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Comparative Toxicity of Two Bio-Insecticides (Spinotoram and Vertemic) Compared with Methomyl Against *Culex pipiens* and *Anopheles multicolor*

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Abstract: Toxicity of two bio-insecticide (Spinotoram 12% and Vertemic 1.8%) compared with Methomyl (Lannete 90% SP) against lab strain of mosquito species (*Culex pipiens and Anopheles multicolor*). The LC_{50} of Spinotoram 12% and Vertemic 1.8% after 24 h of treatment were (0.380; 1.001 and 0.579; 0.266 $mg.L^{-1}$) x^{-3} for *Cx. pipiens* and *An. Multicolo*. On the other hand; the LC_{50} of Methomyl was 2.455 and 2.468 $mg.L^{-1}$ for the two species after 24h. The biochemical assays of Acetylcholine esterase (AChE), Glutathione-S-transferase (GST) and α-and β-esterases were determined at LC_{50} after 24 h of treatment for both two bio-insecticides and Methomyl. The AChE activity was decreased for all tested pesticides and methomyl 90% gave the most potent enhancement of the activity of AChE after 24h with-29.45 and-47.27% for the *Cx. pipiens and An. multicolor* respectively. On the other hand the specific activity of α-and β-esterase in exposed mosquito decreased significantly ($p<0.05$) after 24h of exposure to different pesticides.

Key words: Bio-insecticides · biochemical assays · *Culex pipiens* · *Anopheles multicolor*

INTRODUCTION

Ecologically, mosquitoes are important components of aquatic and terrestrial food chains as they serve as food for a number of animals, such as fish and birds. With respect to the human well-being, mosquitoes are of great economic impact because their bites are annoying and may cause skin allergies and they are vectors for a number of diseases, such as malaria, yellow fever, dengue, filariasis and certain types of encephalitis such as West Nile Fever.

The members of *Culex pipiens* complex are the most widely distributed species in the world Hoogstraal *et al*. [1]. In Egypt, the common house mosquito *Cx. pipiens molesttus* (Forsk) has been recorded from all governorates without exception [2, 3] causing severe morbidity to man and animals. It is the main vector of *Bancroftian filariasis* [4, 5]. It is also the vector of Rift valley fever in Egypt [6] and diseases caused by other viruses [7]. On the other hand, Anopheline mosquitoes were the major vectors of malaria recorded in Oases and other desert regions [8].

Most of the mosquito control programmers target the larval stage in their breeding sites with larvicide's, because adulticides may only reduce the adult population temporarily [9]. The various synthetic products and devices designed to combat such vectors are not successful because of increased resistance developed by various mosquito species. Also the prolonged use of synthetic insecticide has been accompanied by harmful effects on human health and the environment [10, 11]. Gain of mosquitoes different types of resistance against chemical insecticides is also a matter of concern [12]. Insect populations may survive the effect of toxic chemical compounds by different physiological mechanisms including target site sensitivity and detoxifying enzyme production [13]. The primary routes of insecticide resistance in all insects are alterations in the insecticide target site or changes in the rate at which the insecticide is detoxified. So far esterases, glutathione S-transferases and monooxgenases are known to be involved in the detoxification of the four major groups of insecticides [14]. It has been found that the increased activity of these enzymes will help in the development of physiological resistance to insecticides [15]. Esterases and multifunction oxidases are involved in the resistance to pyrethroids [16]. Also acetylcholinesterase (AChE), responsible for neurotransmitter degradation at the cholinergic nerve synapse, is the target of both

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organophosphate and carbamate insecticides. Selection of a modified AChE less sensitive to these insecticides has been shown to be a common resistance mechanism and was observed in numerous arthropod pest species.

The appearance of such problems has been accompanied by growing interest to use new safe bio-insecticide with a new mode of action specially when dealing with water [17, 18]. Spinotoram is a secondary metabolite of the aerobic fermentation of the naturally occurring soil actinomycete *Saccharopolyspora spinosad* which produces a mix of compounds known as spinosyns A and D [20]. Structurally, Spinosad can be described as a macrocyclic lactone containing a unique tetracyclic ring to which two different sugars are attached. Spinotoram is a powerful neurotoxin against certain arthropods [21-24].

Vertemic is a natural product derived from the soil organism *Streptomyces avermitilis* [25] and is effective at a very low dose level (200 $\mu g.kg^{-1}$) in cattle or sheep. Because of its efficiency and broad-spectrum effectiveness, it has been widely used by farmers all over the world.

Pesticides are frequently released into the environment to control agricultural and public health pests worldwide. Often, the release of these chemicals pollutes the environment and affects non-target organisms. It is sometimes difficult to measure the effects of this pollution, especially if the poisoning is low-level chronic or intermittent. There may be measurable bio-chemical changes that are useful as short-term biomarkers, such as the inhibition of acetylcholinesteras [26].

The aim of this research was to investigate the basis of changes in variation in response to environmental stress. Any changes in variation can be attributed to either an increase in the number of genes expressed or an incease in the expression of particular genes. Thus the measurement of increase invariance about the mean will have direct significance to exposure. The aimed of the present study was undertaken to evaluate the effect of two mosquito species against two bio-insecticides Spinotoram 12% and Vertemic1.8% compared with Methomyl (Lannete 90% SP). Investigations were also conducted to correlate the qualitative and quantitative activities of detoxifying enzymes such as Acetylcholine esterase, GST and Ü-β-esterase.

MATERIALS AND METHODS

Pesticides used: Spinetoram 12% is a new member of the spinosad class of insect management tools developed by Dow Agro Sciences. It is derived from fermentation of *Saccharopolyspora spinosa* as are other spinosad, but fermentation is followed by chemical modification to create the unique active ingredient in spinetoram. Spinetoram will proide long-lasting control of a broad spectrum of insect pests in a variety of crops.

- Vertimec or Abamectin is an Acaricide/ Insecticide that is used for the control of mites and insects on a number of crops including cotton, citrus, pome fruit, vegetables and citus. Molecular formula: $C_{48}\ H_{72}\ O_{14}$ (avermectin B_{1a} + $C_{47}\ H_{70} O_{14}$ (avermectin B_{1b}).
- Methomyl, a conventional insecticide named Lannete 90% SP was selected for purpose of comparison only. It is used as insecticide for a number of crops.

Laboratory bioassay: Two mosquito species (*Cx. pipiens* and *An. multicolor*) were reared in our laboratory, Faculty of Agriculture, Suez Canal University. Pure population of 3rd instar larvael of lab strain of *Cx. pipiens and An. multicolor* was used in this study. Three groups of larvae were as signed to each treatment (20 larvae each). Tap water left for 24 hours was referred as de-chlorinated water was used for the test in addition to the untreated water as a control. Mortality responses were recorded 24h later. A larva was classified as dead if it did not move when gently touched with the point of a toothpick. The experiment was preformed three times on different dates.

The susceptibility of mosquito species (*Cx pipiens and An. multicolor*) were exposed to different concentration of Spinotoram 12%, Vertemic 1.8% and Methomyl 90% SP under laboratory condition using a methodology adapted from the Elliot larval test [34]. Three groups of 20 third instar larvae were placed in 300 mL plastic cups containing a solution of Spinotoram at one of the following concentrations of Spinotoram 12% (0.03125, 0.015625, 0.007812, 0.003906, 0.001953, 0.001464 and 0.0009765 $mg.L^{-1}$) and Vertemic 1.8% using one of the following concentrations (0.03906, 0.0195, 0.00975, 0.00487 and 0.00243 $mg.L^{-1}$). Methomyl 90% SP used as standard product at concentrations of 1.8, 2.0, 2.2, 2.5, 2.7, 2.9 $mg.L^{-1}$ for lab strain.

Mortality responses were recorded 24 h later. A larva was classified as dead if it did not move when gently touched with the point of a toothpick. The experiment was preformed three times on different dates.

Biochemical assays: All the tested were done on larvae of both *Culex pipiens and Anopheles multicolor* after 24 h of treatment by LC_{50} of both Spinotoram 12%; Vertemic 1.8% and Methomyl 90% SP and enzyme activity was measured. All the experiments were preformed three times on different dates.

Total protein content, Esterase enzyme assays, Acetylcholine esterase (AChE) and G**lutathione S-transferase (GST).**

Protein assay: Total protein content of the enzyme preparations supernatant was determined by dye binding method [27] as modified by Spector [28] using bovine serum albumin as the standard. Enzyme activities are expressed in units of µmol/min and are presented as specific activites (µmol/min/mg protein).

Enzyme assays: Non-specific esterases: Nonspecific esterase activity from whole body homogenates was determined using α-and β-naphthyl acetate as substrates, using an adaptation of the method used by Penilla *et al*. [29]. Two larvae were homogenized in 400 µl of 1X phosphate buffer (0.01 mol pH 7.2).The homogenates were centrifuged at 10,000 g for 8 min at 4ºC. 20 µl of the supernatant aliquots of homogenate were placed in ependrof tube. 200 µl of 1.5 mM stock solution of α-and β-naphthyl acetate (NA) in acetone and (0.02 M) of phosphate buffer pH 7.2. The reaction mixture was incubated at 25 ^{0}C for 15 min. At the end of this incubation, 50 µl of Fast Blue B Salt in Sodium lauryl sulphate (3 ml of 1% Fast Blue in water + 7 ml of 5% sodium lauryl sulphate) was added to stop the reaction. Transfer 100 µl to microtitre plate wells, the color intensities were measured at 630 nm for Ü-NA and 570 nm for β-NA with a Dyatech ELISA plate reader. The amount of naphthol produced from the esterase reactions was calculated from standard curves of Ü-and β-naphthol and the results expressed as mMol product. min. mg protein^{-1}.

Acetylcholinesterase assay: AChE activity of supernatant was determined as the methodology described by Bourguet *et al*. [30]. Ten larva were homogenized in 400 µl of 100 mM phosphate buffer pH 7.8 containing 1% Triton X-100. Homogenized was centrifuged at 10.000 g for 8 min at 4ºC and the homogenization was carried out on ice. For AChE assay, two replicates of 100 µl of homogenate were transferred to a fresh microtitre plate. 100 µl of 2 mM dithio-bis 2-nitrobenaic acid in 0.1 M phosphate buffer pH7.0 and 2 mM acetylthiocholine iodide in distillate water. The kinetics of the enzyme reaction were monitored continuously at 405 nm for 5 and 15 minutes in a microtitre plate reader. Activity was evaluated as the increase in absorbance between both time points. Absorbance values were converted to units of concentration using a molar extinction coefficient of 13.6 mM^{-1} cm^{-1}for nitrobenzoic acid.The specific activity was expressed as µmol thionitrobenzoic acid produced /min/ml/mg protein.

Glutathion S-transferases assay: GST activity was estimated following the procedure of Habig *et al*. [31] 40 µl from 70 mM 1-chloro-2,4 dinitrobenzene (CDNB); 40 µl 70 mM glutathione reduced (GSH) was added and the volume adjusted to 1.9 ml with 0.1 M (100 mM potassium phosphate buffer pH 6.5). After pre-incubation of the reaction mixture for 5 in at room temperature, 100 µl of the two larval homogenate in 400 µl of 0.1 M phosphate buffer pH 6.5. The change in the absorbance level was noted at 340 nm for 3 min after every 1 min in the UV spectrophotometer. For control, reaction mixture without the enzyme was used as blank.

Statistical analyses: Bioassay data were corrected for control mortality according to Abbott's formula [32] and analyzed with a probity computer program. Biochemical data were subjected to variance analysis (ANOVA) using SAS version 6 [33]. Abbott's formula was used to correct the observed mortality in adult susceptibility tests [34]. The LC_{50} and LC90 values were estimated using dosage mortality regression probit analysis [35]. Observed differences in resistance between generations were analyzed by Student s t-test. A one-way analysis of variance (ANOVA) was used to compare the protein content and enzyme expression levels within and between populations. All levels of statistical significance were determined at <0.05.

RESULTS AND DISCUSSION

For the control group no mortality was observed. In all groups treated with Spinetoram 12%, Vertinec 1.8% and Methomyl 90% the mortality was important and it increased with their concentrations. Results in Table 1 shown the different parameter of LC of the two bio-insecticide (Spinetoram 12% and Vertinec 1.8%) compeer with Methomyl 90% against two mosquito species (*Cx pipiens and Anopheles multicolor*). As shown the LC_{50} after 24 h of treatment were (0.380, 1.001, 0.579 and 0.266 mg.L^{-1}) X^{-3} for *Cx. pipiens* and *An. multicolor* against Spinotoram and Vertemic respectively. On the other hand, the LC_{50} of Methomyl was 2.455 and 2.468 mg.L^{-1} for the two species respectively. Also the slope of regression lines of the two bio-insecticide was high compeer with the Methomyl for both species. Results in Table 2 clearly indicated that the larval instar of both species tested by Methomyl was more susceptible than larval instar tested by the two bio-isecticide the value of the toxicity was less than one x^{-3}. The toxicity index of two bio-insecticide against Methomyl was

Table 1: Toxicity of two potential bio-insecticide and chemical insecticides against 3rd larvae stages of *Cx pipiens and An. multicolor* after 24 h of treated

Toxicity parameter	Spinetoram 12%		Vertimec 1.8%		Methomyl 90%*	
$X10^{-3}$	*Cx. pipiens*	*An. multicolor*	*Cx. pipiens*	*An. Multicolor*	*Cx. pipiens*	*An. Multicolor*
LC_{10}	0.016 (0.003-0.071)	0.044 (0.013-0.144)	0.014 (0.002-0.083)	0.006 (0.001-0.051)	1.621 (1.45-1.81)	1.628(1.457-1.818)
LC_{20}	0.047 (0.016-0.140)	0.128 (0.056-0.291)	0.05 (0.14-0.17)	0.021 (0.004-0.105)	1.868 (1.73-2.02)	1.876 (1.74-2.03)
LC_{50}	0.380 (0.218-0.661)	1.001 (o.56-1.81)	0.579 (0.307-1.09)	0.266 (0.133-0.531)	2.455 (2.35-2.57)	2.468 (2.36-2.59)
LC_{90}	9.180 (2.408-34.996)	22.863 (4.563-114.56)	24.108 (3.544-163.97)	12.248 (2.17-69.13)	3.719 (3.25-4.26)	3.741 (3.27-4.29)
Slope	11.839	11.330	18.065	19.546	1.380	1.381
Alpha (0.05)						
CHI2 (X^2)	3.47<11.08 5, 0.05	0.20<11.08 5, 0.05	0.17<11.08 5, 0.05	0.53<11.08 5, 0.05	4.05<9.5 4,0.05	1.91<9.5 4,0.05

The value LC of Spintoram and Vertimec $x10^{-3}$, *The value LC of Methomyl as its

Table 2: Toxicity index at LC of potential bio-insecticide against Methomyl of 3rd larval stages of *Cx pipiens and An multicolor* after 24 h of treated

	Spinetoram 12%		Vertimec 1.8%	
Toxicity index*	*Cx. pipiens*	*An. multicolor*	*Cx. pipiens*	*An. Multicolor*
LC_{50}	0.155	0.406	0.236	0.108
LC_{90}	2.468	6.148	6.482	3.274

*Percentage = X^{-3}

Table 3: Effects of LC_{50} of two potential bio-insecticide and Methomyl of 3rd larval stages of *Cx pipiens and An multicolor* on total protein (mean±SD) after 24 h of treated

	Cx pipiens	*An. multicolor*
Control	0.2983±0.00800	0.2939±0.00560
Spinototam 12%	0.3159±0.02060	0.3304±0.01711
Vertemic 1.8%	0.2967±0.01162	0.3033±0.01517
Methomyl 90%SP	0.3329±0.03500	0.3106±0.20440

*Protein content µg. g fresh $weight^{-1}$

0.155 and 0.236 for *Cx. pipiens* and 0.406 and 0.108 for *An. multicolor*.

Pesticides are frequently released into the environment to control agricultural and public health pests worldwide. Often, the release of these chemicals pollutes the environment and affects non-target organisms. It is sometimes difficult to measure the effects of this pollution, especially if the poisoning is low-level chronic or intermittent. There may be measurable bio-chemical changes that are useful as short-term biomarkers, such as the inhibition of acetylcholinesteras [26]. The present study, an model to investigate the basis of changes in variation in response to environmental stress. Any changes in variation can be attributed to either an increase in the number of genes expressed or an incease in the expression of particular genes. Thus the measurement of increase invariance about the mean will have direct significance to exposure.

Also the results clearly indicate that Spinetoram 12% and Vertinec 1.8% is highly toxic to both species of the larval of *Cx. pipiens and An. multicolor* compared with Methomyl 90%; These result agree with the results of tested Spinosad against larval of *Cx. pipiens* by Bahgat *et al*. [21] also with other aquatic organisms such as rainbow trout fish (30 ppm), carp fish (5 ppm) *Daphnia magna* (97.7 ppm) and grass shrimp (9.8 ppm) as reported by Smith and Grothe [36], Weinberg *et al*. [37] and Bret *et al*. [38]. Spinosad and other generation Spinotoram is 1,000 to 10,000 times less toxic to fish than many synthetic insecticides such as pyrethroids. Also it was only slightly to moderately toxic to most aquatic invertebrates and is at least 2 to 5 times less toxic than most synthetic alternatives [39-42]. Also, they demonstrates low mammalian and environmental toxicity with reduced risk to humans and other forms of wildlife comparable to traditional biological insecticides [38].

Chemical insecticides play a major role in vector control. However, the continuous and indiscriminate use of insecticide in a population will lead to the development of physiological resistance in the insects. The present results clearly suggest the differential effect of the bio-insecticides on two species of mosquito.

The effect of the tested pesticides on the total protein content in the two mosquito species shown in (Table 3). The results found that no significant differences between susceptible control and the tested pesticides ($p>0.05$).

Table 4: Effects of LC_{50} of Spinotoram 12% on some biochemical analyzes of 3rd stage larval of *Cx. pipiens and An. multicolor* mosquito after 24 h (Mean±SD)

	Cx. Pipiens		***An. multicolor***	
Enzymes analyses	Control	Treated	Control	Treated
α-esterase	13.947±0.724	10.388±0.981	19.153±0.700	17.323±0.0 59
β-esterase	9.244±0.361	5.249±0.488	9.238±0.805	8.01±0.493
GST	0.900±0.095	0.869±0.057	0.893±0.057	0.679±0.010
AChE	0.1291±0.013	0.0906±0.045	0.1652±0.003	0.0873±0.026

Esterase activity (U.mg protein^{-1}), GST (nmol CDNB.min^{-1}.mg protein^{-1}), AChE (mol.min^{-1} mg^{-1} protein

Table 5: Effects of LC_{50} Vertemic 1,8% on some biochemical analyzes of 3rd stage larval of *Cx. pipiens and An. multicolor* mosquito after 24 h (Mean±SD)

	Cx. pipiens		*An. multicolor*	
Enzymes analyzes	Control	Treated	Control	Treated
α-esterase	13.90±0.66	11.5378±0.60	18.325±0.200	16.972±0.418
β-esterase	10.234±0.565	7.2432±0.299	10.523±0.254	8.792±0.477
GST	0.8671±0.014	0.7325±0.073	0.854±0.04	0.6175±0.065
AChE	0.1291±0.013	0.1193±0.011	0.1652±0.003	0.098±0.006

Esterase activity (U.mg protein^{-1}), GST (nmol CDNB.min^{-1}.mg protein^{-1}), AChE (mol min^{-1} mg^{-1} protein)

Table 6: Effects of LC_{50} of Methomyl 90% SP on some biochemical analyzes of 3rd stage larval of *Cx. pipiens and An multicolor* mosquito after 24 h (Mean±SD)

	Cx. pipiens		*An. multicolor*	
Enzymes analyzes	Control	Treated	Control	Treated
α-esterase	12.970±0.188	11.494±0.400	18.325±0.200	8.314±0.25
β-esterase	10.234±0.565	4.597±0.216	10.255±0.459	8.059±0.082
GST	0.623±0.020	0.667±0.050	0.879±0.050	0.767±0.053
AChE	0.1291±0.013	0.109±0.037	0.1652±0.003	0.118±0.009

Esterase activity (U.mg protein^{-1}), GST (nmol CDNB.min^{-1}.mg protein^{-1}), AChE (mol min^{-1} mg^{-1} protein)

The effect of the three pesticides on the activities of α-and β esterase in the two mosquito species shown in (Table 4-6) indicate that the specific activity of each of the two enzymes was decreased in all pesticide-treated mosquito compared with the parallel control. Among the tested pesticides, Methomyl 90% SP gave the most potent enhancement of the activity of both enzymes after 24 h with-54.64 and 50.32% for Ü-esterase and β-esterase, respectively (Table 4-6).

Nonspecific esterases have been reported to be involved in these insecticides metabolism in several insects and could play a role in the metabolism of these insecticides in mosquito species. Even in some cases, there is evidence that esterases are involved in conferring cross-resistance to pesticides in the larvae and adults of *A. albimanus* [43].

As for the effect of tested pesticides on GST, results in Table 4-6 showed that all tested pesticides reduced GST compared to untreated mosquito. The effect of Methomyl 90% SP was more pronounced with-24.13 and 12.5% of GST after 24 h for the *Cx. pipiens and An. multicolor* respectively compared to the other tested pesticides.

The decrease of GST observed after 24 h of exposure can be in relation directly or throughout glutathione to numerous physiological processes, which could be perturbed by this pesticides intoxication. Glutathione is involved in several cellular functions: Phase II metabolism through GST activity and antioxidative defense in relation to redox capacity through GST, GPx and GR activities [44-46]. A decrease of GST activity is observable when the cellular defenses are spillover [47]. So GST decrease can be due to a toxic impact of the used pesticides whatever the other cellular defenses were always available. It can be a first step before subsequent degenerative process induced by this pesticide.

Also the glutathione is the non proteinic thiol the most abundant in the organisms where it plays a crucial

role in intracellular protection against the reactive species of oxygen (mainly through enzymatic activities such as GST, GR, but also glutathione peroxides (GPx): [47, 48]. The glutathione is implied in metabolism of phase II by the activity of the GST and in antioxidant defences compared to its capacity redox by activities of GST, GPx and GRd [46, 49-52]. Moreover the glutathione acts as a source of amino acid for the synthesis of the proteins.

Also GSTs are a group of multifunctional proteins serving several roles in detoxification [53]. Distribution of GSTs is known to be widespread in nature and there is no question about the importance of these enzyme systems for they are essential in explaining selective toxicity and resistance mechanism among various organisms.

The effect of the three pesticides on the activities of AchE in the two mosquito species shown in (Table 4-6) indicate that the specific activity of AchE was decreased in all bio-pesticide treated mosquito compared with the parallel control. Among the tested pesticides, Methomyl 90% SP gave the most potent enhancement of the activity of AchE after 24h with-29.45 and-47.27% for the *Cx. pipiens and An. multicolor*,respectively compared to the other tested pesticides (Table 4-6) Inhibition of AChE by three tested insecticides causes a desensitization of the acetylcholine receptor and leads to eventual death of the organism. Also acetylcholine esterase is an enzyme that occurs in the central nervous system. It functions by removing acetylcholine from its postsynaptic receptor. The result of this action is the hydrolysis of acetylcholine into acetate and prolonged neuroexcitation. Methomyl like the other organophosphate insecticides are generally very long or even irreversible inhibitors of acetylcholinesterase. Organophosphates must be enzyme matically activated to their oxygen metabolites by endogenous oxidize enzymes (the cytochromes P450) before they can serve as effective acetylcholinesterase inhibitors.

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