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The effectiveness of biological mosquito larvicide: Bacteria (*Bacillus thuringiensis israelensis*) in liquid formulation

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Abstract
Mosquitoes are very tiny insects but the impact they brought to humans and society is very huge. An application technique for the control of mosquito larvae under tropical conditions was conducted in Cebu City, Philippines. The study used a bacterium, *Bacillus thuringiensis israelensis* in liquid formulation under laboratory conditions. Five concentrations were prepared in range finding and 4 concentrations in definitive range finding tests. Each of the concentration was tested with 25 3rd and early 4th instar larvae of *Aedes aegypti*. Results obtained from the test have very effective residual effects against the larvae of mosquitoes. The efficacy varies according to the concentrations applied to the mosquito larvae. The high mortality rate of mosquito larvae is associated numerically with higher liquid concentration of the larvicide (1.6 grams) and the low mortality rate of mosquito larvae is associated numerically with lower liquid concentration (0.4 grams) after one (1) hour application of the larvicide. This means that even, with lower level of liquid concentration of the larvicide, the mortality rates among mosquito larvae are still high with the lowest rate of 93.3% after 1 hour of exposure to the biological mosquito larvicide. Further, there is a significant increase in the number of dead mosquito larvae after 2 hours of treatment. It implies that the toxicity level of the larvicide is still there even after 2 hours of treatment. However, 0.4 g concentration is proven to be effective and achieved the same results compared with higher concentrations after 2 hours of application to the mosquito larvae.

Keywords: *Aedes aegypti*, *Bacillus thuringiensis israelensis*, mosquito, biological control, dengue control

1. Introduction
Mosquitoes are very tiny insects but the impact they brought to humans and society is very huge. They are carriers of diseases like dengue and malaria that can kill humans. Dengue is the world's most important mosquito-borne virus disease, with 2500 million people worldwide at risk of infection and 20 million cases a year in more than 100 countries [1]. It is transmitted by the bite of a female *Aedes* mosquito. In the Philippines, dengue is a high-profile and ongoing public health concern together with the newly emerged zika virus. The Philippines experienced the seventh highest number of dengue cases in the world between 2004 and 2010 [2]. There are 179,540 cases that were infected on the virus from January to November 2018 with 907 deaths [3]. In Central Visayas, there are 15,171 dengue cases with the highest infection cases of 2,660 resulting to 14 deaths in Cebu City from January 2018-November 2018 [4]. Although there is already dengue vaccine available in the market however, it poses controversial issues in the society. Due to this, one way of reducing the risk of being infected by mosquito borne diseases is to control the mosquito while they are still in the water or during larval stages. Controlling mosquito larvae in the water is either by the application of Chemical (ex. temephos) or biological way (bacterium). Unfortunately, the widespread use of chemical insecticides has contributed to increasing resistance to these agents among *Aedes aegypti*, especially in the Americas and the Caribbean [5]. Further, one of the disadvantages of chemical application is that there are also other organisms that are affected upon the application. Published international scientific studies found out that temephos is toxic to some bird species and other organisms. Due to this, it cannot be considered as environmentally friendly.

A very promising and really environmentally friendly tool to fight against dengue, which is used internationally and highly recommended by World Health Organization (WHO), is the bacterium *Bacillus thuringiensis israelensis* (B.t.i.). This bacterium is proven to be only toxic to the target enemies, namely: larvae of mosquitoes, black flies and midges (responsible for human diseases) based on international scientific researches and publications. The study evaluates the effectiveness of biological mosquito larvicide *Bacillus thuringiensis israelensis* in liquid formulation as formulated by the author and registered by Cebu Normal University as utility model by the Bureau of Patents, Philippines. Specifically it aims to determine: the mortality rate of mosquito larvae by the application of four concentrations of the larvicide in liquid formulation and; the efficacy of the larvicide after 24 hour of treatment to the mosquito larvae.

2. Materials & Methods

2.1 Mosquito Larvae Collection

Mosquito larvae were collected from clean & stagnant mosquito breeding sites using a water dipper. The collected larvae were kept in half closed jar bottles & transported to the Biology Laboratory, Cebu Normal University. In the laboratory, the mosquito larvae samples were poured into the plastic basin. The larvae in the basin were sorted according to the ages and only the 3rd & early 4th instar larvae of *Aedes mosquito* were used for the experiment.

2.2 Range Finding Test of Utility Model Patent (Assessment of the potency and the minimum effective dosage in the laboratory)

The prototype of the products was provided by the researcher which is the maker of the utility model. The utility model on the process of producing mosquito larvae control comprising the steps of:

The utility model on the process and composition of producing mosquito larvicide in liquid formulation consist of the following process:

a. mixing 5 gram of *Bacillus thuringiensis israelensis* with 1 liter of alkaline water in a mixing vessel;
b. putting 5 gram of fish powder in a container;
c. pouring the mixture of *Bacillus thuringiensis israelensis* and alkaline water in the container containing the fish powder; and
d. stirring thoroughly until the mixture will be properly mixed.

Samples of mosquito larvae were collected in Cebu City. Twenty five mosquito larvae samples were used per concentration and per concentration had triplicates. There were 5 concentrations of the product that were tested under laboratory conditions on the mosquito larvae, namely: 2 g, 4 g, 6 g, 8 g and 10 g. Twenty five 3rd & early 4th instar larvae of *Aedes aegypti* (Linn.) in 2 ml of water were added to plastic disposable cups filled with
148 ml of distilled water. Mortality readings of larvae were taken in five readings after the application of the larvicide (after 1 hr, 2,4,8, & 24 hrs). If the desired mortality rate (100%) was not attained, another batch of mosquito larvae was released to higher concentration until a 100% mortality rate of the larvae was achieved.

2.3 Definitive *Test of Utility Model Patent*

Four different concentrations based on the results from range finding test were tested namely: 0.4g, 0.8g, 1.2g, & 1.6g in triplicates. The same procedure in the range finding test was applied for the application of the different concentrations.

2.4 Data Analysis

Data collected on mortality rate difference of mosquito larvae among the four concentrations were calculated using ANOVA.

3. Results and Discussion

3.1 Range Finding Test of Utility Model Patent (Assessment of the potency and the minimum effective dosage in the laboratory)

Results showed that after 1 hr of the application of the product, average mortality rates of 84%, 88%, 88%, 88%, & 92% in 2g, 4g, 6g, 8g, & 10g were attained, respectively. Further, after 2 hr of the application of the product, 100% mortality rate of mosquito larvae was achieved in 5 concentrations. Based on the results, 2 g concentration was selected to be used for definitive test considering that after 2 hr of the product application, the lowest concentration (2g) was able to attain a 100% mortality rate of mosquito larvae. Figure 1 showed the results of the Range Finding Test.

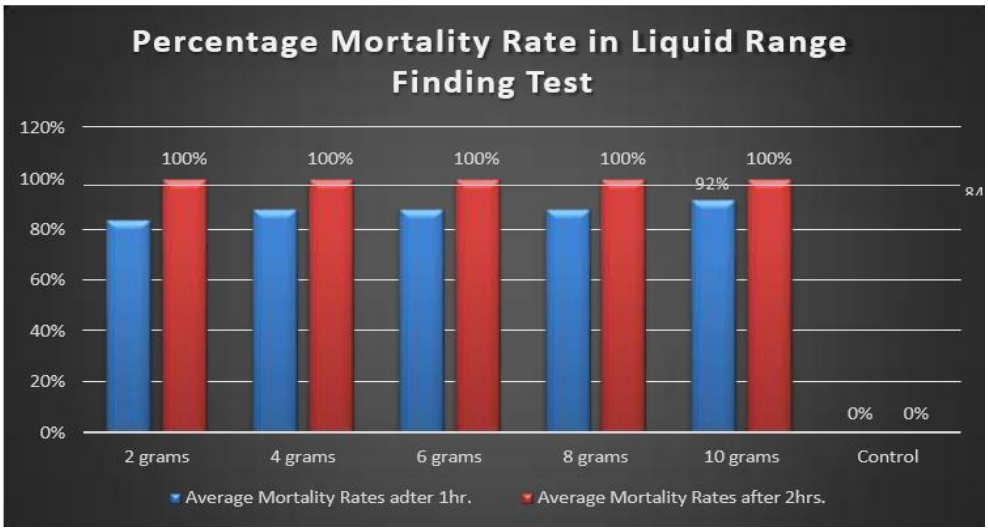

Fig 1: Percentage Mortality Rate of Mosquito Larvae in Liquid Range Finding Test.

3.2. Definitive Test of Utility Model Patent

The product showed a very promising result as shown in Figure 2. After 1 hr of product application, 92%, 96%, 96%, & 100% mortality rates of mosquito larvae in 0.4g, 0.8g, 1.2g, & 1.6g concentration were attained, respectively. Further after 2 hrs. of product application, mortality rate of 100% was attained.

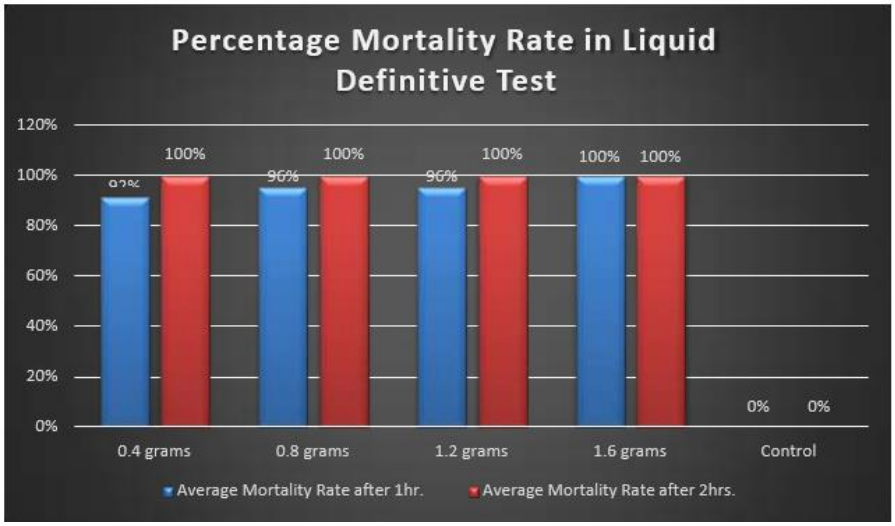

Fig 2: Percentage Mortality Rate of Mosquito Larvae in Liquid Definitive Test

The descriptive statistics associated with the mortality rate of mosquito larvae after the application of four different liquid concentrations of biological mosquito larvicide are reported in Table 1 below. It can be seen that high mortality rate of mosquito larvae (M = 25.0, SD = 0.00) is associated numerically with higher liquid concentration of the larvicide (1.6 grams) and the low mortality rate of mosquito larvae (M= 23.3, SD= 0.58) is associated numerically with lower liquid concentration (0.4 grams) after one (1) hour application of the larvicide.

Table 1: Descriptive Statistics of Mortality Rates of Mosquito Larvae Across Four Different Liquid Concentrations of Biological Mosquito Larvicide

Liquid oncentration	N	M	SD	Mean Mortality Percentage	Levene's	*P* - value
0.4 grams	25	23.3	0.58	93.3%		
0.8 grams	25	24.0	1.00	96.0%	F(3,8) = 2.303	.154
1.2 grams	25	24.3	0.58	97.3%		
	25	25.0	0.00	100 %		

Prior to conducting the ANOVA analysis, the assumption of normality was evaluated and determined to be satisfied as the group's distributions were associated with skewness and kurtosis less than | | and | |, respectively [6]. Moreover, the assumption of homogeneity of variances was tested and satisfied based on Levene's F-test, $F(3,8) = 2.303$, $p = .154$.

In order to test the hypothesis that the level of liquid concentration of biological mosquito larvicide had an effect on mortality rate of mosquito larvae, one-way between-subjects ANOVA was performed as displayed in Table 2. The independent between-groups ANOVA yield a statistically non-significant effect, $F(3, 8) = 3.467$, $p = .071$,Thus, the null hypothesis of no differences in the mean mortality rates of mosquito larvae when exposed to different liquid concentrations of larvicide was not rejected. This means that even, with lower level of liquid concentration of the larvicide, the mortality rates among mosquito larvae are still high with the lowest rate of 93.3% after 1 hour of exposure to the biological mosquito larvicide. However, a moderate 56.5% variance in the mortality rates of mosquito larvae was accounted for by the liquid concentration of the larvicide as indicated by its effect size.

Table 2: ANOVA Summary Table of Different Levels of Larvicide Concentration in the Mortality Rates of Mosquito Larvae

Sources of Variation	Sum of Squares	df	Mean Square	*F* - value	*P* - value
Between Groups	4.333	3	1.444		
Within Groups	3.333	8	0.417	3.467	.071
Total	7.667	11			

Furthermore, another one-way between-subject ANOVA was performed after 2 hours of larvicide application to the mosquito larvae as displayed in Table 3. The independent between-groups ANOVA yield a statistically non-significant effect, $F(3, 8) = 1.000$, $p =$ .441, thus, the null hypothesis of no differences in the mean mortality rates of mosquito larvae after 2 hours exposure to different liquid concentrations of larvicide was not rejected. This means that even, with lower level of liquid concentration of the larvicide, the mortality rates among mosquito larvae are still high with the lowest rate of 98.7% to the highest rate of 100% after 2 hours of exposure to the biological mosquito

larvicide. However, a noticeable 27.3% variance in the mortality rates of mosquito larvae was accounted for by the liquid concentration of the larvicide as indicated by its effect size.

Table 3: ANOVA Summary Table of Different Levels of Larvicide Concentration in the Mortality Rates of Mosquito Larvae after 2 Hours

Sources of Variation	Sum of Squares	df	Mean Square	*F* - value	*P* - value
Between Groups	0.250	3	0.083		
Within Groups	0.667	8	0.083	1.000	.441
Total	0.917	11			

In order to test the hypothesis that the mortality rates of mosquito larvae is different in different time intervals after larvicide treatment, a paired *t*-test was conducted as displayed in Table 4 below. An alpha level of 0.05 was still utilized in this analysis. Descriptive statistics show that there were no violations of normality and homogeneity of variances in the data gathered. A statistically significant difference was evident in the mortality rates of mosquito larvae between 1 hour (M = 96.7%, SD = 3.3%) and 2 hours after (M = 99.7%, SD = 1.2%) larvicide treatment, $t(11) = 3.45$, $p = .003$. This simply means that there is a significant increase in the number of dead mosquito larvae after 2 hours of treatment. It implies that the toxicity level of the larvicide is still there even after 2 hours of treatment. A very large effect size was noted in this study, $d = 0.995$.

Table 4: Differences in the Mortality Rates of Mosquito Larvae Between 1 hour and 2 hours after Treatment of larvicide

No. of hours After Treatment	N	M	SD	Mean Percentage Difference	*t* - value	*P* - value
After 1 hour	12	96.7%	3.3%			
After 2 hours	12	99.7%	1.2%	3.0%	3.45	.003

The results obtained from the test have very effective residual effects against the larvae of mosquitoes. The efficacy varies according to the concentrations applied to the mosquito larvae. It can be seen in the results that high mortality rate of mosquito larvae is associated numerically with higher liquid concentration of the larvicide (1.6 grams) and the low mortality rate of mosquito larvae is associated numerically with lower liquid concentration (0.4 grams) after one (1) hour application of the larvicide.

This means that even, with lower level of liquid concentration of the larvicide, the mortality rates among mosquito larvae are still high with the lowest rate of 93.3% after 1 hour of exposure to the biological mosquito larvicide. Further, there is a significant increase in the number of dead mosquito larvae after 2 hours of treatment. It implies that the toxicity level of the larvicide is still there even after 2 hours of treatment. However, 0.4 g concentration is proven to be effective and achieved the same results compared with higher concentrations after 2 hours of application to the mosquito larvae.

The larvicidal efficacy is due to the parasporal crystal of B.t.i. that composed of four major proteins namely: 27, 65, 128 and 135 kDa [7]. The high efficacy that Bti showed in laboratory and field trials during the early 1980s led rapidly to its development as a commercial bacterial larvicide for control of mosquito and blackfly larvae [8, 9].

Laboratory studies suggest that B.t.i. did not show any resistance to mosquito larvae due primarily to the presence of Cyt1A in the parasporal body [10, 11]. Cyt1A's capacity to synergize endotoxin proteins, including the *B. sphaericus* Bin toxin against resistant and non-sensitive mosquitoes [12].

4. Conclusion

The product used in the study that is registered by Cebu Normal University as utility model by the Bureau of Patents is a very promising tool to combat the problem of *Aedes* mosquitoes which are the carrier of dengue and other viruses.

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