Annual Review & Research in Biology
3(4): 1055-1065, 2013

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Larvicidal Activity and Joint Action Toxicity of Certain Combating Agents on *Culex pipiens* L. Mosquitoes

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Authors' contributions

This work was carried out in collaboration between all authors. Author HEDMZ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author MAK managed the analyses of the study and wrote the final manuscript, Author HAB managed the literature searches. All authors read and approved the final manuscript.

Research Article

Received 13th June 2013
Accepted 26th July 2013
Published 15th August 2013

ABSTRACT

Aims: The main objective of the present study was to investigate the larvicidal effect of some biological control agents like *Beauveria bassiana* and *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) and some natural control agents as Diflubenzuron, Azadirachtin and Emamectin benzoate on *Culex pipiens* mosquito. The toxicity of binary mixtures of these control agents was also assessed.
Methodology: The larval susceptibility test of *C. pipiens* was estimated when the third instar were treated with Azadirachtin, *B. bassiana*, *B.t.i.,* Diflubenzuron, Emamectin benzoate and Deltamethrin (reference compound). Series of concentrations for each compound in addition to control were replicated four times. Mortality counts were carried out after 24, 48 and 72hr of treatment. To determine the joint toxic action of the tested compounds, 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-

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treatment. The joint action of different mixtures in terms of co-toxicity factor (C.F.) was estimated. Data of bioassay were analyzed using Probit program.
Results: Data showed that the LC_{50} of *B.t.i.*, Emamectin benzoate, Azadirachtin, Diflubenzuron and *B. bassiana* were 0.044ppm, 1.24ppm, 3.02ppm, 10.32ppm and 4.122ml/L, respectively on the third instar of *C. pipiens* after 24hrs. Azadirachtin showed time related larvicidal activity. Diflubenzuron induced delayed effect on *C. pipiens* larvae. *B. bassiana*, had the lowest activity against this mosquitoes (LC_{50}= 1.85ml/L) after 72hr of exposure. Data of joint toxic action of some mixtures such as (*B. bassiana* + Diflubenzuron) or (Diflubenzuron+Azadirachtin) revealed antagonistic effect while almost other binary mixtures showed potentiating effects. The mixture of $LC_{12.5}$ *B.t.i.* + $LC_{12.5}$ Deltamethrin recorded the highest potentiating activity.
Conclusion: The study suggests that, the most effective tools for *C. pipiens* larvae eradication included *B.t.i.* followed by Emamectin benzoate, Azadirachtin, Diflubenzuron then *B. bassiana*. The use of some binary mixtures of these tested control measures can get better control, save the amount and reduce control cost.

Keywords: Culex pipiens; microbial agents; natural agents; joint action; antagonistic effect; potentiating effect.

1. 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 [1,2]. 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 organochlorides, organophosphates, pyrethroid and carbamates [3] along with numerous health, environmental and ecological side effects of these agents, guide to the necessity of alternative tools for control [4]. And the current strategy of Integrated Pest Management (IPM) comprises the general approach of environmentally friendly control measures may involve several complements [5,6,7]. Hence the use of microbial insecticides provides alternatives to chemical insecticides and avoids environmental contamination. *Bacillus sphaericus* (*B.s.*) and *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) received increasing attention as mosquito larvicides [8,9,10,11]. The survival rates of *Culex quinquefasciatus* larvae were decreased with the increase of the *B. t. i* concentration [12].

The potency of entomopathogenic fungus *Beauveria bassiana* as an alternative vector control tool against insecticide-resistant mosquitoes under conditions typical of indoor resting environments were discussed by [13]. The blood feeding behavior of wild mosquitoes was reduced by the treatment of *B. bassiana* so it was considered a new mosquito control tool [14].

Insect growth regulators (IGRs) also have high levels of activity and efficacy against various species of mosquitoes in a variety of habitats. IGRs have low mammalian toxicity, are quite safe to fish, birds and most nontarget biota [15]. A number of benzoylphenylurea (BPU) derivatives have been developed such as Diflubenzuron which cause larval and pupal mortality of *Aedes aegypti* [16], and triflumuron, which is described as molt inhibitor through interference with cuticle deposition and chitin biosynthesis of *A. aegypti* adults [17]. Also,

treatment with the Juvenile hormone analog (pyriproxyfen) led to high mosquito larval reduction [18].

Pesticidal active ingredients from the neem tree *Azadirachta indica* A. Juss (Azadirachtin) have been recommended as it was ecofriendly and safe to the non target organisms [19]. Clear larvicidal effect was observed with *C. quinquefasciatus* when exposed to different concentrations of ethanol and methanol leaf extract [20]. Neem seed kernel extract is an ovipositional deterrent for the oriental fruit fly [21]. Neem products are characterized by their effect on oviposition, repellence, size of egg raft, and hatching rate of the eggs of dipterous pests [22].

Emamectin benzoate, the semi-synthetic of abamectin which produced by fermentation of *Streptomyces avermitilis*, is known to have potent toxic activity [23] in parasitic disease and was extremely toxic at low concentrations to a wide range of insects including members of the order Diptera [24].

The main objective of the present study was to investigate the larvicidal effect of *B. bassiana, B. t. i.*, Diflubenzuron, Azadirachtin and Emamectin benzoate. The toxicity of binary mixtures of these control agents was also assessed.

2. MATERIALS AND METHODS

2.1 The Tested Materials

The following commercially formulations were used:

Achook® 0.15% EC Azadirachtin was provided by the Egyptian Agricultural development Co. (Egypt) as natural extract. *Beauveria bassiana* was obtained from Biotech Manufacture, El-Sadat City, Egypt. 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. Embrator®2.5% EC Deltamethrin (DLM), ((S)-α-Cyano-m-phenoxybenzyl (1R, 3R)-3-(2,2-dibromovinyl)-2,2 dimethylcyclopropane carboxylate) was supplied by KZ Co.(Egypt).

2.2 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±1ºC, 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.

2.3 Bioassay Procedures

The larval susceptibility test was conducted according to World Health Organization [25,26]. 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±1ºC and 75± 5%RH. Mortality counts were carried out after 24, 48 and 72hr of treatment. Mortality percentages were calculated and corrected according to [27]. 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 [28].

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

Compound	Concentration range	Time (hr)	Slope	LC_{50}[a]
B. 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)
Azadirachtin	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[b]	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)

[a]*Concentration required killing 50% of the larvae,* [b]*Reference compound*

2.4 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 [29] using the following equation:-

$$\textit{Co-toxicity factor} = \frac{observed\%mortality - \exp ected\%mortality}{\exp ected\%mortality} \times 100$$

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.

2.5 Statistical Analysis

Data of bioassay were analyzed using Probit program [28].

3. RESULTS AND DISCUSSION

3.1 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) which revealed that, the exposure of the third instar of *C. pipiens* mosquito to the different tested control agents resulted in considerable mortality differed according to the agent tested and the time of exposure. *B.t.i.* was more effective, followed by Emamectin benzoate, Azadirachtin, Diflubenzuron and *B. bassiana*, when the larvae treated with each agent for 24 hr.

B.t.i. showed LC_{50} at 0.044, 0.016 and 0.009 ppm which the most close to LC_{50} of Deltamethrin (reference compound) (0.021, 0.004 and 0.003ppm), after exposure for 24, 48 and 72 hr, respectively.

The obtained data are strengthened by other previous reports that demonstrate the efficacy of bacterial pesticides. Treatment with 1 g/m^2 of *Bacillus sphaericus* formulation (VectoLex® WDG) caused 100% mortality rate for the late instar of *Cx. quinquefasciatus* in a sewage habitat, this effect remained for 7 days [30]. Excellent initial control (90-100%) of all larvae were obtained when Vectobac® 12 AS were applied at the rate of 1-1.25 l/ha and Vectobac®G at 7.5-10.0 kg/ha according to the mosquito genera tested under field conditions [31]. Toxicity of *B.t.i*. is referred to its parasporal body which considered as a gut poison, it attacks the midgut epithelium, and the midgut epithelial cells swell and burst, then the gut wall was severely damaged. Also, treatment of larvae with 4 µg/ml *B.t. i*. resulted in cessation of feeding within one hour and reduction in the activity by two hours followed by extreme sluggishness by four hours. In advanced stages general paralysis will be occurred [32].

Applying of the biological control of *Anopheles* characterized with negligible side effects on humans, wild-life, and on the environment. Also, very small cases of mosquito resistant strains to these biological agents were recorded [4].

Our data showed that, median lethal concentration LC_{50} of the formulation of Emamectin benzoate against *C. pipiens* was found to be 1.24, 0.10 and 0.07 ppm after 24, 48 and 72 hr of treatment, respectively (Table 1). Results concerning Emamectin benzoate are agreed with those of [33] who reported that a high mortality was observed in *Anopheles farauti* mosquitoes fed on blood of volunteers treated with ivermectin. And [34] who showed that, loss of mobility, progressive paralysis and high mortality of larvae were recorded on the 3rd and 4th instar of *Aedes aegypti*, after 24 hours when submitted to concentrations of 1, 5 and 10 ppm of ivermectin solution during 5, 15, 10, 60 and 1440 minutes. Also, the increase in ivermectin concentration caused a progressive mortality.

Phytochemicals were considered ideal insecticides for use in the Integrated Pest Management programs, since they are relatively safe, inexpensive and available worldwide [35,36].

Treatment with Azadirachtin (0.15%) resulted in larvicidal activity (LC_{50}= 3.02 ppm after 24 hr post-treatment) on *C. pipiens* larvae and its larvicidal effect increased by time, since, LC_{50} reach to 0.74 after 72 hours of treatment (Table 1). These results are in line with those of [37] who distinguished the linear correlation between the concentration of Azadirachtin and larval and pupal mortality of *C. pipiens* under laboratory conditions.

The action of azadirachtin may due to the deformation happened in the larvae; pupa and adult stages of mosquito, the obvious mortality and the toxic response like sediment lacking, impregnation of some segments with dark substances and loss of respiratory pigments [38] and the inhibition of chitin synthesis [39].

Obtained data indicated that, the IGR Diflubenzuron proved to have a delayed effect on *C. pipiens* larvae for the first 72hr after treatment. The LC_{50} of IGR Diflubenzuron was 10.32, 4.41 and 0.62 after 24, 48 and 72 hr of treatment, respectively (Table 1). On the other hand, the LC_{50} obtained by [15,40,41] after 24 hours of treatment with Diflubenzuron was much lower. The difference in the response may refer to the mosquito genus or species and isolate tested.

Diflubenzuron treatment of larvae, pupae or adults of *Anopheles darlingi* (Root1926) induced some morphological alteration such as elongation and Ecdysis of the third stage larvae according to the exposure time. In addition, tissue extravasation, difficulties to discard the exuvia and mortality were observed [42].

Toxicity of *B. bassiana* was low when compared with all the tested control measures with LC_{50} value reach to 1.85ml/L after 72hr of exposure (Table 1). Morphological abnormalities further explain the virulence of fungus against the pest. Treatment of early instars of *Anopheles stephensi* with *B. bassiana* caused inhibition of chitin synthesis which led to forming delicate body and lengthening of the neck region. Also, fungal growth appeared on the legs and hairs which arrest the larval movement [43,44].

3.2 Joint Action of Some Control Agents Mixtures on *C. Pipiens*

In order to raise the efficiency of the control agents and improve their characters, combined effects were studied. The joint toxic actions of the tested agents have been assessed at different concentrations (Table 2). All mixtures of Diflubenzuron with *B. bassiana* and Diflubenzuron with Azadirachtin, also, $LC_{12.5}$ *B.t.i.* + LC_{50} Diflubenzuron, LC_{25} *B.t.i.*+LC_{50}Diflubenzuron and LC_{50}Diflubenzuron+LC_{25} Deltamethrin resulted in antagonistic effect.

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

LC levels[a] (Bi-mixture)	% mortality		C.F. [b]	Joint action[c]
	Expected	Observed		
$LC_{12.5}$ *B. bassiana* + LC_{25} diflubenzuron	37.50	19.33	-48.45	A
$LC_{12.5}$ *B. bassiana* + LC_{50} diflubenzuron	62.50	23.00	-63.20	A
LC_{25} *B. bassiana* + LC_{25} diflubenzuron	50.00	21.67	-56.66	A

LC_{25} *B. bassiana* + LC_{50} diflubenzuron	75.00	29.00	-61.33	A
$LC_{12.5}$ *B. bassiana* + $LC_{12.5}$ azadirachtin	25.00	59.67	138.68	P
$LC_{12.5}$ *B. bassiana* + LC_{25} azadirachtin	37.50	68.00	81.33	P
LC_{25} *B. bassiana* + $LC_{12.5}$ azadirachtin	37.50	60.00	60.88	P
LC_{25} *B. bassiana* + LC_{25} azadirachtin	50.00	85.67	71.34	P
$LC_{12.5}$ *B. bassiana* + $LC_{12.5}$ Deltamethrin	25.00	71.33	185.32	P
$LC_{12.5}$ *B. bassiana* + LC_{25} Deltamethrin	37.50	75.00	100	P
LC_{25} *B. bassiana* + $LC_{12.5}$ Deltamethrin	37.50	72.67	93.78	P
LC_{25} *B. bassiana* + LC_{25} Deltamethrin	50.00	100	100	P
$LC_{12.5}$ *B. bassiana* + $LC_{12.5}$ emamectin benzoate	25.00	52.33	109.32	P
$LC_{12.5}$ *B. bassiana* + LC_{25} emamectin benzoate	37.50	59.00	57.33	P
LC_{25} *B. bassiana* + $LC_{12.5}$ emamectin benzoate	37.50	54.67	45.78	P
LC_{25} *B. 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.* + $LC_{12.5}$ azadirachtin	25.00	63.33	153.32	P
$LC_{12.5}$ *B.t.i.* + LC_{25} azadirachtin	37.50	68.67	83.12	P
LC_{25} *B.t.i.*+ $LC_{12.5}$ azadirachtin	37.50	87.70	133.90	P
LC_{25} *B.t.i.* + LC_{25} azadirachtin	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 + $LC_{12.5}$ azadirachtin	37.50	0.0	-100	A
LC_{25} diflubenzuron + LC_{25} azadirachtin	50.00	0.0	-100	A
LC_{50} diflubenzuron + $LC_{12.5}$ azadirachtin	67.50	0.0	-100	A
LC_{50} diflubenzuron + LC_{25} azadirachtin	75.00	0.0	-100	A

$LC_{12.5}$ azadirachtin + $LC_{12.5}$ Deltamethrin	25.00	96.00	284	P
$LC_{12.5}$ azadirachtin + LC_{25} Deltamethrin	37.50	100	166.70	P
LC_{25} azadirachtin + $LC_{12.5}$ Deltamethrin	37.50	97.67	160.50	P
LC_{25} azadirachtin + LC_{25} Deltamethrin	50.00	100	100	P

[a]*LC levels = concentration of the tested compounds*
[b]*C.F. = Co-toxicity factor*
[c]*Joint action: P = Potentiate, A = Antagonistic, AD = Additive*

Additive effect can be obtained when the mixture included LC_{25}*B.t.i.* + LC_{25} Diflubenzuron and LC_{50} Diflubenzuron + $LC_{12.5}$ Deltamethrin.

All other binary mixtures resulted in potentiating effects. The highest potentiating effect was gained 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.

These empirical data add support to recent joint action studies suggesting that, the synergistic effect of VectoMax WSP (a mixture of *B.t.i.* and *B. sphaericus*) which reduces the risk of *Culex* and *Aedes Japonicus* [45]. Additionally, treatment of *Anopheles sundaicus* mosquitoe with seaweed extract of *Sargassam wightii* combined with *B.t.i.* toxins had an effect on the gut system, which led to mortality and inhibition in growth [46]. Also, the combination between pyriproxyfen and spinosad showed synergistic effect on the dengue vector *A. aegypti* (L.). The mixture revealed both the larvicidal activity of spinosad and the juvenoid action of pyriproxyfen [47].

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 IPM 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.

4. CONCLUSION

We can be concluded that, the logical first step in Integrated Pest Management, will be defined by utilizes all reasonable methods to achieve pest reduction in a way that has the least negative impact on the environment. The most promising biological control tools for mosquito eradication included *B.t.i.* followed by Emamectin benzoate, Azadirachtin, Diflubenzuron then *B. bassiana*. The use of some binary mixtures of the tested control measures can get better control, save the amount and reduce control cost. Applying of some of these agents in mixture resulted in different effect in control the *C. pipenes* larvae. The variations in the levels and types of interaction among the tested mixtures may be attributed to the differential mode of action of the present compounds and the concentration tested.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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