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Impacts of emamectin benzoate and lemon oil on silkworm *Bombyx mori* (Lepidoptera: Bombycidae)

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Abstract

Unexpected random application of insecticides on fields and farms near mulberry plantations or rearing houses of mulberry silkworm *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) causes a huge loss in the cocoon crop. The aim of this study is to shed light on some chemical compounds used in pest control to show the extent of their impact on economic insects, the most important of which is the mulberry silkworm, which is a model by which to measure the impact of different chemical materials on human health. It estimated the spraying of different concentrations of emamectin benzoate insecticide and lemon oil on mulberry leaves as the only food of mulberry silkworms and the effect of these treatments was evaluated on larval mortality, biological aspects and some enzymes activity of *B. mori*. The results showed that emamectin benzoate has the highest mortality percentage of larvae when treated with concentrations of 0.3 and 0.4 ml/L. However, treatment with lemon oil had the least mortality percentages of larvae when treated with concentrations of 3 and 2ml/l. respectively. Also, LC50 and LC90 were lower for emamectin benzoate than that lemon oil. The toxicity index and probability of emamectin benzoate were higher than that lemon oil. The duration of 5^{th} instar larvae silkworm was treated with emamectin benzoate and lemon oil increased the larval duration of 5^{th} instar larvae as compared to the control. Emamectin benzoate and lemon oil treatment at the highest concentrations cause the death of the larvae before cocooning process and inside the formed cocoons and no pupation happened. The results showed that a high inhibitory effect on amylase, invertase and trehalas activity of 5^{th} instar larvae of silkworms was recorded with emamectin benzoate treatment, while lemon oil treatment caused low inhibition. The same trend happened in acetylcholine esterase activity, ß-esterase activity and a-esterase activity where emamectin benzoate recorded at the highest inhibition rate, while lemon oil recorded low inhibition. Also, total carbohydrate content recorded the lowest values with emamectin benzoate concentrations compared to lemon oil concentrations and the untreated group. It was found that the difference in total protein content was not significant with emamectin benzoate and lemon oil treatments. So, it was recommended that prevent using of these insecticides around mulberry trees and silkworm rearing houses to protect cocoons crops and the silk industry.

Introduction

Mulberry silkworm *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) is a very important insect because of its production of natural silk threads. China is considered the highest production country of silk around the world followed by India. Silkworm is very highly susceptible to chemicals and insecticides sprayed near mulberry trees or behind the sericulture of silkworm. Silkworm, in Egypt, feed on mulberry leaves collected daily from mulberry trees found around and between treated field crops and feeding on previously treated leaves by chemical insecticides or their alternatives cause high mortality in silkworm populations (Elyamani *et al.*, 2017).

Feeding the larvae on these polluted mulberry trees will kill or at least harm the mass rearing of *B. mori* (Bohidar and Choubey, 2005). Insecticides are usually applied at the minimum concentrations to avoid a high mortality in silkworm (Naseema and Shivanandappa, 2003). Hence, mulberry plantations for silkworms must be free of insecticide. However, the complete elimination of insecticides drift is impossible, especially those that are used through aerial spraying, which can damage sericulture activity.

Emamectin benzoate is used against several species of lepidopteran such as *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), *Spodoptera littoralis* (Boisduval), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (El-Sheikh, 2015 and Moustafa *et al.*, 2016), with low toxicity to non-Lepidoptera and most beneficial insects (Jansson *et al*., 1997). Emamectin benzoate, belongs to the avermectin family of 16 –membred macrocylic microorganism, *Streptomyces avermitilis* (Crouch *et al*.,1997). It is composed of ~ 90% avermectin B1a and ~10% of avermectin B1b (Mushtaq *et al*., 1997). Emamectin benzoate could directly active a7nACHR, auß2nAChR, a1ß2y2 GABAA receptor and P1GABAC receptor concentration dependently, with similar potencies for each channel (Xiaojun *et al*., 2016).

Affecting the GABA gated chloride channels, simulating the flow of chloride ions into a neuronal cell with hyperpolarization, the sweep of signal transmission, and disruption of nerve impulses, which leads to death in the end (Jansson *et al*.,1997).

Lemon oil is extracted from the leaves of lemon trees in Egypt. It is used as a natural insect repellant because it contains para-menthane-3, 8, -doil (PMD). The essential oil of some citrus species has been reported to have insecticidal properties against insect pests (Elhag, 2000). Using 1% limonene mixture was safe for most plants and good control of scale insects. Whereas Lemon oil controlled 100 % of mealy bugs and scale insects (Hollingsworth, 2005).

The present study, highlights the extent of the possible toxicity of emamectin benzoate insecticide and lemon oil as natural oil on silkworm performance, also the aim of the work was to analyze biochemical parameters (Changes in the activities of some important enzymes and changes in the total proteins and total carbohydrates of 5^{th} instar larvae of silkworms after treated with emamectin benzoate and lemon oil.

Materials and methods

The present work was carried out in laboratories of Sericulture Research Department, Plant Protection Research Institute and Central Laboratory of Organic Agriculture, Agricultural Research Center, Egypt.

1. Silkworm rearing technique:

The mulberry silkworm *B. mori* (**Local** hybrid) eggs were obtained from Sericulture Research Department of Plant Protection Research Institute (PPRI), Agricultural Research Center, Giza, Egypt**.** The newly hatched larvae were fed on chipped fresh clean mulberry leaves (Variety Balady) until 3rd larval instar and the whole leaves were offered to the mature larval instars (4th and 5th instars). Mulberry silkworm larvae were reared under the laboratory conditions of 26 ± 2 ° C and 75 ± 5 % RH. according to the techniques of Krishnaswami (1978). The 5th larval instar was employed in this experiment. After 4th moulting the larvae were divided into control and two treated groups which were divided into four subgroups each of three replicates.

2.The treatments:

2.1. Emamectin benzoat: It was used as Mix Mikora 7.5 % WDG.

2.2. Lemon oil: It was used as natural oil.

3.Treatment method:

Four concentrations of emamectin benzoate and lemon oil with three replicates were used for each treatment. Fifty individuals of 5th instar larvae were used for each replicate. Mulberry leaves have been sprayed with emamectin benzoate (0.1,0.2,0.3, and 0.4 ml/L.) and with lemon oil (2,3,4,5ml/L.). The treated mulberry leaves were offered once during the first meal of the second day for the 5th instar larvae. After 24 hours the death rate was calculated according to Abbott (1925). LC_{50} values were determined by using the probity statistical analysis method of Finney (1971). The toxicity index for LC_{50} was calculated according to Sun (1950). The 5th instar larvae duration and pupation rate of silkworm were calculated for the treated groups with different concentrations of emamectin benzoate, lemon oil and control.

4. Biochemical analysis:

4.1. Preparation of haemogenate samples of insects for enzyme activities:

Insects were homogenized in distilled water and collected in cold tubes (On ice), then centrifuged at 6000 rpm for 10 min. at 5℃ using (Beckman GS-6R Centrifuge). After centrifugation, the supernatant fluid was divided into small aliquots (0.5 ml) and stored at -20℃ until analysis of enzyme activities. Three replicates were carried out for each enzyme activity.

4.2. Determination of enzyme activities:

4.2.1. Determination of carbohydrates hydrolyzing enzymes activities:

The method was based on the digestion of trehalose, starch and sucrose by trehalase, amylase and invertase, respectively, according to the method described by Ishaaya and Swirski (1976). The free aldehyde group of glucose formed after trehalose, starch and /or sucrose digestion was determined using 3,5 dinitrosalicylic acid reagent, using standard curve of glucose.

4.2.2. Determination of esterases:

Detoxification enzymes such as alpha esterases (a-esterases) and Beta esterases (ß- esterases) were evaluated according to the method of (Van Asperen,1962) using a-and ß-naphthyl acetate as substrates, respectively. The activity of the EST was presented as ug a- *naphthol*/min/g.b.wt.

4.2.3. Determination of acetylcholinesterase (AchE):

Acetylcholinesterase (AchE) activity was measured according to the method described by (Simpson *et al*., 1964) using acetylcholine bromide (AchBr) as a substrate and expressed as ug AchBr/g.b.wt.

4.2.4. Determination of total protein content:

Total proteins were determined by using Coomassie Brilliant blue G-250 reagent and bovine albumin as a standard according to the method of (Bradford, 1976).

4.2.5. Determination of total carbohydrates content:

Total carbohydrates were determined by the method described by (Singh and Sinha,1977) using *anthrone* reagent. Total carbohydrate is expressed as mg glucose/gm larval fresh weight.

5. Statistical analysis:

Statistical analysis was performed using analysis of variance (ANOVA), means were compared using Duncan's test (≤ 0.05) according to Snedecor and Cochran (1982) using Costat V.6.311 (2005) Software.

Results and discussion

1. Larval mortality:

The data in Table (1) indicated that, emamectin benzoate caused a higher negative effect on silkworms than lemon oil specially at the highest concentration 0.4 %. The mortality rates range from 44.00% to 96.00%, respectively for the treated groups with concentrations from 0.1 to 0.4ml/l. While the treatment with lemon oil was less impact on silkworms, it was registered mortality rates ranged from 33.00% to 80.80%, respectively for the treated groups with concentrations from 2 % to 5%.

Table (1): Effect of emamectin benzoat and lemon oil on 5th instar larvae of silkworms after 24 hrs. under laboratory conditions.

Treatments	Con. ml/l.	Observed Response%(Mortality)	Linear Response%	Linear Probit
Emamectin benzoate	0.1	44.00	41.76	4.79
	0.2	70.00	72.05	5.58
	0.3	80.40	85.26	6.04
	0.4	96.00	91.56	6.37
Lemon oil	2	33.00	27.93	4.41
	3	40.40	49.93	4.99
	4	66.00	66.00	5.41
	5	80.80	76.83	5.73

2.Toxic effect:

Table (2) showed that LC_{50} was 0.119 ml/l and 3.00 ml/l for emamectin benzoate and for lemon oil. Also, LC_{90} was 0.368 ml/l and 7.318 ml/l for emamectin benzoate and lemon oil, respectively. The toxicity index was recorded at 100 and 0.039 for emamectin benzoate and lemon oil. The probability was recorded as 0.0876 for emamectin benzoate and 0.551 for lemon oil.

Table (2): Lethal concentrations of emamectin benzoat and lemon oil on 5th instar larvae of silkworms.

Treatments	LC_{50} ml/l.	LC_{90} ml/l.	Slope±S.D.	LC_{50}/LC_{90} Ratio	Toxicity index	Relative potency	P	R
Emamectin benzoate	0.119	0.368	2.63±0.319	0.323	100	25.21	0.0876	0.9560
Lemon oil	3.00	7.318	3.31±0.4526	0.409	0.039	1.00	0.551	0.954

P: Probability. R: Regression.

3. Biological aspects:

Table (3) assure that duration of 5th instar larvae silkworm was 7.2 days for control group, while the treatment with emamectin benzoate and lemon oil elongated the duration of 5th instar larvae. Treatment with 0.2% emamectin benzoate elongated the 5th instar larval duration to 9.3 days followed by 8.9 days for 0.1 ml/l. treatment, 8.6 and 8.5 days for 4 ml/l. and 2ml/l. concentrations of lemon oil.

Table (3): Biological aspects of silkworm treated with emamectin benzoate and lemon oil.

Treatment	Con. ml/l.	Duration of 5th instar larvae (Days)	Pupation rate%
Emamectin benzoate	0.1	8.9 a	52.9% d
	0.2	9.3 a	29.3% e
	0.3	0.0 c	0.0% f
	0.4	0.0 c	0.0% f
Lemon oil	2	8.5 a	80.4% b
	3	8.2 ab	77.3% c
	4	8.6 a	0.0% f
	5	0.0 c	0.0% f
Untreated	-	7.2 b	92.4% a
L.S.D 0.05	-	1.131	1.88
F	-	126.32	3850

Additionally, the application of tested treatments at the highest concentrations 0.4ml/l and 0.3ml/l of emamectin benzoate and 4ml/l and 5ml/ of lemon oil caused full death for the larvae and no cocoons have formed. Farther more, pupation percentages were recorded at 80.4 and 77.3%, respectively for the treatment of 2 and 3ml/l. of lemon oil compared to 92.4% for the control group. We found that the treated larvae with 0.2 and 0.1ml/l. of emamectin benzoate soun thin-layered cocoons and after cocooning failed to pupate and most larvae died inside the cocoons. Similar observations were recorded by Munhoz *et al*. (2013) and their results confirm the toxicity of chlorantraniliprole in silkworm larvae. They found that treated larvae also formed thin-shelled cocoons, which establishes a serious economic problem because this type of cocoon is not suitable for the silk industry.

Also, Tao *et al.* (2007) studied the toxicity of emamectin benzoate on four types of non-target organisms (Partridge, bee, zebrafish and silkworm), the result of the experiment showed that the stomach toxicity by emamectin benzoate for silkworm LD50 was 0.01109 mg/kg after 96 hrs. Moreover, Yanyan *et al*., (2015) found that the emamectin benzoate was consistently the most toxic insecticide among the other five insecticides tested against silkworm as follows: Emamectin benzoate > lambda-cyhalothrin > imidacloprid > chlorpyrifos > dimethoate.

Sunil *et al.*, (2019) revealed that among the eight insecticides tested, exhibited that Lambda-cyhalothrin and emamectin benzoate was highly toxic to silkworms with 100 % mortality even after 30 days after spraying (DAS). Wei *et al*. (2008) found that emamectin benzoate was extremely toxic to silkworms. The similar results to Kordy (2014) who said that the highest mortality of silkworm larvae occurred by the recommended half dose of Abamectin followed by emamectin benzoate after 24 hrs.

Also, Chun (2010) showed that three insecticides, 20% chlorantraniliprole EC, 5% fipronil SC and 50% nitenpyram SG, had a very high level of stomach poisoning toxicity but low level of fumigant toxicity. On the other hand, two insecticides 5% emamectin benzoate WP and 48% chlorpyrifos EC, had high levels of stomach poisoning toxicity and fumigant toxicity**.** As for the use of essential or fixed oils the previous studies proved its useful impact on silkworm specially with low concentrations.

Similar results were recorded by Youssef and Mona (2014) who evaluated the effect of the essential oils (EOs) of fennel (Foeniculum vulgare) and (*Carum carvi*), on instars of

silkworms by using a leaf-dipping bioassay. Their results indicated that the highest larval duration was recorded with increasing concentration of essential oil compared with control, but lowest concentrations had no adverse effects on the growth rate and silk production of the larvae.

4. Biochemical effects:

4.1. Amylase, invertase and trehalas activity:

Table (4) showed that a high inhibitory effect on amylase, invertase and trehalas activity of 5th instar larvae of silkworms with emamectin benzoate was recorded 59.48±2.85, 100.76±3.21 and 499.33±22.60 respectively, while lemon oil and untreated (Control) there is no significant difference. Lemon oil causes low inhibition was recorded 272.71±18.95, 538.55±29.26 and 547.02±8.16, respectively.

Table (4): Changes in amylase, invertase and trehalas activity of 5th instar larvae of silkworms after 24 hrs. with LC 50 under laboratory conditions:

Treatments	Amylase activity µg glucose/min/g body weight Mean±SD	Invertase activity µg glucose/min/g body weight Mean±SD	Trehalase activity µg glucose/min/g body weight Mean±SD
Emamectin benzoate	59.48±2.85	100.76±3.21	499.33±22.60
Lemon oil	272.71±18.95	538.55±29.26	547.02±8.16
Untreated	262±14.58	431.17±54.97	548.47±3.72

4.2. Acetylcholine esterase activity, ß-esterase activity and a-esterase activity:

Table (5) indicated that emamectin benzoate was recorded the highest inhibition rate in acetylcholine esterase activity, ß-esterase activity and a-esterase activity were recorded at 66.83±4.7, 2.88±0.4 and 8.76±0.2, respectively. While Lemon oil was low inhibition was recorded at 164.53± 22.3, 9.74±1.3 and 22.7±0.4, respectively.

Table (5): Changes in Acetylcholine Esterase activity-esterase activity and a-esterase activity of 5th instar larvae of silkworms after 24 hours with LC 50 under laboratory conditions:

Treatments	Acetyl Choline Esterase activity µg acetylcholine bromide/min/g body weight Mean±SD	ß-esterase Activity µg ß naphthol/min/g body weight Mean±SD	a-esterase Activity µg a naphthol/min/g body weight Mean±SD
Emamectin benzoate	66.83±4.7	2.88±0.4	8.76±0.2
Lemon oil	164.53± 22.3	9.74±1.3	22.27±0.4
Untreated	186.9±11.4	4.65±0.9	21.36±3.3

4.3. Total proteins and total carbohydrates:

Table (6) accentuates those total proteins was decreased with emamectin benzoate and lemon oil compared with control, the total proteins were 3.1± 0.2 and 3.4± 0.1(mg/gm body. wt), while total carbohydrates were at the lowest rate with emamectin benzoate compare lemon oil and Untreated.

Table (6): Effect of abamectin benzoat and lemon oil on total proteins and total carbohydrates of 5th instar larvae of silkworms after 24 hours with LC 50 under laboratory conditions.

Treatments	T.proteins (mg/gm body wt.) Mean±SD	T.carbohydrates(mg/gm body wt.) Mean±SD
Emamectin benzoate	3.1±0.2	6.9±0.1
Lemon oil	3.4±0.1	8.1±0.2
Untreated	4.77±0.1	11.4±0.2

These results are similar to Vyjayanthi and Subramanyam (2002) said that changes in the activities of digestive enzymes with fenvalerate-20EC (As contact and stomach action insecticides) in the late stages of multivoltine silkworms. Also, Jiu-Sheng *et al*. (2008) found that sub-lethal dosages (LC5, LC10 and LC20) of abamectin on the 5th instar larvae of silkworm cause inhibited the growth of larvae, the body mass and its increase rate as well as their relative growth rate being lower than control. The decreases in the activity of AChE due to occur as are blocking the action potential of the nervous system caused by the toxic effect of larvae treated with oraganophosphorus insecticides but emamectin benzoate caused inhibits muscle concentration due to the continuous flow of chlorine ions in GABA and H-Glutamate receptor sites (Fanigliulo and Sacchetti, 2008). Our results are in harmony with Abd-El-Aziz (2014) reported that emamectin benzoate inhibited all enzyme activities of the 4th instar larvae of *S. littoralis*. Also, said that emamectin benzoate and indoxacarb high reduction in a-esterases activity. It is come to an end that non-specific esterase plays an important role in the metabolites of insecticides.

Muthusamy *et al*., (2011) said that high doses of insecticides (Temephos, dichlorvos and lambdacyhalothrin) under laboratory conditions caused changes in AChE enzyme activity in *S. litura* from South India. Also, AChE may be due to the decrease in body weight defense against insecticide stress. Carbohydrates are an important role in the growth of insects such as metabolism, reproduction and embryonic (Chapman, 1998).

The reduction of carbohydrate content may be due to the effect of antifeedant and increased metabolism under toxicant stress. The carbohydrate reduction be passed to active glycogenolysis and glycolytic pathway to provide excess energy in stress conditions (Remia *et al.,* 2008). These results are polyphony with Nedal and Dahi (2009) said that larvae of *S.littoralis* collected from fields in Egypt sprayed with spinetoram changes occurred to carbohydrate metabolism

Proteins are important for individual-level fitness-associated traits such as body size, growth rate, fecundity and even biological diversification (Fagan *et al.,* 2002). These results harmony with Assar *et al. (*2016) said that total protein content was decreased with emamectin and spinetoram as bioinsecticides on the 4th instar larvae of *S. littoralis*. Wilkinson (1976) said that protein helps to synthesize microsomal detoxifying enzymes which assist in the detoxification of toxicants that enter the insect body. In general, the problem of protein synthesis is inseparably related to metabolism of nucleic. Frouzan *et al.* (2014) observed a significant difference between the sub lethal concentration (LC10, LC20, LC30 and LC40) from spinosad on *Glyphodes pyloalis* larvae and untreated in the content of carbohydrates.

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