Ansari (2013), stated that the increased LPO may be due to the intervention of a toxic substance such as pesticides, which matches the outline of the present condition, wherein, increase in LPO was clearly found to be based on the concentration of investigated toxicant CPF. The present findings of increased LPO and decreased antioxidant enzyme activity can reaffirm a strong support to suggested hypothesis stating induced LPO could be the consequence of decreased antioxidant enzyme status or vice versa (Grim *et al.*, 2011).

Table 3: Acetylcholinesterase activity in control and exposed tadpoles of *D. melanostictus* following exposure to chlorpyrifos.

Parameter/Group	C	E1	E2	E3
Acetylcholinesterase	7.81 ± 0.24	6.64 ± 0.2*	5.02 ± 0.25*	2.15 ± 0.16*

Values are means ± SE are significantly (*$p < 0.05$) according to One-Way ANOVA with Tukey's post hoc test.

From the outcome of the present investigation, it could be noted that i*n vivo* exposures to sub-lethal lethal concentrations of CPF led to a concentration-dependent inhibition of AChE activity (Table 3). The significant decline in activity of AChE in exposed tadpoles was compared with the previous study of Richards and Kendall (2003), who states that *X. laevis* exposed to chlorpyrifos showed the significant inhibition of ChE at the 100 and 10-g/L. Bonfanti *et al.* (2004), also examined the ChE inhibiting potential of chlorpyrifos on early stage *X. laevis* embryos at 100 and 250 g/L chlorpyrifos for 5 days of an exposure period. Further, this study line up with Colombo *et al.* (2005), who observed a significant reduction of ChE activity in early stage *X. laevis* embryos exposed to 100 g/L chlorpyrifos. Inhibition of AChE activity in the tadpoles leads to the deprived neurotransmission and swimming performance thereby increases the susceptibility to predators (Peltzer *et al.*, 2013). The findings clearly promote categorizing the insecticide CPF, under, "xenobiotics with potentials for neurobiochemical toxicity and oxidative stress in tadpoles of *D. melanostictus* under given sub-lethal doses at 5 days of exposure tenure".

CONCLUSION

The present investigation suggests the toxic potentials of

commercial formulations of organophosphate CPF as evidenced by increases the oxidative stress through a decline in antioxidant enzyme activity in *D. melanostictus* during metamorphosis. The selected sub-lethal lethal doses of CPF were also known to demonstrate antagonistic conduct against acetylcholinesterase activity in exposed tadpoles. Thus, endpoints that biochemical perspective may provide a sensitive indication of sub-lethal toxicity. Therefore, it is suggested that necessary precautions are required prior to its use and disposal under the proximity of active amphibian habitats, in order to conserve the vanishing amphibian species. Further, the assays included in present study validate the feasibility to measure the toxicity of CPF in terms of antioxidant stability, anticholinesterase potentials in the course of regulatory surveillance and monitoring the waters with suspected CPF contamination.

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CONFLICT OF INTEREST

The authors hereby declare no conflict of interest.

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