Toxicity and Joint Toxic Action of Some Control Agents on *Culex pipiens* L. Larvae

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ABSTRACT

The present study aimed to assess and to evaluate some of the sustainable and safe measures against *Culex pipiens* mosquito larva that can be implemented in areas at risk of contacting elephantiasis. The joint toxic action of these control measures was also studied. Data showed that Deltamethrin (LC_{50}=0.021ppm) was more toxic than *Bacillus thurengiensis* var. *israelensis*, diflubenzuron, emamectin benzoate and azadiractin by 2.0, 491.42, 59.0 and 143.8 fold, respectively, after 24hr. Also, after 48hr Deltamethrin (LC_{50} = 0.004 ppm) was 4.0, 1102.5, 25 and 232.5 times more toxic than Vectobac G, diflubenzuron, emamectin benzoate and azadiractin, respectively, after 48hr. After 72hr the toxicity of Deltamethrin (LC_{50}=0.003 ppm) was 3.0, 206.66, 23.33, 246.66 fold more toxic than *B.t.i.*, diflubenzuron, emamectin benzoate and azadiractin, respectively. Toxicity of *Beauveria bassiana* was very low compared with all the tested control measures with LC_{50} value 1.85ml/L after 72hr of exposure. Data of joint toxic action revealed that, all mixtures of *Beauveria bassiana* with diflubenzuron and all mixtures of diflubenzuron with azadiractin resulted in antagonistic effects. Approximately, all other binary mixtures resulted in potentiating effects. The highest potentiating effect was obtained when the mixture of $LC_{12.5}$ *B.t.i.* + $LC_{12.5}$ Deltamethrin was used. Finally, the use of some binary mixtures of the tested control measures can achieve better control, reduce control cost and induce lesser environmental pollution.

INTRODUCTION

Mosquitoes, one of the major arthropods carriers, spread diseases and cause havoc for millions of people in developing countries both among urban and rural populations. The loss in terms of human's lives is irrevocable. It is estimated that every year, at least 600 million people suffer from malaria, filariasis, encephalitis, dengue and recently chikungunya (Ravikiran and Sita Devi 2007). *Culex quinquefasciatus* (Say) is a major disease transmission vector in Africa as well as in other tropical regions of the world and has been shown to be directly responsible for 80 million annual lymphatic filariasis of which 30 million cases exists in chronic infection. The present proliferation of this disease is not only due to higher number of breeding places in urban area, but also due to increasing resistance of mosquitoes to current commercial insecticides such as organo-chlorides, organophosphates, pyrethroid and carbamates (Das and Amalraj, 1997).

The integrated vector control (IVC), is an ecologically based approach that may involve several complementary interventions used in combination or singly (Lacey and Lacey, 1990). Control measures against filariasis vectors are primarily with conventional adulticides, but the vectors became resistant especially to organophosphorus insecticides due to repeated applications (Juan *et al.*, 1997). So, the use of microbial larvicides such as *Bacillus sphaericus* (*B.s.*) and *Bacillus thuringensis* var. *israelensis* (*B.t.i.*) as mosquito larvicides is receiving increasing attention (Nielsen-Leroux, *et al.*, 1995).

Insect growth regulators (IGRs) also have high levels of activity and efficacy against various species of mosquitoes in a variety of habitats. They are likely to supplementing microbial larvicides, pyrethroids and organophosphorus larvicides (Mulla *et al.*, 1989). A number of benzoylphenylurea (BPU) derivatives have been developed such as triflumuron, which is described as molt inhibitor through interference with cuticle deposition and chitin biosynthesis (Belinato *et al.*, 2009).

Also, insecticidal active ingredients from the neem tree (*Azadirachta indica* A. Juss) have been recommended in IVC (Parmar, 1993). Neem products are characterized by their effect on growth disrupting (Dua *et al.*, 2009). For dipterous pests, their effects on oviposition, repellence have been reported (Chen *et al.*, 1996). Neem products are known for their fast breakdown of azadirachtin under field conditions (Schmutterer, 1997), so it was used in mixtures for prolongation of its duration of action.

Additionally emamectin benzoate, the semi-synthetic of abamectin which produced by fermentation of *Streptomyces avermitilis*, is known to have potent toxic activity (Miller *et al.*, 1979) in parasitic disease and was extremely toxic at low concentrations to a wide range of insects including members of the order Diptera (Putter *et al.*, 1981).

The main objective of the present study was to investigate the toxicity of five control agents on the 3rd instar larvae of *C.pipiens* L. Also, this work was carried

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out to study the toxicity of binary mixtures of these control agents aiming to reduce the doses of these insecticides as a primary purpose.

MATERIALS AND METHODS

The tested materials:

The following commercially formulations were used:

Biofly® (*Beauveria bassiana*) was obtained form Biotech Manufacture, El-Sadat City. Spore count was done in haemocytometer and was 3×10^7 conidia /ml. Vectobac® G (*Bacillus thuringiensis* var. *israelensis* 5000 ITU/mg) was provided by Abbott laboratories, North Chicago IL, USA, as a corncob formulation. Dudim®4%G (diflubenzuron; DML), 1-(4-Chlorophenyl)-3-(2,6-difluorobenzoyl) urea was supplied by Duphar B.V., Weesp (Holland). Proclaim® 5% SG (emamectin benzoate) was supplied by Syngenta. Achook® 0.15% EC (azadiractin) was provided by the Egyptian Agricultural development Co. (Egypt) as natural extract. Embrator®2.5% EC (Deltamethrin (DLM)), ((S)-α-Cyano-m-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2dimethylcyclopropane carboxylate) was supplied by KZ Co.(Egypt). Embrator® served as a reference compound for mosquito control based on WHO guidelines.

Insects:

The used *Culex pipiens* L. (Diptera: Culicidae) colony was maintained in the laboratory of Medical and Veterinary Insects, Department of Applied Entomology, faculty of Agriculture, Alexandria University, for more than 10 years. Mosquitoes were held at 27±1 °C, 70±5% RH, and a photo regime of 14:10 (light:dark) hr. Adults were provided with a 10% sucrose solution as food source. A pigeon was introduced twice a week to the adults for blood feeding. Larvae were reared in dechlorinated water under the same temperature and light conditions and were fed daily with baby fish food.

Bioassay procedures:

The larval susceptibility test was conducted according to WHO guidelines (WHO; 1975, 1981). Third instar larvae were used for assessment of the larval susceptibility to the tested compounds. Sufficient numbers of larvae in the 3rd instar were kept in the same breeding water till the test was carried out. Series of concentrations for each compound in addition to control were replicated four times (range of concentrations is shown in Table 1). Lots of 30 larvae were distributed in each replicate (glass beaker), containing 100ml of water. All the experiments were conducted at 27±1C° and 75± 5%RH. Mortality counts were carried out after 24, 48 and 72hr of treatment. Mortality percentages were calculated and corrected according to Abbott, (1925). The larvae that had pupated during the test were discarded. If more than 10% of control larvae pupate in the course of the experiment, the test was discarded. The LC-p lines were plotted on log-probit sheets. Values of $LC_{12.5}$, LC_{25}, LC_{50}, Confidence limits and slope functions were calculated and ascertained using Probit program (Finney, 1971).

The joint action of the tested insecticides mixtures:

To determine the joint toxic action of the tested compounds on *C.pipiens* L., the calculated $LC_{12.5}$, LC_{25} and LC_{50} (after 72hr) were used alone (to calculate the expected mortalities) and in bi-mixtures. For each treatment, four replicates of 30 larvae/replicate were used. Percent mortalities of larvae were recorded after 72hr post-treatment.

The joint action of different mixtures in terms of co-toxicity factor (C.F.) was estimated according to Mansour *et al.*, (1966) using the following equati

$$Co\text{-}toxicity\,factor = \frac{observed\,\%\,mortality - expected\,\%\,mortality}{expected\,\%\,mortality} \times 100$$

A positive factor of 20 or more is considered potentiation, a negative factor of 20 or more means antagonism and intermediate values between -20 and +20 indicate only additive effect.

Statistical analysis

Data of bioassay were analyzed using probit program (Finney *et al.*, 1971).

RESULTS AND DISCUSSIONS

Susceptibility of C. *pipiens* to some control agents:

The intension of the statistical analysis proved the insignificant heterogeneity of the results and the goodness of fit of the drawn LC-p lines, as the experimental $(Chi)^2$ values were less than those of the tabulated ones at 5% probability levels. The median lethal concentration (LC_{50}) values with their fiducial limits and the slope of the lines were summarized in Table (1). Data have disclosed that Deltamethrin (LC_{50}=0.021ppm) was more toxic than *B.t.i.*, diflubenzuron, emamectin benzoate and azadiractin by 2.0, 491.42, 59.0 and 143.8 fold, respectively, after 24hr. Also, Deltamethrin (LC_{50}= 0.004 ppm) was 4.0, 1102.5, 25 and 232.5 times more toxic than *B.t.i.*, diflubenzuron, Proclaim and azadiractin, respectively, after 48hr. After 72hr the toxicity of Embrator was 3.0, 206.66, 23.33, 246.66 fold more toxic than *B.t.i.*, diflubenzuron, emamectin benzoate and azadiractin. Toxicity of *Beauveria bassiana* was very low compared with all the tested control measures with LC_{50} value 1.85ml/L after 72hr of exposure.

Table 1. Susceptibility of 3rd instar larvae of *C.pipiens* to different insecticidal compounds

Compound	Concentration range	Time (hr)	Slope	LC_{50}*
Beauveria bassiana	1.5-3.5 ml/L	24	2.07	4.122(3.525-5.365)
		48	2.71	2.968(2.693-3.325)
		72	2.64	1.85(1.21-2.72)
B.t.i.	0.01-5.0 ppm	24	1.12	0.044(0.036-0.054)
		48	1.96	0.016(0.013-0.019)
		72	2.04	0.009(0.007-0.012)
Diflubenzuron	1-120 ppm	24	1.73	10.32(7.41-15.64)
		48	1.65	4.41(0.246-33.147)
		72	1.84	0.62(2.674-16.166)
Emamectin benzoate	0.05-20 ppm	24	1.35	1.24(1.01-1.51)
		48	1.50	0.10(0.07-0.14)
		72	1.60	0.07(0.05-0.09)
Azadiractin	0.5-5.0 ppm	24	1.99	3.02(2.41-3.74)
		48	2.11	0.93(0.78-1.08)
		72	2.04	0.74(0.61-0.90)
Deltamethrin **	0.001-10 ppm	24	1.97	0.021(0.051-0.028)
		48	1.85	0.004(0.003-0.006)
		72	2.00	0.003(0.002-0.005)

*Concentration required killing 50% of the larvae.
**Reference compound.

The obtained data are strengthened by other previous reports that demonstrate the efficacy of bacterial pesticides. The *B.t.i* formulation; Vectobac G was found to be effective in reducing larval populations of *Culex pipiens* and *Aedes aegypti* at the 0.01 mg/liter level and susceptibility varied according to different species and strains of mosquito larvae (Chui *et al.*, 1993).

Regarding *Beauveria bassiana*, larvae may have rejected the spore mass as food most likely because of large clump size and the density of the mass made spore attachment avoidable (Prasad and Veerwal, 2010). After inoculation with *Beauveria bassiana*, fungal isolation, the hypha penetrated the integument inside the whole body cavities then reached all cells like fat, neural and muscle tissues and damage them. Even though, *Beauveria bassiana* spores act as midgut toxins, when applied over the water surface they are readily available to the larvae (Hajek and Leger, 1994).

The high efficiency of microbial agents is due to its specificity in action where its toxic protein molecules (delta-endotoxins; 25-KDa proteins in case of *B.t.i.*) attach to the accessible mosquito midgut cell membrane receptor, most likely phospholipids and glycoconjugates in a progressive and irreversible manner to generate small pores. The creation of these spores will lead to colloid-osmotic lysis, i.e. cell swelling and eventual lysis (Chilcott, *et al.*, 1990).

Therefore, the most toxic tested formulations; *B.t.i.* and deltamethrin (reference compound) are recommended for control of *C.pipiens*, however the hazardous effect of classical chemical insecticide; deltamethrin on the beneficial insects and the surrounding ecosystem should be taken into consideration if it will be applied in the field on large scale. The obtained data indicated that IGR Dudim (diflubenzuron) proved to have a delayed effect on *C. pipiens* larvae for the first 72hr after treatment. The obtained findings are in agreement with a previous study (Kawada *et al.*, 1993) that discussed the larvicidal efficacy of IGR Dudim (diflubenzuron) on anopheline mosquitoes (*A. sergenti, gambiae, albimanus*). In all species, no cross resistance between IGRs and the other kinds of insecticides, such as organophosphate, organochlorine, carbamate and pyrethroid, was observed. The mosquitocidal effects observed in triflumuron-treated larvae were similar to those commonly induced by diflubenzuron (Seccacini, *et al.*, 2008).

Achook (0.15% azadirachtin) was found to have larvicidal activity (LC_{50}= 3.02 ppm after 24 hr post-treatment) on *C. pipiens* larvae. The larvicidal effect of

Table 2.The joint-action of different bi-mixtures of the tested compounds against the 3rd instar larvae of *C.pipiens* after 72hr post-treatment

LC levels[a] (Bi-mixture)	% mortality		C.F. [b]	Joint action [c]
	Expected	Observed		
$LC_{12.5}$ *Beauveria bassiana* + LC_{25} Diflubenzuron	37.50	19.33	-48.45	A
$LC_{12.5}$ *Beauveria bassiana* + LC_{50} Diflubenzuron	62.50	23.00	-63.20	A
LC_{25} *Beauveria bassiana* + LC_{25} Diflubenzuron	50.00	21.67	-56.66	A
LC_{25} *Beauveria bassiana* + LC_{50} Diflubenzuron	75.00	29.00	-61.33	A
$LC_{12.5}$ *Beauveria bassiana* + $LC_{12.5}$ Azadiractin	25.00	59.67	138.68	P
$LC_{12.5}$ *Beauveria bassiana* + LC_{25} Azadiractin	37.50	68.00	81.33	P
LC_{25} *Beauveria bassiana* + $LC_{12.5}$ Azadiractin	37.50	60.00	60.88	P
LC_{25} *Beauveria bassiana* + LC_{25} Azadiractin	50.00	85.67	71.34	P
$LC_{12.5}$ *Beauveria bassiana* + $LC_{12.5}$ Deltamethrin	25.00	71.33	185.32	P
$LC_{12.5}$ *Beauveria bassiana* + LC_{25} Deltamethrin	37.50	75.00	100	P
LC_{25} *Beauveria bassiana* + $LC_{12.5}$ Deltamethrin	37.50	72.67	93.78	P
LC_{25} *Beauveria bassiana* + LC_{25} Deltamethrin	50.00	100	100	P
$LC_{12.5}$ *Beauveria bassiana* + $LC_{12.5}$ Emamectin benzoate	25.00	52.33	109.32	P
$LC_{12.5}$ *Beauveria bassiana* + LC_{25} Emamectin benzoate	37.50	59.00	57.33	P
LC_{25} *Beauveria bassiana* + $LC_{12.5}$ Emamectin benzoate	37.50	54.67	45.78	P
LC_{25} *Beauveria bassiana* + LC_{25} Emamectin benzoate	50	78.33	56.66	P
$LC_{12.5}$ *B.t.i.*+ LC_{25} Diflubenzuron	37.50	50.00	33.33	P
$LC_{12.5}$ *B.t.i.* + LC_{50} Diflubenzuron	62.50	33.33	-46.67	A
LC_{25} *B.t.i.*+ LC_{25} Diflubenzuron	50.00	43.33	-13.34	AD
LC_{25} *B.t.i.*+ LC_{50} Diflubenzuron	75.00	56.67	-24.44	A
$LC_{12.5}$ *B.t.i.* + $LC_{12.5}$ Azadiractin	25.00	63.33	153.32	P
$LC_{12.5}$ *B.t.i.* + LC_{25} Azadiractin	37.50	68.67	83.12	P
LC_{25} *B.t.i.*+ $LC_{12.5}$ Azadiractin	37.50	87.70	133.90	P
LC_{25} *B.t.i.* + LC_{25} Azadiractin	50.00	89.00	78.00	P
$LC_{12.5}$ *B.t.i.* + $LC_{12.5}$ Deltamethrin	25.00	89.33	257.20	P
$LC_{12.5}$ *B.t.i.* + LC_{25} Deltamethrin	37.50	100	166.7	P
LC_{25} *B.t.i.*+ $LC_{12.5}$ Deltamethrin	37.50	94.00	150.70	P
LC_{25} *B.t.i.*+LC_{25} Deltamethrin	50.00	100	100	P
$LC_{12.5}$ *B.t.i.* + $LC_{12.5}$ Emamectin benzoate	25.50	58.00	132	P
$LC_{12.5}$ *B.t.i.* + LC_{25} Emamectin benzoate	37.50	81.67	117.80	P
LC_{25} *B.t.i.* + $LC_{12.5}$ Emamectin benzoate	37.50	67.33	79.50	P
LC_{25} *B.t.i.* + LC_{25} Emamectin benzoate	50.00	82.00	64.00	P
LC_{25} diflubenzuron + $LC_{12.5}$ Deltamethrin	37.50	73.33	95.50	P
LC_{25} diflubenzuron + LC_{25} Deltamethrin	50.00	90.00	80.00	P
LC_{50} diflubenzuron + $LC_{12.5}$ Deltamethrin	62.50	60.00	-4.00	AD
LC_{50} diflubenzuron + LC_{25} Deltamethrin	75.00	89.00	18.66	A
LC_{25} diflubenzuron + $LC_{12.5}$ Emamectin benzoate	37.50	96.67	157.80	P
LC_{25} diflubenzuron + LC_{25} Emamectin benzoate	50.00	100	100	P
LC_{50} diflubenzuron + $LC_{12.5}$ Emamectin benzoate	62.50	53.33	147	P
LC_{50} diflubenzuron + LC_{25} emamectin benzoate	75.00	100.00	33.33	P
LC_{25} diflubenzuron + $LC_{12.5}$ Azadiractin	37.50	0.0	-100	A
LC_{25} diflubenzuron + LC_{25} Azadiractin	50.00	0.0	-100	A
LC_{50} diflubenzuron + $LC_{12.5}$ Azadiractin	67.50	0.0	-100	A
LC_{50} diflubenzuron + LC_{25} Azadiractin	75.00	0.0	-100	A
$LC_{12.5}$ azadiractin + $LC_{12.5}$ Deltamethrin	25.00	96.00	284	P
$LC_{12.5}$ azadiractin + LC_{25} Deltamethrin	37.50	100	166.70	P
LC_{25} azadiractin + $LC_{12.5}$ Deltamethrin	37.50	97.67	160.50	P
LC_{25} azadiractin + LC_{25} Deltamethrin	50.00	100	100	P

LC levels = concentration of the tested compounds C.F. = Cotoxicity factor

[c] Joint action: P = Potentiator A = Antagonistic AD = Additive

neem increased by time. Neem products are known for their fast breakdown of the active component under field conditions (Schmutterer, 1997), so it is often used in mixtures for prolongation of its duration of action.

Most of literatures suggested that the wide spread use of proclaim in veterinary and human medicine showed a highly potential larvicidal effect with unrecognized mode of action on mosquito populations. Recently, Foley *et al.*, (2000) reported that a high mortality was observed in *Anopheles farauti* mosquitoes fed on blood of volunteers treated with ivermectin (22,23 dihydroavermectin B1; 250 µg). Ivermectin treatment of animals could have an important role in malaria control when *A. farauti* is the vector. In most ivermectin-treated *Aedes aegypti* females (0.1 µg/mL) within 1hr after ingestion of blood containing this chemical, uncoordinated movements, paralysis and death were observed (Mahmood *et al.*, 1991). The most striking effect of ivermectin on *A. aegypti* at this dosage was on ovarian development.

It is worthy to mention that there is seasonal concentration and expansion of the elephantiasis vector population, where during winter the vector population is localized and the larval cycle of the mosquitoes increases from approximately 8 days in summer to about 44 days during winter. Consequently, the production of adult mosquitoes is reduced and the population becomes concentrated in the larval stages. Hence, larval breeding sites during winter could be targeted for supplementary control measures, thereby enhancing the overall control efforts (Abd-Allah *et al.*,2002).

Joint action of some control agents mixtures in *C. pipiens*

In order to raise the efficiency of the tested control agents and improve their characters, in current study, we have tested the joint toxic action of them at different concentrations which is shown in Table (2). It is clear that, all mixtures of *Beauveria bassiana* with diflubenzuron and all mixtures of diflubenzuron with azadiractin resulted in antagonistic effects. On the other hand, all other binary mixtures resulted in potentiating effects. The highest potentiating effect was obtained when the mixture of $LC_{12.5}$ *B.t.i.* + $LC_{12.5}$ Deltamethrin was used. This means that the dosages of these compounds can be reduced when they are used in mixtures. The strong synergistic effect observed by Darriet and Corbel (2006) between pyriproxyfen and spinosad allows a reduction in both pyriproxyfen and spinosad amounts by 5 and 9 fold to kill almost 100 % mosquitoes.

Although the current study proved the larvicidal potency of the tested compounds especially when used in mixtures, the choice of target-specific, environmentally safe and economically cost-effective combinations will be the end point determinant in IVC programs and strategies for mosquito control. Further complementary testing under semi-field and full field conditions are needed to specify the strategy that can be implemented in risky areas.

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الملخص العربي

السمية والتأثير السام المشترك لبعض المواد المستخدمة في مكافحة يرقات بعوض الكيولكس بيبيتز

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تهدف هذه الدراسة إلى تقييم مجموعة من المركبات الآمنة ضد يرقات بعوضة الكيولكس بيبيتز والتي يمكن تطبيقها في المناطق التي ينتشر فيها داء الفيل. وكذلك تم دراسة التأثير السام لخلط المبيدات. و أظهرت النتائج أن الـ Deltamethrin (LC_{50} = ٠,٠٢١ جزء في المليون) كان أكثر سمية من الـ *B.t.i.* ، Diflubenzuron ، Emamectin benzoate ، Azadiractin بنحو ٢، ٤٩١,٤ ، ٥٩ ، ١٤٣,٨ ضعفاً على الترتيب بعد ٢٤ ساعة. كما أن الـ Deltamethrin (LC_{50}=٠,٠٠٤ جزء في المليون) كانت سميته بمقدار ٤، ١١٠٢,٥، ٢٥، ٢٣٢,٥ ضعفاً سمية من الـ *B.t.i.*، Diflubenzuron، Emamectin benzoate، Azadiractin على الترتيب وذلك بعد ٤٨ ساعة من بدء المعاملة.

بعد ٧٢ ساعة من بدء المعاملة فإن سمية الـ Deltamethrin (LC_{50} = ٠,٠٠٣ جزء في المليون) كانت اكثر بمقدار ٣، ٢٠٦,٦٦ ، ٢٣,٣٣ ، ٢٤٦,٦٦ مرة من الـ *B.t.i.*، Diflubenzuron، Emamectin benzoate، Azadiractin.

وكانت سمية الـ *Beauveria bassiana* منخفضة جدا مقارنة بباقي المركبات المختبرة حيث كانت قيمة الـ LC_{50} الخاصة به تساوي ١,٨٥ مل/لتر و ذلك بعد ٧٢ ساعة من المعاملة.

أظهرت نتائج التأثير السام المشترك أنه على الرغم من ان كل مخلوطات الـ *Beauveria bassiana* مع الـ Diflubenzuron وكذلك كل مخلوطات الـ Diflubenzuron مع الـ Azadiractin أدت إلى ظهور تأثير تثبيط للفعل الإبادي الا ان كل المخلوطات الثنائية الأخرى أدت إلى ظهور تنشيط للفعل الإبادي.

وأعلى تأثير منشط تم الحصول عليه عند خلط التركيز تحت المميت $LC_{12.5}$ من الـ *B.t.i.* مع التركيز تحت المميت $LC_{12.5}$ من الـ Deltamethrin .

وأخيرا فإن إستخدام المخلوطات الثنائية من المركبات المختبرة يمكن أن تؤدي إلى مكافحة أفضل ليرقات بعوض ال *Culex pipiens* L. كما أنها تقلل تكاليف المكافحة و تقلل من تلوث البيئة.