TOXICOLOGICAL AND BIOCHEMICAL EFFECTS OF CHLORPYRIFOS, CHLORFLUAZURON AND OXYMATRINE ON LARVAE OF BOMBYX MORI

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ABSTRACT

The toxicity effect of Oxymatrine, Chlorfluazuran and Chlorpyrifos on the economic characteristics of *Bombyx mori* and their inhibitory effect on acetyl choline esterase and phenol oxidase activity were determined. The data showed that, Oxymatrine was superior in its effect with LC_{50} value of 11.89 mg/l. While, Chlorpyrifos had the lowest effect as their corresponding LC_{50} value was 145.92 mg/l. Chlorpyrifos was the most effective one in the life span (Longevity days) against of both stages followed by Chlorfluazuran against larvae. The data indicated that, treated with Oxymatrine, Chlorfluazuran caused an increased in larval weight at different concentrations. There are no significant different in cocoon weight between control and all concentrations of Oxymatrine, the values were 1.01, 1.16, 1.05 and 1.07g respectively. The obtained data for determination of ChE and phenoloxidase activity revealed that, the data were found to be significant for the decreased activities in all tested insecticides.

INTRODUCTION

Mulberry (Morus spp) is infested by several pests. These pests affect the growth of mulberry and cause considerable damage to the plant and loss in the yield. The insecticides applied for the control of mulberry pests have greater impact on silkworm. Pesticides leave residues in mulberry leaves which intern affect the sensitive silk worm. To overcome this problem, safe waiting period should be followed for leaf harvest (Yokayama, 1962). Field observations in India have indicated loss of yield of cocoons from silk worms fed on mulberry leaves harvested in farms that were sprayed with insecticides (Narasimhanna, 1988). Exposure to the residue of insecticides in the mulberry leaves could affect growth, reproduction. The quality of economic characteristics of cocoons, eclosion and fecundity (Bhosale *et al.*, 1988). *B. mori* had a weak resistance to insecticide, and its production was reduced by more than 30% annually because of insecticide poisoning in china (Bing *et al.*, 2010).

Environmental pollutants, like pesticides have been found to be destructive on different aspects of life. Silkworms, as beneficial insects, are no exception. Due to this many problems have appeared in sericulture as a result of the pesticide applications to cultivations, especially when mulberry tree grow next to cultivated plants. There are few studies that have focussed on the effect of insecticides on

Bombyx mori deal with toxicity, retardation of development and growth, fecundity, mortality, food utilization and economic parameters (Kuribayashi, 1988; Kumar *et. al.*, 1992; Vyjayinthi and Subramanyam 2002, Datta *et. al.*, 2003). Sik *et. al.*, (1976) reported that more than 1.4% of yield reduction in sericulture is due to side effect of pesticide application. 49.4% was due to the application of different pesticides in rice fields, 21.2% in fi-uit gardens and 12.3% in oleri culture. Sharma *et. al.*, (2006) studied the biochemical aspect of pest infested mulberry varieties and their impact on silkworm. Synthetic chemicals have problem of residue in the mulberry leaves which in turn affect sensitive silkworm. To overcome this problem, safe waiting period should be followed for leaf harvest (Yokayama, 1962). Safe period for utilization of insecticide sprayed leaves for silkworm rearing was found to vary from 10- 15days (Ullal and Narasinthamma, 1981; Munivenkatapa *et. al.*, 1989).

The organophosphorus insecticides have been of interest for years because of their toxicological activities in a wide variety of organisms, including the overall changes observed in acetylcholine esterase activity (Nath et,al., 1997). AChE is one of the targets of organophosphate (OP) and carbamate (CB) insecticides. Structural alternation of AChE, resulting in insensitive enzyme, is one of the major mechanisms of the OP and CB resistance in more than 25 arthropod species (Fournier and Mutero 1994). Also Organophosphorus insecticides (OPs) are widely used for a variety of agricultural and public health applications (Maria et al., 2000). OPs produce a wide range of toxicity in mammals by inhibiting acetylcholine esterase (AChE), and the consequent accumulation of the neurotransmitter acetylcholine (ACh) in synaptic junctions leads to excessive stimulation of postsynaptic cells leading to cholinergic toxicity (Radhakrishna and Delvi 1992).

All the insecticides initiated dose dependent changes, resulting in delayed metamorphosis with abnormal wing development no moulting and finally death with in 24 - 48 hrs after treatment (Kodandaram et. al, 2008). The neem based pesticides were highly detrimental to first, second and third instar larvae of *B. mori* causing complete mortality and most of the larvae died with out moulting (Jothi et. al., 1999). Toxicity of cartaphydrochloride, diflubenzuron and lufenuron were detrimental to the growth and development of *B. mori* worms at the lowest concentration of 0.1 ppm leading to significantly higher mortality. (Abhay, *et. al.*, 2008)

The feeding behaviour of pbytophagous insects may change when it feeds on insecticide treated plant as well as when it got exposed to sub lethal dose of insecticides. Alterations in feeding leading to changes in conversion of both ingested and digested food into their body matter which may eventually cause changes in their further development, metamorphosis and also fecundity of resultant adults (Srinivasa and Rao 2003). The larval and pupal critical weight which is an important factor for metamorphosis may be affected by the insecticide treatment. (Nagapasupathy, 2005). Growth was more severely reduced than food intake, and the reduction in growth also occurred during period when food intake was not affected, possibly due to post ingestive effect. (Martinez and VanEmden, 1999). Food intake by larvae, as well as the quality of food ingested affects growth rate, development time, final body weight and survival (Slansky and Scriber, 1985).

B. mori plays an important role during growth, development and the feeding behaviour of phytophagous insects may change when it feeds on insecticide treated plant as well as when it got exposed to sub lethaldose of insecticides. Alterations 4 in feeding leading to changes in conversion of both ingested and digested food into their body matter which may eventually cause changes in their further development, metamorphosis and also fecundity of resultant adults (Srinivasa and Rao 2003). The larval and pupal critical weight which is an important factor for metamorphosis may be affected by the insecticide treatment. (Nagapasupathy, 2005).

The present study determined the effect of the pesticides chlorpirifos oxymatrine and Chlorufluazuran on the economic characteristics of *B. mori* and their inhibitory effect on acetyl choline esterase and phenol oxidase activity.

MATERIAL AND METHODS

Insect culture:-

Eggs of silkworm were obtained from Thailand hybrids and reared during spring season. Patches of 22.00 eggs were hatched on mulberry leaves at 25± 2°C temperature and 75 ± 5 RH. Hatched larvae were distributed in cartoon boxes. The eggs were incubated and larvae were reared following the method of previous workers (Krishnaswami *et. al.*, 1971).

Treatments:-

Freshly fifth instars Leaf discs were thoroughly washed with water and later dipped in the liquid concentrations of insecticides for 20 seconds and then shade dried. The treated leaves were transferred to plastic trays. In each tray, 20 prestarved (for half an hour) silkworm larvae (fifth instar of F I generation) were placed and each treatment was replicated four times. The control was treated with water. The treated leaves were fed to the experimental silk worm larvae only one time on the first day after wards normal feeding schedule was followed). The worms were carefully monitored every day and allowed to spin consumed leaves. Surviving larvae were kept for pupation and observations on various parameters like larval weight during fifth instar, pupal weight, larval duration, and larval, pupal and adult abnormalities were recorded. The recorded data were subjected to analysis to determine the significant difference between the various parameters of the treated and untreated insects.

The pesticides tested

1- Chlorfluazuron (Capris5% EC) 1-[3,5-dichloro-4-(3-chloro-5-trifluoromethyl-2pyridyloxy)phenyl]-3-(2,6 difluorobenzoyl) urea, supplied by Help Co., New Domiat City, Egypt.

2- Oxymatrine (KingBo 0.6% SL) matrine N-oxide ammothamnine, C15H24N202, a natural plant extract from quinolizidine alkaloid; produced from the roots of Sophora flavescens, supplied by EGD Co., Giza Governorate, Egypt.

3- Chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)-phosphorothioate)

Cocoon Pupal and Shell weights:

a) Cocoon weight: The randomly selected cocoons were taken and weighed using an electronic balance and the weight was expressed in grams.

(b) Pupal weight: After removing the floss, the cocoons were cut open and the pupae were taken out without causing any damage to them. Then the pupae were weighed using an electronic balance.

(c) Shell weight: The shell of the cocoon after removing the floss and pupa was weighed using an electropic balance

Biochemical determination:

The activity of Acetylcholinesterase was determined according to the method of Ellman *et al.,* (1961). Determination of phenoloxidase activity was based on a method described by Ishaay *et al.,* (1972). Catechol (Sigma Chemical Co.) at 0.25% was used as a substrate. The frozen dried enzyme powder was dissolved in a phosphate buffer. The enzyme solutions together with the substrate were then incubated for 3 min at 25 'C. The developing color was measured 2 min later using a spectrophotometer model Spekol I I at 470 nm.

$$\text{Inhibition of enzymes \%} = ((SA^o - SA)/ SA^o)*100$$

SA^o=Specific activity without inhibitor (control).

SA= Specific activity in the presence of inhibitor (treated).

RESULTS AND DISCUSSION

Toxicity of tested insecticides to silkworm *B. mori* larvae in the laboratory:

The susceptibility of *B. mori* larvae to Oxymatrine, Chlorfluazuran and Chlorpyrifos were determined and the data are illustrated in Table (1). Based on these data, it is quite clear that the percent mortalities of larva are dosage-dependant. The obtained results of LC_{50} values

(Table 1), it could be noticed that Oxymatrine was superior in its effect with LC_{50} value of 11.89 mg/l. While, Chlorpyrifos had the lowest effect as their corresponding with LC_{50} value of 145.92 mg/l. Based on LC_{50} values (Table 1), the effect of tested pesticides in descending order was Oxymatrine > Chlorfluazuran > Chlorpyrifos. Concerning the obtained slope values, the data represented in table (1), showed that the highest degree of populations homogeneity was obtained towards Oxymatrine which had the highest slope value (3.44).

This result confirmed the previous findings of Kodandaram *et al.*, 2008, reported that all the insecticides initiated dose dependent changes, resulting in delayed metamorphosis with abnormal wing development no moulting and finally death with in 24 - 48 hrs after treatment. Also, the toxicity of cartaphydro chloride, difluberizuron and lufenuron were detrimental to the growth and development of Bombyxntori worms at the lowest concentration of 0.1 ppm leading to significantly higher mortality (Abhay, *et. al.*, 2008)

Table (1): LC_{50} values, their confidence limits deduced from Ld-p lines of the initial toxicity of Oxymatrine, Chlorfluazuran and Chlorpyrifos to *B. mori* larvae.

Concentration (mg/l)	Larvae Mortality (%)	LC_{50} values	Confidence limits		Slope value
			Lower	Upper	
Oxymatrine					
7.5	23.91±5.19 e	11.89	10.54	13.26	3.44
15	64.78±3.72 c				
30	89.07±1.82 a				
Chlorfluazuran					
50	44.92±7.08 d	58.94	45.94	70.30	2.28
100	67.29±2.10 c				
200	89.94±1.79 a				
Chlorpyrifos					
250	73.66±1.05 b	145.92	57.90	199.66	2.72
400	88.66±2.24 a				
500	92.53±2.10 a				

Effect of Oxymatrine, Chlorfluazuran and Chlorpyrifos on life span (Longevity days) of *B. mori* fifth larvae instar

The tested larvae and pupa showed different response to the insecticides effects. Chlorpyrifos was the most effective one in the life span (Longevity days) agents of both stage followed by

Chlorfluazuran agents larvae. All concentrations of Chlorpyrifos caused death for tested Pupa (Table 2).

The data also revealed that although no significant difference was found between control and Chlorfluazuran at 200 mg/l against larvae, the values were 9.66 and 9.33 respectively. Also, Oxymatrine at 7.5 and Chlorfluazuran at 100 mg/l against the tested pupa, the values were 7.00 and 7.66 respectively. On the other hand, there are significant differences between the control and the other concentrations. The period from larvae and pupa was extended after treated with different concentration of Oxymatrine and Chlorfluazuran. Yamanoi (1981) and Kuribayashi (1988) have also reported similar resultes in the effects of insecticides on silkworm life spam.

Table (2): Effect of Oxymatrine, Chlorfluazuran and Chlorpyrifos on life span (Longevity days) of *B. mori* fifth larvae instar

Concentration (mg/l)	Larvae Longevity/day	Pupa Longevity/day
Control	9.66±1.15 d	6.66±1.15 d
Oxymatrine		
7.5	14.33±1.15 a	7.00±1.00 cd
15	11.66±1.52 bc	8.00±1.00 bc
30	10.33±0.57 bcd	9.00±0.10 ab
Chlorfluazuran		
50	12.00±1.73 b	9.66±0.57 a
100	10.00±1.00 cd	7.66±0.57 cd
200	9.33±0.57 d	8.00±0.01 bc
Chlorpyrifos		
250	4.33±1.15 e	0.0
400	0.0	0.0
500	0.0	0.0
LSD0.05	1.78	1.07

Effect of Oxymatrine, Chlorfluazuran and Chlorpyrifos on larvae mean weight (g) consumed leaves (g)/larvae, cocoon, shell and pupa weight (g) of *Bombyx mori*

The larvae mean weight of *Bombyx mori* were recorded in Table (3); it was found out that, there was change in the body weight due to the consumption of tested pesticides. The data indicated that, treated with Oxymatrine, Chlorfluazuran caused an increased in larval weight at different concentration. On the other hand, Chlorpyrifos decreased the weight of tested larval at 250 mg/l compared with control, the values was 1.38 and 3.61 g, respectively. Treated the larval with 400 and 500 mg/l of Chlorpyrifos were Led to the death of all larvae in each treatments.

Table (3): Effect of Oxymatrine, Chlorfluazuran and Chlorpyrifos on larvae mean weight (g) consumed leaves (g)/larvae, cocoon, shell and pupa weight (g) of *B. mori*

Concentration (mg/l)	Larvae Mean weight	Consumed leaves	Consumed Index	Cocoon (g)	Shell weight	Pupa Weight
Control	3.61±0.36 d	19.35±0.98 d	0.55	1.01±0.05c	0.12±0.03a	0.89b
Oxymatrine						
7.5	4.74±0.21 a	26.35±1.50a	0.39	1.16±0.11bc	0.003±0.005c	1.157b
15	4.28±0.34 bc	21.84±1.16 bc	0.44	1.05±0.10c	0.11±0.06a	0.94b
30	4.11±0.13bc	19.51±0.89 d	0.46	1.07±0.07c	0.07±0.02b	1.00b
Chlorfluazuran						
50	4.63±0.33 ab	23.11±1.74 b	0.42	0.78±0.12d	0.0	0.78c
100	4.27± 0.15 bc	20.39±1.37 cd	0.48	1.35±0.18a	0.0	1.35a
200	4.49±0.20 abc	19.15±0.43 d	0.46	1.26±0.10ab	0.0	1.26b
Chlorpyrifos						
250	1.38±0.27 e	9.35±1.99 e	1.57	0.0	0.0	0.0
400	0.0	0.0		0.0	0.0	0.0
500	0.0	0.0		0.0	0.0	0.0
LSD0.05	0.40	2.05		0.16	0.04	

The data of this study shown in Table (3) indicated that, all concentrations of Oxymatrine and Chlorfluazuran caused increase in consumed leaves, except for the high concentration of each.

The Consumed Index was decreased in all treatments of Oxymatrine and Chlorfluazuran compared with control, the control value was 0.55. While the treatment with Chlorpyrifos increased the consumed index compered with control. It is obvious that the quality of mulberry leaves has predominating influence on the development of the worms. This contension is supported by the literature on the influence of quality of mulberry leaves on the growth and development of silk worm, (Ito and Arai 1963; Radha *et. al,* 1978; Pillai and Jolly, 1985) it is therefore concluded that feeding of larvae with pesticides treated leaves had a negative response on silkworm in respect of growth. The impact of pesticide was greater at the beginning of larval stage.

There are no significant different in cocoon weight between control and all concentration of Oxymatrine, the values were 1.01, 1.16, 1.05 and 1.07g respectively. On the other hand, the results showed that there are significant in all treatment with Chlorfluazuran, the cocoon weight were 0.78, 1.35 and 1.26 respectively.

The use of different concentrations of Oxymatrine has resulted in a significant decrease in the shell weight of *B. mori* compared with control. The mean weights of shell were 0.12, 0.003, 0.11 and 0.07g respectively. While other treated with Chlorfluazuran and Chlorpyrifos caused no shell can be weighed (Table 3).

The data presented in table (3) showed that, the mean weight of pupa in control (0.89 g) and Oxymatrine insecticide, the values were 1.157, 0.94 and 1.00 g respectively. There are no significant different between control and all concentration of Oxymatrine. While there was significant different between control and all concentrations of Chlorfluazuran. Dose dependent inhibition of larval growth and morphogenesis in silkworm *B. mori* could be accounted due to reduced physiological change brought about by reduced food intake and weight gains (Joseph, 2000).The present findings demonstrate that the pesticides have the potency to interrupt development and metamorpbosis of *B. mori*.

Effect of some insecticides on Acetylcholinesterase (AChE) and phenoloxidase activity:

The enzymatic reactions are of two types: phase I: reactions, involving oxidation, reduction and hydrolysis, phase II: reactions, consisting of conjugation or synthesis. Phase I reactions generally convert foreign compounds to derivatives that can undergo phase II reactions (Neal, 1980). Some toxicological effects of Oxymatrine, Chlorfluazuran and Chlorpyrifos were determined as in vivo experimental model. The activity of Acetylcholinesterase, phenoloxidase and total protein were determined in fifth larvae instar.

Effect on acetyl choline esterase:

Acetylcholinesterase (AChE) is one of the hydrolytic enzymes for acetylcholine. The inhibition of the enzyme disturbs the normal nervous function (Eto and Olkaw, 1970). Significant inhibitory effect was noticed with Oxymatrine, Chlorfluazuran and Chlorpyrifos with % of inhibition 57.21, 34.61 and 55.91 at different concentration 30, 200 and 500 (mg/l) respectively. Reviewing the results presented in Table (4), one could noticed that, Oxymatrine caused significant inhibition of the I_{50} of AchE, its value was 26.90. On the other hand, Chlorfluazuran was the last one, the I_{50} was 279.57.

The obtained data for determination of ChE activity in *B. mori* fifth larvae instar (Table 4) revealed that, the specific activities of 16.75, 14.87, and 8.22 in case of Oxymatrine at 7.5, 15 and 30 (mg/l) respectively. These data were found to be significant for the decreased activities in all tested insecticides.

The medium inhibition concentrations (I_{50}) of Oxymatrine, Chlorfluazuran and Chlorpyrifos for acetylcholinesterase were 26.90, 279.57 and 175.68 respectively. The specific activities (µmole/mg protein/min) of Oxymatrine, Chlorfluazuran and Chlorpyrifos were (16.75, 14.87 and 8.22), (18.68, 15.30 and 12.56) and (9.21, 8.90 and 8.47) respectively. Based on these data, it is quite clear that the specific activities of larva are dosage-dependant. These results are in agreement with the results of Lang *et al.*, (2010) reported that, several organophosphorus (OP) can selectively kill the silkworm and inhibition the AChE with different concentrations.

Table (4): In vivo effect of Oxymatrine, Chlorfluazuran and Chlorpyrifos on acetylcholinesterase activity of *B. mori* fifth larvae instar

Concentration (mg/l)	Specific activity µmole/mg protein/min	% Inhibitions	I_{50}	Confidence limits		Slope value
				Lower	Upper	
Control	19.22±1.00 a					
Oxymatrine						
7.5	16.75±0.66 b	12.85±3.43f	26.90	22.46	35.21	2.26
15	14.87±0.33 c	22.6±1.71e				
30	8.22±0.40 ef	57.21±2.11a				
Chlorfluazuran						
50	18.68±0.20 a	2.77±1.08g	279.57	215.32	452.76	2.24
100	15.3±0.15 c	20.39±0.82e				
200	12.56±0.08d	34.61±0.43d				
Chlorpyrifos						
250	9.21±0.03 e	52.06±0.18c	175.68	115.32	452.76	0.3
400	8.90±0.06 ef	53.65±0.31bc				
500	8.47±0.02 ef	55.91±0.13ab				
LSD0.05	0.72	2.64				

Effect on phenoloxidase activity:

Phenoloxidase (PO) is a major component of the insect immune system. The enzyme is involved in encapsulation and melanization processes as well as wound healing and cuticle sclerotization (Fournier, 2005).

The activity of Phenoloxidase was recorded in Table (5). In general Phenoloxidase (OP) activities were significantly decreased compared with control. The values were 22.20, 21.5 and 17.96, respectively for Oxymatrine, 25.57, 23.95 and 20.02, respectively, for Chlorfluazuran and20.94, 18.12 and 16.04, respectively for Chlorpyrifos. Based on these data, the specific activities of larva are dosage-dependant.

The results for the I_{50} of Oxymatrine, Chlorfluazuran and Chlorpyrifos on phenoloxidase in *B. mori* fifth larvae instar (Table, 5) were indicative for inhibitory effects in all treatments. Significant reduction was noticed in the enzyme activity, the I_{50} values were 26.90, 279.57 and 175.68 respectively. These resultes are in agreement with the results of Seino *et al.*, 2007.

Table (5): In vivo effect of Oxymatrine, Chlorfluazuran and Chlorpyrifos on phenoloxidase activity of *B. mori* fifth larvae instar

Concentration (mg/l)	Specific activity µmole/mg protein/min	% Inhibitions	I_{50}	Confidence limits		Slope value
				Lower	Upper	
Control	28.14±1.00a					
Oxymatrine						
7.5	22.20±0.05d	21.1±0.19g	26.90	22.46	35.21	2.26
15	21.5±0.10e	23.59±0.37f				
30	17.96±0.11g	36.17±0.39b				
Chlorfluazuran						
50	25.57±0.07b	9.12±0.27I	279.57	21.5.32	452.76	2.24
100	23.95±0.15c	14.87±0.54h				
200	20.02±0.05f	28.83±0.20d				
Chlorpyrifos						
250	20.94±0.07e	25.56±0.25e	175.68	115.32	452.76	0.3
400	18.12±0.03g	35.60±0.12c				
500	16.04±0.04h	42.97±0.14a				
LSD0.05	0.55	0.52				

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الملخص العربى

السمية والآثار البيوكيماوية للمبيدات الاوكسيمترين و الكلورفلازورين و الكلوربيرفوس على يرقات ديدان الحرير.

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تم تقدير التاثيرات السامه لكل من الاوكسيمترين و الكلورفلازورين و الكلوربيرفوس على الخصائص الاقتصادية لديدان الحرير وكذلك التاثير التثبيطى على انزيم الاسيتايل كولين استريز وانزيم الفينول اكسيديز. وقد اوضحت النتائج أن الاوكسيمترين كان اعلى المركبات فى احداث الفعل السام وقيمة الجرعة القاتلة لـ ٥٠% من الافراد المعاملة ١١,٨٩ في حين، الكلوربيريفوس كان أدنى تأثير كما كان قيمتها ١٤٥،٩٢ الكلوربيريفوس كان الأكثر فعالية في التاثير على طول العمر اليرقى (أيام طول العمر)

اوضحت النتائج ان المعاملة بكل من الاوكسيمترين و الكلورفلازورين احدثت زيادة فى وزن اليرقات فى جميع المعاملات. كما اوضحت النتائج انه لا يوجد فرق معنوى فى اوزان الشرنقه وذلك بالمقارنه بين المعاملات بالاوكسيمترين والكنترول وكانت الاوزان كالاتى ١,٠٥،١,١٦،١,٠١ و ١,٠٧ جرام.

اوضحت نتائج التاثير على الانزيمات ان كل المعاملات قد احدثت خفض معنوى فى نشاط الانزيمات محل الدراسة الاسيتايل كولين استريز والفينول اكسيدز.