Asian J. Exp. Sci., Vol. 33, No. 1, 2019; 47-52

Online available on : www.ajesjournal.com, ISSSN : 0971-5444

Response and Recovery of Liver AChE of the Fish *Catla catla* as Response to *Datura stramonium* Leaf Extract and Chlorpyriphos-contaminated water.

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Received : November 10, 2018 ; Revised : November 25, 2018; Accepted: December 15, 2018

Abstract : Present study was conducted to determine the *in vivo* effects of short term exposures of ethanolic leaf extract of *Datura stramonium* (*D.s.* 100 mg/l) and chlorpyriphose (0.0034 mg/l) separately and combined for 96 hours on acetylcholinesterase (AChE) activity, inhibition, and enzyme kinetics of the liver of *Catla catla.* This was followed by a recovery period in clean water (120 h more). Enzyme inhibitory activity was carried out by using Ellman's method (1961). Enzyme kinetics was estimated by Line Weaver Burk's plots. The results demonstrated inhition in AChE activity in the liver were ranging from 32% to 51%. Following 120 hours of recovery, AChE activity in catla liver was still different from that of controls and recovered 24.3% at the end of experiment.

Introduction

Organophosphates and carbamates are major agrochemicals that adversely affect different neuroenzymes and the growth of various fish species. Exposure to pesticides affects the functions of organs and neurotransmission to various extents. Acetylcholinesterase (EC 3.1.1.7; AChE), which is abundant in brain tissue, plays a role in signal termination at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine. This enzyme is also present in the liver to act in detoxification (Brimijoin and Koenigsberger, 1999). The inhibition of cholinesterase with nerve agents, especially pesticides, causes the accumulation of acetylcholine at the synaptic cleft and interrupting the nervous transmission, eventually leading to paralysis and death (Fulton and Key, 2001). In the aquatic environment pesticides and other xenobiotics can attach to suspended matter, sediments in bed of water body or be absorbed by the aquatic organisms where they undergo detoxification or bioaccumulation (Nimmo, 1985). Thus, AChE from aquatic organisms has been used due to its ability to assess the environmental impact when these compounds are not present in the water (Morgan *et al.,* 1990). Among these organisms are fish (Fulton and Key, 2001). According to the Food and Agriculture Organization (FAO, 2007), 20% inhibition of brain AChE activity is considered the endpoint to identify the no observed-adverse-effect-level (NOAEL) in organisms, while signs and symptoms appear when AChE is inhibited by 50% or more. Death occurs above 90% inhibition.

Therefore, search of some alternatives of harmful pesticides are required which should be environmentally safe, biodegradable and less expensive. Many medicinal plants and their products have been reported to be used for controlling unwanted fish population not only in India but across the world. (Kualakkattolickal, 1989; Bhatia, 1970; Tiwari, 2003).Due to biodegradability, herbal extracts attracted much interest of investigators to explore possibility of their potential AChE inhibitory powers in order to replace pesticides use in the environment. The AChE inhibitory effects of 255 herbal medicines were evaluated using an AChE assay of which 8 herbal medicines significantly inhibited the AChE activity more than 50 % (Chung *et al.,* 2015). The natural compounds have always been served as a useful source to study inhibitory effect on AChE activity. A number of phytochemicals, namely, alkaloids, pregnane glycosides (cynanchoides), stilbenes, triterpenes (Gurovic et *al.,* 2010), ursane (Mukherjee *et al.,* 2007), and xanthones, have shown AChE inhibitory activity.

In the present study, AChE activities, inhibition, recovery and kinetics in liver were studied after exposing fish to ethanolic leaf extract of *Datura stramonium* and chlorpyriphose individually and in combination and it was found that AChE activities can be used as a biomarker for observation of this contamination in natural water.

Materials and Methods

Plant materials and preparation of extracts

Datura stramonium leaves were collected from the outskirts of Bhopal and identified in the Botany

Department of M. L.B. Girls College, Bhopal (India). Voucher specimens of plant have been deposited in the Laboratory. 500 g shade dried and coarsely powdered leaves of *Datura stramonium* was subjected to Soxhlet extraction with 90% ethanol for about 12 hrs following standard method (Harborne, 1973 alcohol was evaporated at room temperature from the obtained extract and the semi solid mass was stored at 4^0C for further use in the experiment.

Experimental Fishes

Fingerlings of *Catla catla (*median weight 100 ± 10 gm) collected from the fishpond near Kolua village, Raisen road, Bhopal and stocked in glass aquaria of 60 liters supplied with tap water (temperature 22.7 + 0.61 ^{0}C, hardness as $CaCO_3$ 212 + 4.8 3 ppm, pH 7.3 + 0.05, chlorides 87.62 + 2.39, total alkalinity as $CaCO_3$ 165 + 1.15 ppm). They were acclimatized for 15 days. The commercial dry feed pellets (Tokyu, Spirulina, Japan) were supplied daily to the fingerlings. Water in the aquarium was renewed daily. Physico-chemical property of water was monitored according to APHA/AWWA/WEF (2005). During the treatment, water in each aquarium was aerated. The feeding was stopped before 24 hrs prior to and during the exposure period, which extended 96 hrs.

Animal Treatment

Four groups of thirty fishes each were made. Group-I: served as control without toxicants; Group-II: Daily exposure for 96 hrs to 0.00073 mg/l based on 96 h LC^{50} (0.0034 mg/l) of CPF for *Catla catla).* Group-III: Daily exposure for 96 hrs to 100 mg/l of *D.s.* leaf extract. ; Group- IV: The fishes were pre-treated with 100 mg/l of *D.s.* extract for 96 hrs followed by the exposure to 0.00073 mg/l CPF for 96 hrs. At the end of the experiment, five fish were removed from each group to study the effect of CPF and D. s extract. However, remaining fishes were transferred to toxicant free water to study recovery of AChE. Water was changed after every 24 hrs. Five fishes from this stock were removed and dissected at the end of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs.

Sample Preparation

At the end of experiments the treated fish were euthanized, dissected and the livers were removed quickly and washed in 0.9% saline. A 10% (w/v) tissue homogenate was prepared in Elvehjem potter homogenizer and centrifuged at 5000 rpm for 10 min at 4^0C in cooling centrifuge (Remi). The supernatants were kept in deep freeze for AChE assay, kinetics and Protein content.

AChE Assay

AChE activity was measured spectrophotometrically according to the method of Ellman's *et al. (1961*) in the liver of Catla catla. Samples of homogenate were diluted with 2.6 ml 0.1 M Sodium phosphate buffer (pH 7.4) to which 100 mM DTNB. The reaction was initiated by addition of 75 mM AChI (acetylcholine iodide). The rate of color production was measured at 412 nm in SL 164 UV-VIS spectrophotometer. All measurements were done in duplicate. Specific activity was expressed in nmol/min/mg protein.

Protein Determination

Protein was estimated by the method of Lowry *et al.* (1951) using BSA as the standard. Samples of homogenate were diluted with reagents then 0.5 ml Folin's reagent was added and after 20 min read at 620 nm against a reagent blank. Measurements were done in duplicate.

AChE Kinetics

Kinetic parameters (Km & Vmax) were determined by using four different concentrations of substrate i.e 0.66 mM, 0.44 mM, 0.33 mM, 0.26 mM. Lineweaver-Burk plot was made by plotting the reciprocals of velocity and substrate concentration.

Statistical Analysis

Significant differences were analyzed by t tests to determine which individual groups were significantly different from the control. The level for the accepted statistical significance was $P>0.05$ and $P > 0.01$.

Results

No fish died during the toxicants exposure and recovery period. Treated fish showed some abnormal behavior such as loss of balance, and irregular swimming first day compared to control group.

AChE activity in the liver of control fish was 13.74 $\pm$ 2.6 nmole/ min/ mg protein which was (Table:1 and Fig: 1)**.** The acute effect of chlorpyriphos (0.0731 ppm) was more pronounced in the liver as its Specific activity reduced to 6.61 $\pm$ 1.41 nmole/ min/ mg protein in comparison to control value showing 51.8% inhibition in the liver AChE was determined**..** *D.s* extract was also found to be toxic to liver AChE. The AChE activity in the liver was declined to 9.25 $\pm$ 3.64 nmole/ min/ mg protein. The inhibition in AChE activity was calculated to be -32.6% **(**Table: 1; Fig: 1). The exposure of *D.s* extract followed by chlorpyriphos did not produce synergistic effect. The AChE activity in this group was 7.27 $\pm$ 2.91 nmole/

min/ mg protein. The % inhibition in AChE activity was 47% which was less than % inhibition of AChE in the liver due to chlorpyriphos (Table:1; Fig:1).

Fish were allowed to recover in toxicant-free water for 120 days of exposure to chlorpyriphos and ethanolic extract of *Datura stramonium* leaves. Liver AChE activity after exposure to chlorpyriphos recovered slowly up to 72 hrs and rapidly after 96 hrs. Our study demonstrated 4.3% recovery of inhibited AChE at 48 hrs, however it was 6.8% at 72 hrs, 26.1% at 96 hrs and 26.6% at 120 hrs. Similarly, comparatively the slow recovery was observed in AChE after exposure to ethanolic extract of *D.s* leaves viz. 2.1% recovery was observed at 48 hrs while 10.4% and 20.3% recovery was observed at 72 and 96 hrs respectively. At120 hrs there was 24.3% recovery in AChE was observed (Table: 2; Fig: 2).

The AChE Kinetics study in the liver revealed that the Km value of liver AChE of control group was 0.2 x 10^{-3} M. However, CPF-induced AChE inhibition resulted into increase in Km to 0.33 x 10^{-3} M. *D.s* extract also increased Km value of liver AChE to 1.42 x 10^{-3} M. Further, increment in Km value to 2.5 x 10^{-3} M was calculated in *D.s* extract and CPF group of fish. However, Vmax value remained constant in control and treated groups. The Vmax was calculated to 0.25 activity /min/mg protein showing competitive inhibition in the liver (Table: 1; Fig: 3).

Table:1- Effect of 96 hrs exposure of chlorpyriphos and *Datura stramonium* on acetylcholinesterase activity, inhibition, Km and Vmax of the liver of *Catla catla.* The Specific activity is expressed in nmole/ min/ mg protein.

Parameters / Group	Specific activity of AChE	% inhibition of AChE	Km x 10^{-3} M	Vmax (Abs/min/mg protein)
Control	13.74 ± 2.6	-	0.2	0.25
CPF (0.0731 ppm)	6.61 ± 1.41**	51.8%	0.33	0.25
***D. s* (100mg/L)**	9.25 ± 3.64**	32.6%	1.42	0.25
Pretreatment of *D. s* (100 mg/L) *post* exposure of CPF (0.0731 ppm)	7.27 ± 2.91**	47%	2.5	0.25

Each value is mean ± SD of five individual observations. Significant: P > 0.05*; highly significant: P > 0.01**

Group / Parameter	Control	CPF (0.0731 ppm)	Recovery				*D.s* (100 mg/L)	Recovery			
			48 hrs	72 hrs	96 hrs	120 hrs		48 hrs	72 hrs	96 hrs	120 hrs
AChE activity (nmole/min/ mg protein)	13.74 ±2.6	6.61±1.41**	6.9± 0.92**	7.06± 1.6**	8.34± 1.4**	8.37± 2.1**	9.25 ± 3.64**	9.4± 0.11**	10.2± 1.24**	11.1± 0.71**	11.5± 0.44**
% inhibition of AChE	-	51.8%	49.7	48.6	39.3	39	32.6%	31.5	25.7	19.2	16.3
% recovery of AChE	-		4.3	6.8	26.1	26.6		2.1	10.4	20.3	24.3

Each value is mean ± SD of five individual observations. Significant: P > 0.05*; highly significant: P > 0.01**

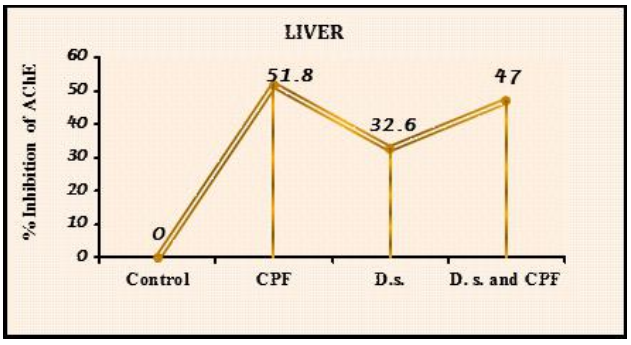

Fig.: 1 - Percentage inhibition of AChE activity in the liver of *Catla catla* of control group, CPF group (0.0731 ppm), *D.s* group (100 mg/L) and pre treatment of *D.s* (100mg/L) and post exposure of CPF (0.0731 ppm) for 96 hrs.

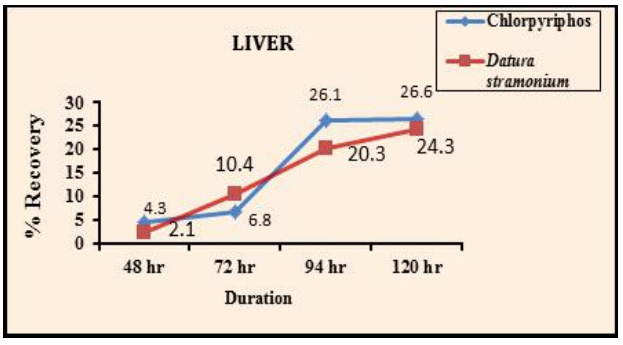

Fig. :2 - Recovery of inhibited AChE after 48,72, 96 and 120 hours in the liver of *Catla catla* exposed to 0.0731 ppm chlorpyriphos and 100 mg/L *Datura stramonium* for 96 hours.

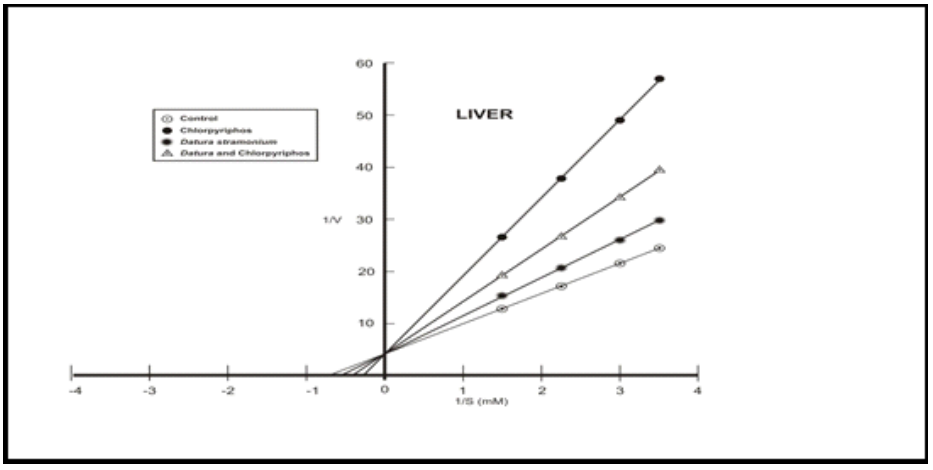

Fig. : 3 - Line-Weaver Burk plot of inhibition of the liver AChE of *Catla catla* exposed to CPF (0.0731 ppm), D. s extract (100 mg/L) for 96 hrs and pretreatment of D. s extract (100 mg/L) and post exposure of CPF (0.0731ppm) for 96 hrs. Each point represents the mean of five assays. ATChI used as a substrate

Discussion

In the present investigation significant AChE inhibition was observed due to CPF and ethanol extract of *Datura stramonium* in liver. A higher inhibition was observed with CPF than *D. s.* extract in liver, however there was a synergistic inhibitory effect observed with the combination of CPF and *D. s.* extract. Singh and Kumar (2000) reported that sub-chronic exposure of malathion causes 63% AChE inhibition in the liver of *Catla catla.* When newly synthesized organophosphate compounds RPR-II, RPR-V and monocrotophos was studies on acetylcholinesterase in liver of *Channa punctatus,* Monocrotophos produced more potent inhibition Rahman *et al.* (2004). Tiwari and Singh (2004) reported that exposure to alcoholic leaf extract of *Nerium indicum* caused significant reduction in AChE activity in the liver of *Channa punctatus* by 44%.

We studied the recovery of inhibited AChE by CPF and D.s. extract alone and found 26.6% and 24.3% recovery in AChE activity respectively at 120 hours. Carr *et al.* (1997) determined the level of recovery of inhibited acetylcholinesterase (AChE) and aliesterase (ALiE) in the liver of several species of fish including largemouth bass, bluegill sunfish, golden shiners and mosquitofish exposed to chlorpyriphos. They observed that the liver AChE activity was recovered to about 50% of control values. Rao *et al.* (2005) demonstrated the brain

acetylcholinesterase activity in mosquitofish, *Gambusia affinis* in response to the sub-lethal exposure to chlorpyriphos. Experiments were carried out at day 4, 8, 12, 16 and 20 to determine AChE activity. They reported that the brain AChE activity was inhibited maximum (55%) on day 4 and slightly recovered and exhibited 40% inhibition on day 20. Dembele *et al.* (1999) studied the recovery of inhibited acetylcholinesterase activity in common carp treated with organophosphate pesticide chlorfenvinphos and carbamate pesticide carbofuran. Their experiment revealed that the brain AChE activity was almost completely recovered within one day after exposure to carbofuran and 15 days after exposure to chlorfenvinphos.

To substantiate the AChE inhibition by CPF and D.s. extract further study on AChE kinetics were investigated by analyzing Km and Vmax. Our findings exhibits that both CPF and D.s. extract produce competitive inhibition in liver AChE. This was indicated by increasing Km values with CPF and D.s. extract concentrations as compared to control value. Rahman *et al.* (2004) conducted *in vitro* acetylcholinesterase kinetics study in RBC, brain and the liver of *Channa punctatus* treated with monocrotophos, RPR – II and RPR-V. They showed decreased Km and Vmax values with all the three compounds. They suggested that these three compounds produced mixed inhibition in acetylcholinesterase. Crowch and Okello (2009) observed inhibition kinetics of acetylcholinesterase with two medicinal plants, *Acacia niloticus* and *Rhamnus prinoides.* The aqueous extracts of *Acacia niloticus* and *Rhamnus prinoides* both exhibited non-competitive uncompetitive mixed inhibition. They also reported that galanthamine induced a mixed type of inhibition.

Conclusions

The present investigation has shown that CPF and *D.s.* ethanolic extracts from the leaves could inhibit the activity of AChE in liver of *Catla catla*. The extracts of *Datura stramonium leaves* was proved to have a great potential and should be considered for further studies to identify the constituents responsible for the AChE inhibitory activity, which can be eventually utilized in the controlling of insects and pest to minimize the application of synthetic OP and carbamate pesticides for the environment protection.

Acknowledgements

The authors are thankful to Principal, MLB Govt Girls College, Bhopal, India for the research facility.

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