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Immunomodulatory effect of chlorpyrifos formulation (Pyrifos- 20 EC) on *Philosamia ricini* (Lepidoptera: Saturniidae)

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Abstract
In present study immunotoxic effect of organophosphate pesticide, chlorpyrifos formulation (Pyrifos-20 EC) on *Philosamia ricini* (eri silkworm) was evaluated. Herein, two sub lethal concentrations of chlorpyrifos (1.5 mg/L and 2.0 mg/L) were administered to eri silkworm and the effect on Phenoloxidase (PO), lysozyme enzyme activity and hemocyte immunity were investigated at 24-96 h time interval. Results showed at 72-96 h 2.0 mg/L concentration showed significant inhibition in enzyme activity. Likewise, after 24 h of exposure a significant increase in lysozyme activity was recorded in both pesticide exposed groups, however at 72-96 h significant decrease in lysozyme activity was measured. Moreover, after 48-96 h significant reduction in hemocyte abundance was observed (p<0.05). Changes in proportional counts of hemocytes showed that sub lethal chlorpyrifos exposure caused an increase of granulocytes and plasmatocytes in a concentration-dependant manner. However, prohematocytes, spehrulocytes and oenocytes abundance was decreased significantly (p<0.05) in chlorpyrifos exposed groups.

Keywords: Chlorpyrifos, Eri silkworm, Phenoloxidase, Lysozyme, Total and differential hemocyte count

1. Introduction
Pesticide is any substance or mixture of substances used for preventing, destroying, repelling or mitigating any pest (insects, mites, nematodes, weeds, rats, etc.) in agricultural practices. Pesticides include insecticide, herbicide, fungicide and various other substances used to control various pests. Organophosphate is the most earliest organic synthesized pesticides along with carbamate and organochlorine insecticides. Organophosphates are widely employed in natural as well as synthetic applications because of its low cost and faster degradability than the organochlorides. Chlorpyrifos (CPF) [O, O-diethyl-O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate one of the broadly used organophosphate pesticides both in agriculture and household pest control practices. Although chlorpyrifos is toxic but due to its cost efficiency and degradable nature it is extensively used worldwide [1].
The use of pesticide is so indispensable in agricultural production and about one-third of the agricultural production is based on pesticide application. Previous study results stated that without pesticide application serious loss of fruits, vegetables and cereals would reach 78%, 54% and 32% respectively [2] and crop loss from pest's declines in the range of 35% to 42% due to pesticide application [3]. Although use of pesticide sounds beneficial for mankind, nevertheless the risks of pesticides use cannot be ignored [4]. Most of the pesticides are highly toxic to the environment, non targeted flora and fauna and humans. Pesticide residues and their degraded products enter into ecosystem from site of application and contaminate the surroundings. It is estimated that over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species. As a result of this accumulation, biomagnifications of pesticides takes place and thereby threaten health of non target organisms. Like other pesticides, chlorpyrifos also contaminates the ecosystem by various processes like direct application, spray drifts or crop run off. There are several reports of chlorpyrifos induced toxicity in non-target organisms starting from microorganisms, plants, other invertebrates including arthropods, aquatic organisms to higher mammals [5-7].
In this regard silkworms are also considered as a non target organism for pesticide toxicity.
Previous reports showed the impact of pesticide pollution on sericulture industry of china and it

was stated that almost 30% reduction in annual silk production was taken place in China due to pesticide application [8]. The silkworm larvae get exposed to pesticide residue contaminated food plants mainly by spray drift method from the site of application. The accumulation or exposure of pesticide induce toxicity in *Bombyx mori* silkworm and as a result of this larval health get disturbed resulting less production of silk [9]. *Philosamia ricini* is also a commercially beneficial non mulberry silkworm of North Eastern region of India. This variety of silkworm is also under the threat of pesticide pollution as the food plants (castor plants) grow nearby agricultural area and thereby exposed to pesticide residues. However, no report of pesticide contamination in *P. ricini* is available. The present study aims to evaluate the immunotoxic effect of chlorpyrifos formulation in *P. ricini* by immune enzyme activity assay and cell mediated immune alteration assay.

2. Material and methods

2.1 Silkworm

Disease free layings of eri silkworm were obtained from Mangaldoi sericulture farm, Assam. After hatching the larvae were reared in laboratory condition under recommended condition at 25-27 ^{0}C, 75 ± 5% relative humidity and 12 h light/12 h dark condition. The larvae were fed with castor leaves harvested from IASST silkworm food plant garden and the study was carried out during March – April (spring season) of 2015 and 2016.

2.2 Pesticide and other chemicals

Commercial formulation of chlorpyrifos, Pyrifos-20 EC was purchased from Assam fertilizer house, Guwahati, Assam. All other chemicals used were of analytical grade.

2.3 Pesticide stock solution preparation

100 mg/L stock solution was prepared for chlorpyrifos formulation by dissolving 0.05 ml of Pyrifos- 20 EC in 99.95 ml of acetone. Subsequent dilution of the stock solution was prepared in distilled water to determine the LC_{50} value and sub lethal concentrations.

2.4 LC_{50} value determination

Acute toxicity of chlorpyrifos formulation was determined in eri silkworm by adopting the procedure of Zhang *et al.* (2008) [9]. Herein, castor leaves were sprayed with different chlorpyrifos concentrations and air dried. After two minutes the leaves were fed to 5th instar eri silkworm larvae and mortality was recorded. The LC_{50} value of chlorpyrifos formulation was calculated by Probit analysis and recorded as 2.35 mg/L at 96 h time period (unpublished data). Two sub lethal concentrations (1.5 mg/L and 2.0 mg/L) were selected for further assays.

2.5 Immune response assay

The 5th instar larvae of eri silkworm were orally administered with sub lethal concentrations of chlorpyrifos formulation (1.5 mg/L and 2.0 mg/L) contaminated leaves. Larval hemolymph was collected by cutting the pro leg at 24 h, 48 h, 72 h and 96 h exposure period and used for immune response assay.

2.6 Phenoloxidase (PO) activity assay

PO enzyme activity was studied by following the method of Ashida and Soderhall (1984) [10]. Briefly, 10 µl of hemolypmh samples were taken and mixed with 20 µl of PBS and centrifuged at 5000 rpm for 5 min at 4 ^{0}C for removal of hemocytes. The supernatant of hemolymph plasma were used for PO enzyme assay. Thereafter 10 µl hemolymph plasma solution was mixed with 200 µl of 10 mM L-DOPA (L-3, 4-dihydroxyphenylalanine dissolved in sterile water) and incubated for 30 min at room temperature. After incubation, absorbance was measured at 490 nm and PO enzyme activity was expressed in unit (1U defined as change in absorbance of 0.001 per minute per µl of enzyme sample).

2.7 Lysozyme activity assay

The lysozyme activity in silkworm of both control and pesticide exposed groups was measured by following standard protocol of Azambuja *et al.* (1991) and Drayton *et al.* (2011) [11]. Herein, 50 µl of cold PBS was added to 10 µl of hemolymph and centrifuged at 5000 rpm for 10 min. After centrifugation the supernatant was collected and 30 µl of supernatant was added to 200 µl of Micrococcus lysodeikticus ATCC No. 4698 cell suspension (Sigma) and incubated at room temperature for 10 min. Thereafter, 200 µl of aliquots were taken in the microplate reader and the change in absorbance was measured at 450 nm at 1 min interval for 30 min. The enzyme activity was expressed in unit (1U defined as change in absorbance of 0.001 per minute per µl of enzyme sample). A lysozyme standard from chicken egg whites (Sigma) was used simultaneously along with the hemolymph samples to confirm the progress of the assay.

2.8 Total and differential hemocyte count (THC and DHC)

Total and differential hemocyte count was performed in both control and pesticide exposed 5th instar silkworms at regular time intervals (24, 48, 72 and 96 h). The total number of hemocyte was counted by following the method of Jones (1962) [12]. Briefly, hemolymph was drawn to 0.5 mark in WBC pipette and diluted by Tauber-Yeager Fluid [13]. Thereafter, the cell number was counted by Neubauer hemocytometer. For DHC, initially hemolymph samples were diluted to 5 times with 1X PBS buffer (pH 7.4), smeared uniformly on slides and air dried. After drying the slides were stained with 10% giemsa stain for 10 min. The stained slides were washed with 1X PBS (pH 7.4) for two times, air dried and mounted with DPX. 200 cells per slide (10 replications) were taken into account for both control and chlorpyrifos exposed groups [14].

3. Results

3.1 Phenoloxidase activity assay

PO enzyme activity was measured at different time intervals and results were presented in Fig. 1. At 24 h exposure period no significant decrease was observed at 1.5 mg/L concentration (F = 0.369, P>0.05), however at 2.0 mg/L the reduction was significant (F = 34.79, P<0.05). At 48 h of exposure both the experimental groups showed significantly less enzyme activity (F = 11.08, 195.37; P<0.05). Although at 72 and 96 h higher concentration showed significant decrease in enzyme activity (F = 32.83, 349.72; P<0.05) but at 1.5 mg/L no significant reduction was observed (F = 0.53, 0.20; P<0.05).

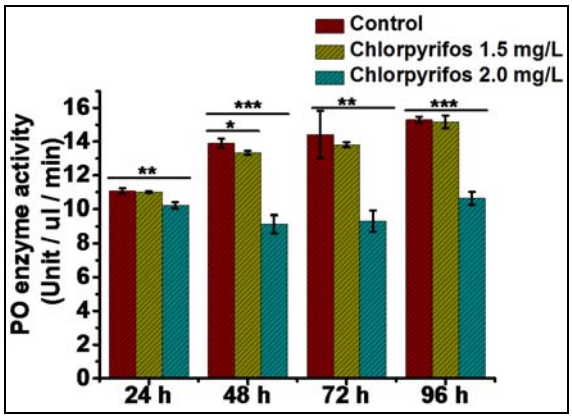

Fig 1: Phenoloxidase enzyme activity in control and chlorpyrifos exposed eri silkworm at different time intervals

3.2 Lysozyme activity assay

Lysozyme activity was measured in response to exposure time and result was presented in Fig. 2. Herein after 24 h of exposure a significant increase in lysozyme activity was recorded in both pesticide exposed groups (F = 8.34, 104.8; P<0.05). However in respect to 24 h, at 48 h the enzyme activity was decreased in pesticide exposed groups but significantly more than the control group (F = 15.83, 10.15; P<0.05). In contrast to this, at 72 h of exposure significant decrease in lysozyme activity was measured at 2.0 mg/L (F = 235.22, P<0.05) but no significant difference was observed at 1.5 mg/L (F = 6.85, P>0.05). Similar type of result was also observed at 96 h of exposure where the enzyme activity was inhibited to 7-18 % (F = 36.16, 158.65; P<0.05).

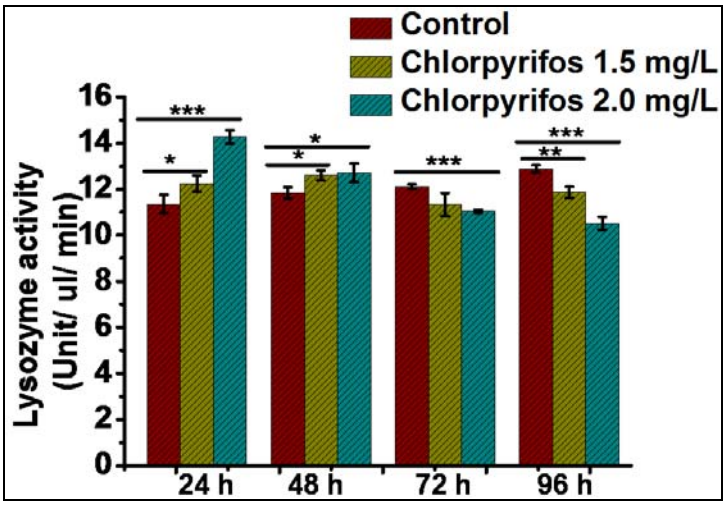

Fig 2: Lysozyme enzyme activity in control and chlorpyrifos exposed eri silkworm at different time intervals

3.3 Total and differential hemocyte count

Hemocyte abundance was measured in function of time and chlorpyrifos concentration and presented in Table 1. Herein at 24 h of exposure smaller concentration (1.5 mg/L) showed no significant difference with the control group; however at 2.0 mg/L decrease in hemocyte number was significant. After 48-96 hr of exposure 1.5 mg/L concentration showed significant reduction in hemocyte abundance and at 2.0 mg/L the reduction was more significant (Table 2).

Similarly differential hemocyte percentage was also studied and presented in Table 3. Results showed that chlorpyrifos induced a significant alteration in different hemocyte abundance (Table 4). Granulocyte percentage increased significantly in chlorpyrifos exposed groups and at 96 h the increase in percentage was 18.80% in 2.0 mg/L chlorpyrifos. Likewise, plasmatocyte percentage was also increased significantly with sub lethal concentration of chlorpyrifos. Prohemocyte percentage decreased to 17.8 – 21.1% (24–96 h) at 1.5 mg/L concentration, whereas at 2.0 mg/L the reduction was 15 – 19% (24–96 h). Similarly spherulocyte percentage was also decreased significantly in chlorpyrifos exposed groups. In control eri silkworm oenocytes abundance was least and in both chlorpyrifos exposed groups percentage got reduced significantly in comparison to control group.

Table 1: Total hemocyte count of *P. ricini* in cells/mm3 in control and chlorpyrifos exposed groups in the time interval of 24h.

	Average no of hemocytes/mm^3			
	Exposure period	→		
	24 h	**48 h**	**72 h**	**96 h**
Control	11618.10 ± 536.44$_a$	11513.50 ± 398.50$_a$	11598.70 ± 568.94$_a$	11567.50 ± 399.09$_a$
Chorpyrifos 1.5 mg/L	11451.40 ± 370.9$_a$	11021.70 ± 505.53$_b$	10830.50 ± 608.5 $_{bb}$	9930.10 ± 336.05$_{bbb}$
Chlorpyrifos 2.0 mg/L	11037.50 ± 479.13$_b$	9950.50 ± 471.15$_{ccc}$	9151.30 ± 243.46$_{ccc}$	8104.50 ± 348.15$_{ccc}$

Values not sharing a common subscript letter within each column differ significantly at P<0.05 level.

Table 2: Analysis of variance (ANOVA) for total hemocyte count in chlorpyrifos exposed groups.

	24 h		**48 h**		**72 h**		**96 h**	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
1.5 mg/L	0.65331	0.42949	5.83704	0.02654	8.5025	0.00922	98.49346	1.00362E-8
2.0 mg/L	6.51594	0.01999	64.15455	2.40898E-7	156.40247	2.58786E-10	427.55898	5.41789E-14

Table 3. Differential hemocyte count of *P. ricini* in percentage in control and chlorpyrifos exposed groups at 24 h exposure time

		Percentage of different hemocytes (% mean ± sd)		
Types of hemocyte	**Time (hr)**	**Control**	**1.5 mg/L chlorpyrifos**	**2.0 mg/L chlorpyrifos**
Granulocytes	24	34.5 ± 0.52 $_{a}$	35.1 ± 0.73 $_{a}$	41.2 ± 1.98 $_{bbb}$
	48	34.3 ± 0.67 $_{a}$	42.1 ± 2.07 $_{bbb}$	50.5 ± 1.84 $_{ccc}$
	72	34.6 ± 0.84 $_{a}$	41.0 ± 1.15 $_{bbb}$	51.9 ± 2.76 $_{ccc}$
	96	34.5 ± 0.70 $_{a}$	44.9 ± 1.19 $_{bbb}$	53.3 ± 3.23 $_{ccc}$
Plasmatocyte	24	27.1 ± 1.10 $_{a}$	27.4 ± 1.07 $_{a}$	38.2 ± 3.81 $_{bbb}$
	48	26.7 ± 1.05 $_{a}$	34.5 ± 2.17 $_{bbb}$	40.7 ± 1.15 $_{ccc}$
	72	27.3 ± 0.82 $_{a}$	36.4 ± 1.26 $_{bbb}$	45.0 ± 0.66 $_{ccc}$
	96	27.1 ± 1.66 $_{a}$	39.1 ± 2.60 $_{bbb}$	40.3 ± 4.13 $_{ccc}$
Prohemocyte	24	22.8 ± 0.42 $_{a}$	21.1 ± 0.87 $_{bbb}$	19.0 ± 0.81 $_{ccc}$
	48	22.9 ± 1.10 $_{a}$	19.9 ± 0.73 $_{bbb}$	17.0 ± 0.66 $_{ccc}$
	72	23.1 ± 1.37 $_{a}$	18.7 ± 0.82 $_{bbb}$	16.3 ± 0.94 $_{ccc}$
	96	23.0 ± 0.47 $_{a}$	17.8 ± 0.78 $_{bbb}$	15.4 ± 0.51 $_{ccc}$
Spherulocyte	24	14.1 ± 0.31 $_{a}$	12.1 ± 1.19 $_{bbb}$	11.0 ± 0.66 $_{ccc}$
	48	13.8 ± 0.63 $_{a}$	11.5 ± 0.70 $_{bbb}$	10.0 ± 0.47 $_{ccc}$
	72	14.1 ± 0.56 $_{a}$	11.5 ± 0.52 $_{bbb}$	10.0 ± 0.47 $_{ccc}$
	96	14.2 ± 0.63 $_{a}$	10.6 ± 0.51 $_{bbb}$	8.7 ± 0.67 $_{ccc}$
Oenocyte	24	9.6 ± 1.17 $_{a}$	9.5 ± 0.70 $_{b}$	8.6 ± 0.51 $_{ccc}$
	48	9.3 ± 1.60 $_{a}$	9.6 ± 1.05 $_{bbb}$	7.9 ± 0.56 $_{ccc}$
	72	9.5 ± 2.04 $_{a}$	9.0 ± 0.81 $_{bbb}$	7.6 ± 0.69 $_{ccc}$
	96	9.0 ± 2.34 $_{a}$	8.8 ± 0.78 $_{bbb}$	6.6 ± 0.51 $_{ccc}$

Values not sharing a common subscript letter within each row differ significantly at $P<0.05$ level.

Table 4: Analysis of variance (ANOVA) for differential hemocyte count in chlorpyrifos exposed *P. ricini* silkworm

	1.5 mg/L chlorpyrifos		2.0 mg/L chlorpyrifos	
	F	***P***	***F***	***P***
Granulocytes				
24 h	4.37838	0.05084	106.03937	5.677E-9
48 h	127.33953	1.34884E-9	682.6474	8.88178E-16
72 h	200.34783	3.38952E-11	357.71713	2.52132E-13
96 h	559.44828	5.21805E-15	322.61258	6.10845E-13
Plasmatocyte				
24 h	0.38028	0.54517	86.29494	2.74201E-8
48 h	104.09886	6.55045E-9	794.59459	2.22045E-16
72 h	363.5561	2.1938E-13	2791.69307	0
96 h	151.04895	3.43169E-10	87.6067	2.44747E-8
Prohemocyte				
24 h	30.6	2.98151E-5	171	1.25039E-10
48 h	51.26582	1.14566E-6	210.26174	2.26895E-11
72 h	75.75652	7.2273E-8	166.464	1.55775E-10
96 h	320.21053	6.51146E-13	1181.45455	0
Spherulocyte				
24 h	26.08696	7.36059E-5	176.5102	9.64145E-11
48 h	58.77778	4.46872E-	232.07143	9.94727E-12
72 h	112.66667	3.54013E-9	248.91429	5.52058E-12
96 h	194.4	4.35086E-11	353.57143	2.78666E-13
Oenocyte				
24 h	11.52	0.00371	40	1.01019E-5
48 h	16.98824	7.99385E-4	63.4717	5.85154E-7
72 h	124.13793	5.98241E-9	271.38462	1.85942E-11
96 h	37.78313	1.4055E-5	131.8806	3.87926E-9

4. Discussion

Similar to vertebrates, insects have a capable immunity against microbial infections exposing in their environment. This immunity based on involved components known as cellular and humeral defenses [15]. Pesticides have prominent effect on the immune system of different non target organisms [16-19]. Galloway and Handy (2002) [20] in their review article focused the immunotoxic effect of organophosphate pesticides and stated that over last 20 years various study proved that organophosphate can interfere with the immune system and exert immunotoxic effect on laboratory organisms. Exposure to high doses of organophosphate pesticides can cause direct damage to cells and organs of immune system and therefore a general decrease in immune function is obvious.

Circulating hemocytes of insect hemolymph plays important role in both cellular as well as humoral immune system. In insect five basic types of hemocytes have been observed named as prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytes. The smallest and basic ones are

prohemocytes which developed into plasmatocytes and granulocytes when an infectious challenge appeared in the hemolyph [18]. Plasmatocytes and granulocytes provide immune responses to pathogens via phagocytosis, nodule formation and encapsulation [21]. In the present study a prominent difference in abundance of circulating hemocytes was observed in chlorpyrifos exposed eri silkworm indicating an alteration in cell mediated immunity of eri silkworm. The increase in plasmatocyte and granulocyte percentage in chlorpyrifos exposed groups signified the immune signal in response to pesticide; however decrease in prohemocyte percentage might be the reason of weak immune system in later periods. Moreover earlier studies showed that prophenoloxidase (PO) system gets activated in insect hemolymph as a stimulus of immune response [22-24]. The melanization reaction is a common response to foreign particle entry in invertebrate animals is due to the activity of phenoloxidase. Herein, at higher concentration of chlorpyrifos PO activity was inhibited in eri silkworm, which indicated the disturbance in PO enzyme activation cascade. This result is in agreement with previous studies wherein exposure to sub lethal concentration of pesticides alters PO enzyme activity and thereby interferes with the immune system [16, 25, 26]. Similarly, lysozyme (EC 3.2.1.17) is a lytic enzyme which plays an important role in the immune defense system of both vertebrates and invertebrate organisms [27, 28]. It can kill microbial cells via both lytic and nonlytic mechanisms [29-31] and also plays role in activation of proPO system. Present study results showed an increase in lysozyme activity in chlorpyrifos exposed silkworm at early period, however after long time exposure the activity of this enzyme inhibited. The alteration in lysozyme activity might be the probable cause of alteration in PO enzyme activity in silkworm as a result of which the defense system is affected. This result is supported by previous study where a sub lethal concentration of pesticide alters lysozyme activity and act as an immunotoxic agent [17].

Conclusion: This study suggests that the chlorpyrifos in the assayed concentrations had a high immunotoxic effect in silkworm. The results clearly showed that silkworm exposed to chlorpyrifos leads to modulation of the immune system involving impairment of humoral and cellular immune functions. However, further research is needed in order to understand the immunomodulation mechanisms of this pesticide in eri silkworm. This might be helpful for safe use of pesticides for sustainable growth of non targetted insect like silkworm for development of seri culture industry.

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