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Production, design, and evaluation of *Bacillus thuringiensis* serov. *israelensis* formulations against *Aedes aegypti* larvae

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Abstract
Bacillus thuringiensis israelensis (Bti) was cultured on three culture media: a reference medium and two new media. Then, spore–crystal complexes were recovered and evaluated against *Aedes aegypti* larvae. After two formulations, tablets and granulates, were prepared, then irradiated with ultraviolet light and evaluated for larval mortality and residual activities. The most toxic extract was obtained in soluble medium 1 showing the lowest LC_{50} of 0.3748 mg/L. When Bti extract was formulated and tested in the laboratory, the granulated formulations, presented approximately 90%–100% mortality, during the first 3 days post-application, whereas tablets showed <60% mortality. At 14 days post application, only granulates 2 and 3 showed >40%, whereas unformulated extract presented 22% mortality. The formulations showed reduced mortality of 5%–12% (tablets) and granular (6%-11%) after 72 hours of exposure to UV light; otherwise, reductions in the unformulated Bti extract were higher (32%).

Keywords: *Bacillus thuringiensis israelensis*, formulations, larval control, mosquitoes, *Aedes aegypti*

1. Introduction
Mosquitoes are widely distributed insects worldwide, mainly in tropical and subtropical regions, where larvae and pupae are aquatic [1]. Among the various species of mosquitoes, *Aedes Aegypti* Linnaeus (Diptera: Culicidae) is the most common and vector of viral diseases such as yellow fever, dengue classic and hemorrhagic, Chikungunya, and Zika. The global dengue cases are estimated as 390 million infections/year; 96 million of these are considered clinical infections [2]. In the Americas, the Pan American Health Organization (PAHO) reported 693,489 suspected cases of Chikungunya in 2015, with 37,480 confirmed cases. The majority of these cases were reported from Colombia, with 356,079 suspected cases [3].

In 2016, the WHO Regional Office in the Americas reported 349,936 suspected cases, with 146,914 confirmed. Most cases occurred in Brazil (265,000 suspects), followed by Bolivia and Colombia (19,000 suspects). In contrast, a large outbreak of exanthematous disease was reported in Brazil in March 2015, which was quickly identified as a result of infection with the Zika virus, and in July 2015, an outbreak was described to be associated with Guillain–Barré syndrome. In October 2015, the association between this infection and microcephaly was also described in Brazil. Outbreak and evidence of transmission in the Americas, Africa, and other regions of the world soon occurred. To date, 86 countries and territories have reported cases of infection by the mosquito-borne Zika virus [4].

Dengue is one of the most serious public health problems that cause a major impact on the economy of the affected populations, requiring vector control as the most important actions both in their larval and adult states, which has been traditionally performed using chemical larvicides such as Temephos (Abate) that yield good results. However, cases of resistance have already been reported [5, 6, 7]. In addition, these chemicals have an effect of the ecosystem due to their lack of selectivity, causing damage to other life forms.

Based on these drawbacks, other alternatives for vector control such as the use of several

biological control agents, such as *Bacillus thuringiensis israelensis* (Bti), have been introduced in some countries for more than three decades already, with target insects not presenting resistance [8, 9].

To date, different preparations of Bti have been used, including liquids, powders, granules, pellets, microgranules, and others, which are applied to breeding sites depending on the species of the mosquito [10].

Studies have reported several formulations, i.e., wettable powders or emulsifiable liquids, which have shown efficiency but reduced the activity for periods of not >2–3 weeks [11, 12], whereas granular formulations have presented residual activity between 30 and 78 days [13, 11]. Other formulations such as tablets have been developed with variable residual activities [14].

In this study, the production of Bti strain 225 bioinsecticide was carried out in three economical and available culture media, after recovering the larvicidal extracts; in addition, these extracts were evaluated against *A. aegypti* larvae: first, elaboration of two formulations, tablets and granulates; then the formulations were exposed to ultraviolet (UV) light; and finally, its residual activity was evaluated in the laboratory.

2. Material and Methods

2.1 Strain

The 225 Bti strain was obtained from our collection of *Bacilli* Entomopathogenic from the Institute of Biotechnology; Biological Sciences Faculty, Autonomous University of Nuevo León (FCB-UANL) and was selected in a previous study [15].

Table 1: Composition of culture media for Bti production of larvicidal extracts.

	Ingredients (g/L)		
	Reference Medium	**Soluble Medium 1**	**Medium 2 Sunflowers**
Soybean meal	10	0	0
Corn steep solids	10	10	0
Molasses	10	0	0
Corn syrup	0	10	0
Yeast Extract	0	10	0
Sunflower seed flour	0	0	10
Sucrose	0	0	10
$CaCO_3$	1	1	1
$MgSO_4$	0.3	0.3	0.3
$ZnSO_4$	0.02	0.02	0.02
$FeSO_4$	0.02	0.02	0.02
$MnSO_4$	0.02	0.02	0.02
pH	7	7	7

2.2 Fermentation for the production of the larvicidal extract

The Bti strain, maintained in slants with nutritive agar, was activated in tubes with the same medium and incubated at 30 °C for 24 h. Subsequently, each three different culture media (shown in Table 1), i.e., 1) reference medium, 2) soluble medium 1, and 3) sunflower medium, were inoculated with Bti strain, incubated in agitation at 200 rpm and 30 °C for 72 h (Lab-Line environ shaker, model 3527). This procedure was repeated four times. At the end of fermentation, spores and crystals were harvested following the methodology described previously [16] using lactose and acetone. The final product, Bti extract, was dried, ground in mortar, and then the yield obtained (g/L) was calculated. Then, extracts were evaluated for larvicidal activity.

2.3 Mosquito species tested

The late third-stage *A. aegypti* larvae, San Nicolas strain, was used for bioassays. The strain has been reared for several years at the Institute of Biotechnology (FCB-UANL) at 25± 3 °C, 14:10 h light: dark photoperiod and 70%–80% relative humidity.

2.4 Evaluation of larvicidal extracts produced in the three-culture media

The extracts obtained from the three culture media were evaluated against late third-stage larval *A. aegypti* according to methodology described previously [17]. For the experiment, stock solution containing 5000 mg/liter of the Bti extract was used. Ten glass beads were added to the stock solution, and each batch was vortexed for 2 min. Then, 1 and 0.1 ml were taken to prepare 1:10 (500 mg/L) and 1:100 (50 mg/L) dilutions, respectively, to obtain the corresponding quantities (in microliters) and test concentrations of 0.5 and 0.05 mg/L to apply to plastic cups containing 150 mL of distilled water. Each dose was applied to groups of 25 larvae, with 4 replications per dose. Four cups with only distilled water were used as untreated controls. The cups were kept at 25± 3 °C and 14.10 h light/dark photoperiod. The mortality data were recorded after 24 and 48 h. The experiments were repeated 3 times on different days.

The dose range to obtain the median lethal concentration (LC_{50}) was selected according to the results of previous experiments. For this, serial dilutions ranging from 1.0 to 0.1 mg of the extract per liter were used (5–6 doses). These treatments, in the same way, were applied to cups containing 25 late third-stage larvae and 150 mL of distilled water with four repetitions per dose and for untreated control. These bioassays were repeated four times on different days.

Table 2: Ingredients (as % of total weight) used for preparation of Bti formulations (tablets and granules)

Tablets (T)					**Granules (G)**		
Ingredient (% w/w)	T1	T2	T3	T4	G1	G2	G3
Acacia gum	0	0	10	5	0	5	6.6
Pectin	10	5	0	0	10	5	3.3
$CaCO_3$	40	40	40	40			
Starch	40	40	40	40			
β carotene					1	1	1
Ground cork					79	79	79
Unformulated Bti extract	10	15	10	15	10	10	10
Total weight	100	100	100	100	100	100	99.99

2.5 Preparation of formulations

Two types of formulations were designed: 1) tablets and 2) granules, with compositions shown in Table 2. For tablets, Bti extract was included as an active ingredient at 10 and 15% w/w and two polymers: pectin (food grade) and acacia gum (Desarrollo de Especialidades Químicas, S.A. de C.V., Monterrey, N. L. Mexico). Other ingredients included were calcium carbonate (Tecnología Industrial Química, S. A. de C.V. Escobedo, N. L. Mexico) and soluble starch (Jalmek, S. A. de C.V. San Nicolás de los Garza, N. L. Mexico). For granular formulations, polymers and floating materials (ground cork, Intergasket S. A. de C. V. San Nicolás de los Garza, N. L. Mexico) as well as a solar protectant (β carotene obtained from Industrial Orgánica S. A. de C.V: Monterrey, N. L. México) were used. Formulations were prepared by dissolving each one of the polymers in distilled water; then, the Bti extract was incorporated and homogenized with agitation; subsequently, the protectant was added in the granulated formulations and mixed and dried at room temperature. For tablets, the mixture was pressed to achieve a compressed form. Besides, was included unformulated Bti extract (UBE) for comparison in tests.

2.6 Evaluation of formulations before and after UV irradiation

To test the effects of UV light on the larvicidal activity of unformulated and formulated Bti extracts, the formulations prepared as tablets and granules and unformulated Bti extract were irradiated for 72 h with a Spectroline UV light lamp of 75 mW intensity (Model ENF-280 C, Spectronics Corp. Westbury, N. Y.) at 254 nm λ. Bioassays were performed using 50 mg of the Bti larvicidal extract irradiated and non-irradiated and were suspended in 10 mL distilled water (5,000 mg/L) after was taken 1 mL to prepare 1:10 dilution (500 mg/L). From this dilution were taken the corresponding quantities (as microliters) in order to obtain the test concentration of 1.6 mg/L and then placed in polystyrene containers (15.6 × 13.8 cm and 9 cm high) filled with 1 liter of dechlorinated water. The same dose of active ingredients (1.6 mg/L) was also applied for formulations prepared with Bti extract, irradiated and nonirradiated, corresponding to 16 mg of formulation containing 10% of Bti extract and 10.6 mg from the formulation containing 15% of Bti extract. Groups of 25 late third-stage larval *A. aegypti* were placed into each container, four repetitions for the treatment and untreated control. Bioassays were performed thrice. Larval mortality in each treatment was recorded after 24 h. Results were expressed as mortality percentages.

2.7 Evaluation of residual activity of the formulations

Bioassays were carried out in laboratory (25 ± 3 °C temperature and 14.10 h light/dark photoperiod), and 7 formulations (4 tablets, T1 to T4 and 3 granulates, G1 to G3) prepared with the most toxic extract were tested. In addition, an unformulated Bti extract (UE) was also included for comparison and tested at the same concentration (1.6 mg/L) and conditions. The bioassay method was similar to that reported previously [18] and modified as follows: 25 late third-stage larval *Ae. aegypti* were placed into each container following the same steps in testing the effect of UV irradiation. Then, different treatments were administered. Unformulated Bti extract at 1.6 mg/L dose, with formulations containing 10% Bti extract (16 mg), as well as those containing 15% Bti extract (10.6 mg), were used at the same proportion of the active ingredient. Four repetitions per treatment were applied, and an untreated control was included. Larval mortality in each treatment was recorded after 24 h, and all living or dead larvae from each treatment were carefully removed using a dropper and then counted and discarded. A new batch of 25 larvae was introduced into each treatment container at 1, 2, 3, 7, 10, 14, 18, 21, 24, and 28 days post-treatment, mortality was evaluated 24 h after the introduction in each case, and data were recorded.

2.8 Statistical analysis

Yield means (g/L) of larvicidal extracts obtained from the three culture media were analysed through a one-way analysis of variance (ANOVA) and least significant difference method at 0.05 level. The number of dead larvae (average) in bioassays was recorded after 24 h and analysed using a one-way ANOVA, and means were compared with the least significant difference (LSD) method at $p \leq 0.05$ to determine differences in the mortality of larvae produced by different larvicidal extracts obtained from three tested media.

Subsequently, LC_{50} and LC_{90} were determined using the PROBIT analysis program [19]. To test the effects of UV light on the larvicidal activity of formulations, the number of dead larvae were analyzed using the Student's *t*-test at 0.05 level, for each sample before and after UV irradiation. To evaluate the residual activity, the average dead larvae obtained in each sampling day was analysed using a one-way ANOVA, and means were compared using the least significance difference method at $p \leq 0.05$.

3. Results

3.1 Yield and toxicity of larvicidal extracts

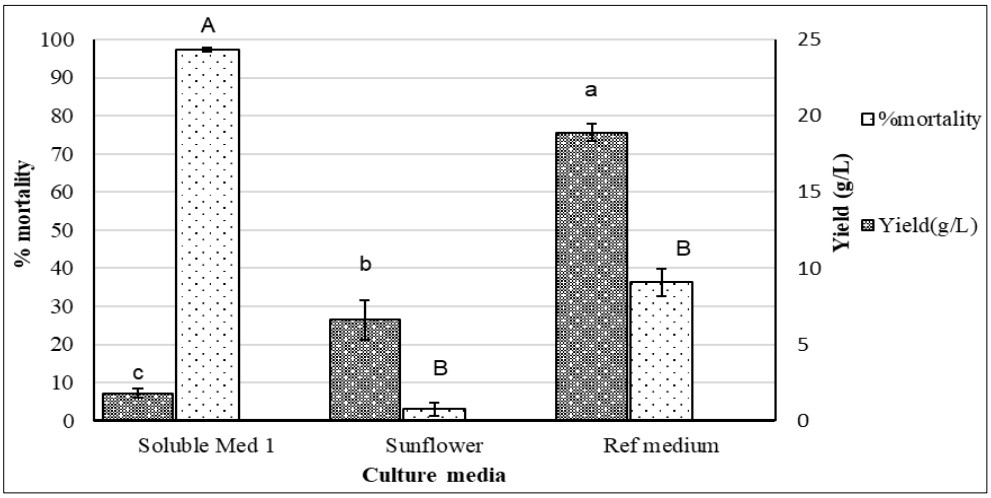

Fig 1: Means of yield (g/L) and toxicity (% mortality) ± standard error, presented by Bti larvicidal extracts produced on different culture media. Means (4 repetitions) followed by the same letter are not significantly different (ANOVA, LSD 0.05). Uppercase letters correspond to comparison of mortality and lowercase letters to comparison of yield.

Yields obtained in the fermentation media varied, with an average of 18.9 g/L for the reference medium composed of molasses and soybean meal; approximately 1.8 g/L for the soluble medium 1 composed of corn syrup, corn steep solids, and yeast extract; and 6.6 g/L for the medium 2 composed of sunflower seed flour and sucrose. However, the mortality percentage presented in Bti larvicidal extracts, tested to 0.5 mg//L concentration against *A. aegypti* larvae, was higher for those obtained in soluble medium 1 than for medium 2 and reference medium (Fig. 1). The preliminary test of mortality against *A. aegypti* showed high mortality for Bti extracts obtained in soluble medium 1 with corn steep solids and yeast extract, whereas extracts obtained in the reference medium and sunflower seed flour presented low mortality, when applied at 0.5 mg/L concentration.

Table 3 [1]Larvicidal activity of Bti extracts produced in two culture media against *Aedes aegypti* larvae (95% confidence limits in parentheses)

Culture Media	Exposure Time	Number tested larvae	[2]LC_{50}	[2]LC_{90}
Reference Medium	24	2400	0.9296 (0.07-11.53)	1.6633(0.007-20.60)
Medium Soluble 1	24	2400	0.3748 (0.19-0.70)	1.1954(0.43-6.42)

[1] in mg/L
[2] Average of four bioassays

3.2 Determination of LC_{50}

The laboratory activity shown by two Bti extracts, 1) obtained from the reference medium and 2) soluble medium 1, is presented in Table 3. Bti extract obtained in medium 2 did not determined the LC_{50} because of low mortality. Bti extract produced in the reference medium was less toxic than that produced in soluble medium 1. Averages of four bioassays were compared using a *t* test (Student) at 0.05 level, demonstrating a highly significant difference between them (t value = 21.3185, $t_{0.05}$= 2.447, $t_{0.01}$= 3.707, df =6) where the most toxic extract was obtained from soluble medium 1.

3.3 Irradiation of formulations and Bti extract with UV light

After 72 h of UV light exposure, the mortality was reduced by 5%–12% in tablets and 6%–11% in granules; otherwise, the reduction was higher in unformulated Bti extract (32%), as shown in Table 4.

Table 4: Mortality means ± standar error of *Aedes aegypti* caused by Bti formulations (tablets as T and granules as G), after 72 h exposure to uv light (irradiated) compared with nonirradiated

Formulation	% Mortality[1]		% Reduction
	Non-Irradiated	Irradiated	
T1	92 ± 5.6	80 ± 16.3	12
T 2	99 ± 2	93 ± 9.4	6
T 3	97 ± 3.8	85 ± 16.4	12
T 4	96 ± 3.2	91 ± 8.8	5
G1	100	92 ± 5.6	8
G 2	100	89 ± 8.2	11
G3	100	94 ± 5.1	6
Unformulated Bti	100	68 ± 5.6	32
Untreated Control	0	0	0

[1] Average of 4 repetitions

3.4 Evaluation of the residual activity of formulations

Formulations and unformulated Bti extract tested against *A. aegypti* larvae, as presented in Fig. 2, showed that tablet 2, granulate 3, unformulated Bti extract, presented nearly 100%

mortality on the first post-application day, while the remaining granulates and tablets reached >90% mortality, without significant differences between treatments (F = 1.9193; df = 7, 24; P =*0.110;* coefficient of variation [C.V:] = 6.14%). On the second post-application day, granulate 3, unformulated Bti extract, retained nearly 100% mortality, whereas tablet 2 and granulate 1 showed >90% mortality; however, tablets 1, 3, and 4 had decreased toxic activity to approximately 80% mortality (F = 4.9714; df = 7, 24; P =*0.002*; C.V. = 10.40%). On the third post-application day, only granulates 1, 2, and 3, along with the unformulated Bti extract, retained approximately 90% mortality, whereas tablets showed <60% mortality, except for tablet 2 that showed nearly 80% mortality (F= 11.5278; df = 7, 24; P <0.0001; C.V.= 13.83). On the 7 post-application day, granulate 3 presented residual activity of approximately 60% mortality, whereas unformulated Bti extract still showed 77% mortality; and all tablet formulations presented <50% mortality (F = 6.4433; df = 7, 24; P < 0.0001; C.V.= 23.58%). In the following sampling days, 14 (F = 13.3487; df = 7, 24; P < 0.0001; C.V.= 31.93%) and 21 (F = 6.6911; df = 7,24; P < 0.0001; C. V.= 51.82%) post-application, granulates 2 and 3 showed 48-32% mortality, whereas the unformulated Bti extract showed only 22-10% mortality. At the end of the test, the formulations lost their residual activity within 28 days (F = 13.0714; df = 7,24; p < 0.0001; C.V.= 61.54%).

4. Discussion

Yields obtained from the production of larvicidal extracts widely varied. Yields obtained in the soluble medium 1 were lower than that of the reference and sunflower media; however, the toxicity presented was more than three times greater than those from the reference medium, which is mostly used for the production of other varieties of Bt. In addition, this spore–crystal complex was free of impurities, such as unused residues of culture media. Other researchers [20] obtained 9.1–15.7 g/L from a medium containing 10 g/L molasses, corn steep liquor (10 g/l), powder paste (30 g/L), and mineral salts, whereas in another works [21] reported 7.8 g/L from in a medium containing 2.5% soybean meal. In another report [22] obtained 1.64–4.78 g/L of spores and crystals in the media with peptone and yeast extract at different concentrations. In a complex medium containing 30 g starch/L, 25 g soybean/L, and 5 g yeast extract/L, they [23] obtained 3.06 g/L of the spore and crystal complex. Similarly, other report [24] produced 6.85 g/L of spores and crystals in a medium containing 56 g glucose/L.

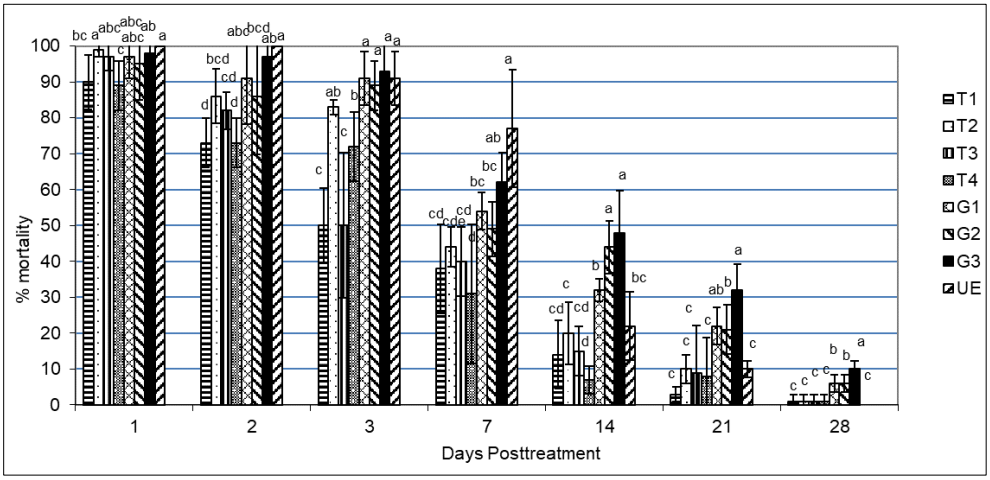

Fig: 2 Residual activity (means of 4 repetitions) of Bti formulations as Tablets (T) and Granules (G), besides unformulated Bti extract (UE) tested against late third instar *Aedes aegypti* larvae in laboratory conditions. (Different letters indicate significant differences on each sampling day determined through ANOVA and LSD at P≤0.05Meanwhile, in a new work, [25] obtained 1,672 mg/L of Bti toxin variety *kurstaki* from a medium composed of wastes from the starch industry.

When LC_{50} was determined, 0.9296 mg/L was found for the extract produced by the reference medium containing molasses and soybean meal and 0.3748 mg/L for the extract produced in soluble medium 1 containing syrup and corn steep solids, both LC_{50} at 24 h; this means that the larvicidal extract produced in the soluble medium 1 was 2.4 times more toxic than that produced by the reference medium.

Compared to our results, in a previous work [20] obtained LC_{50} values between 0.2675 and 0.0685 mg/L from a culture medium containing 10 g/L molasses, corn steep liquor (10 g/L), powder paste (30 g/L), and mineral salts, whereas other authors have obtained 0.26 ± 0.10 mg/L from studies using a tablet [26]. However, in some reports [14] have obtained even smaller LC_{50} for the technical powder (4 ng/mL) used in the preparation of an experimental tablet, XL-47, whereas other researchers [22], also obtained 18 ng/mL from a medium composed of 2% peptone and 2% yeast extract. Although the values obtained for LC_{50} were not the most toxic in this study, they are still comparable to other values obtained by some researchers [20, 26].

As regards the results of toxicity and residual activity assessments of the formulations in the first two days post-treatment, all formulations presented >80% mortality, except for tablets 1 and 4. On the third post-application day, all granulated formulations, along with unformulated Bti extract, retained approximately 90% mortality, which was not similar with those of tablets that decreased to <60%. Thus, the residual activity was better for granulated than for tablet formulations; granulate 3 retained approximately 60% mortality at 7 days post-application, and the unformulated Bti extract started to decline from 10 days post-application, which was surpassed by the granulated formulations: therefore, these showed >40% mortality in 14 days, whereas the unformulated Bti extract only presented approximately 25% mortality;

however, all formulations were losing their activity gradually. Compared to our results, other report [26] obtained much higher residual activity of up to 180 days in conditions without exposure to sunlight; however, the quantities used for their formulations (tablets) were also higher (250 mg/tablet) as compared to the amount used (1.6 mg) in this study. Other work [14] reported the preparation and evaluation of a tablet (190 mg) containing 4.8% of active ingredient, with reduced pupae formation from 59.2% to 100%, depending on the partial exposure to sunlight conditions to full exposure for 16–12 weeks; however, no larval mortality was reported in those periods. The amount of active ingredient on each tablet represented approximately 9 mg of the technical powder/tablet, and considering that 1, 2, 3, 5, or 8 tablets were added per container, each container could have contained 9–72 mg of active ingredient; values that were also much higher than our tablets containing only 1.6 mg.

Recently, experiments conducted with "fly ash" Bti formulations [27] tested against larval mosquitoes reportedly used the preparation of 16 formulations, where the formulation containing Bti, 4.5% lignite, and 1% carboxymethylcellulose was the most effective, with LC_{50} values of 0.0417, 0.0462, and 0.1091 mg/L against *Cx. quinquefasciatus*, *A. aegypti*, and *An. stephensi*, respectively; however, the residual activity of these formulations was not reported.

Regarding to irradiation of UV light on formulations, granulated and tablet formulations presented smaller reductions in toxic activity (6–11 and 5–12%, respectively) as compared to the unformulated Bti extract, which showed greater reductions (32%) after 72 h of irradiation. In a previous work [28], they also obtained protection for Bti's toxin with various photo protectants, such as malachite green, and gelatin and acacia gum as encapsulating polymers. After 72 h of UV irradiation, larval mortality was reduced by 40% for the unformulated Bti extract and only 4–9% in prepared formulations. Thus, the inclusion of a photo protectant could to have a positive effect on the toxic activity. New formulations should be developed to improve the residual activity of products for an extensive use worldwide, primarily in developing countries.

5. Conclusions

The results of this study showed that larvicidal extracts more toxic can be obtained improving the composition of culture media, as those obtained from the soluble medium 1, which was higher than that from the reference and sunflower media. However, the yield obtained was less than reference medium, which indicate that is necessary a balance between toxicity and yield. The formulations prepared against *A. aegypti* larvae in the laboratory, presented nearly 90%-100% mortality, whereas tablets showed less 60% mortality, during the first 3 days post-application, After, the toxic activity was gradually decreasing, at 14 days post application, when granulated formulations presented approximately 40% mortality in comparison with the unformulated extract showing almost the middle of mortality. The addition of protectans for UV irradiation, friendly with the environment in formulations, prevents the loss of toxic activity of formulations.

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