OPEN ACCESS Plant Protection, 10 (05) 2026. 973-981 DOI:10.33804/pp.010.05.6268

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Available Online at EScience Press

Plant Protection

ISSN: 2617-1287 (Online), 2617-1279 (Print)

http://esciencepress.net/journals/PP

Research Article

Multi-Stage Bioefficacy of *Bacillus thuringiensis* Extract against *Culex quinquefasciatus*: Effects on Mortality, Egg Hatching and Adult Emergence

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ARTICLE INFO

Article history

Received: 8th August, 2026

Revised: 17th October, 2026

Accepted: 20th October, 2026

Keywords

Bacillus thuringiensis

Culex quinquefasciatus

Ovicidal activity

Larvicidal activity

Mosquito control

ABSTRACT

Mosquito control requires effective alternatives to conventional insecticides. This study evaluated the insecticidal activity of *Bacillus thuringiensis* extract against different developmental stages of *Culex quinquefasciatus*. The extract exhibited significant, concentration-dependent ovicidal, larvicidal, and pupicidal activities. The highest concentration (10^{-1}) caused 100% egg mortality and completely inhibited hatching, while the 10^{-2} and 10^{-3} concentrations resulted in 97.67% and 91.33% mortality, respectively. Against fourth-instar larvae, mortality reached 70.00% at the highest concentration, compared with 56.67% and 43.33% at the 10^{-2} and 10^{-3} concentrations, respectively. Exposure also caused substantial delayed mortality during pupation (62.33–68.33%) and markedly reduced adult emergence to 10.00–23.33%, compared with 100% in the untreated control. Direct pupal treatment resulted in 60.00–80.00% mortality, with adult emergence decreasing to 20.00% at the highest concentration. Probit analysis further demonstrated high toxicity, with LC_{50} values of 3.13×10^{-3} for fourth-instar larvae and 1.44×10^{-4} for pupae. The models showed satisfactory goodness of fit ($P > 0.05$). Overall, the *B. thuringiensis* extract demonstrated substantial toxicity across multiple developmental stages and disrupted mosquito development and adult emergence. These findings support its potential as a biological tool for managing *C. quinquefasciatus*, although further studies are needed to establish field efficacy, environmental safety, and practical application.

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Introduction

The southern house mosquito, *Culex quinquefasciatus* (Say), is a prominent species in urban and peri-urban areas and is a major vector of many debilitating diseases. It is a significant vector for *Wuchereria bancrofti* (which causes lymphatic filariasis) and a number of arboviruses such as West Nile virus (WNV) (Larrick and Connelly, 2009). Therefore, control of its populations is an important public health issue worldwide. Mosquito control has been heavily dependent on synthetic chemical insecticides in the past. But the widespread and unselective use of these chemicals has led to serious ecological problems (Karunaratne and Surendran, 2022). These include environmental pollution, toxicity to non-target organisms, and, most importantly, the general emergence of physiological and biochemical insecticide resistance in mosquito populations. This growing problem calls for immediate action to move towards sustainable and environmentally friendly approaches in the context of Integrated Vector Management (IVM) (Abbasi, 2025).

Biological control with entomopathogenic microorganisms is a very promising alternative. *Bacillus thuringiensis* (Bt) is a soil bacterium that forms spores and is a highly successful biopesticide used around the world. It is primarily entomocidal, by means of parasporal crystalline inclusions (Cry and Cyt toxins) formed during sporulation (Guerrero, 2023). These protoxins are solubilized and activated in the highly alkaline midgut of susceptible larvae when ingested. The active toxins interact with specific epithelial receptors, leading to pore formation, paralysis of the midgut and ultimately death of the larvae by starvation and septicemia (Ma et al., 2023).

In addition to lethal toxicity, Bt extracts can have strong sub-lethal effects on surviving mosquito populations. Sub-lethal concentrations can cause abnormal physiological processes, with extended development times, decreased pupation and adult emergence rates, morphological abnormalities and decreased fecundity. These sub-lethal effects are important to understand because they can have a significant effect on the vectorial capacity and reproductive success of the population over successive generations (Akhtar et al., 2022).

Hence, the present study was designed to investigate the efficacy of the extract of *B. thuringiensis* bacteria against *C. quinquefasciatus* comprehensively. Specifically, this research examined the acute larvicidal effect of the extract and its sub-lethal effects on various biological and developmental parameters, for a better understanding of its possible use as a sustainable and environmentally friendly tool in mosquito management programs.

Material and methods

Samples of *C. quinquefasciatus* mosquitoes were collected from drainage water in the Abu Ghraib area, Baghdad. Fourth-instar larvae and pupae were collected from the aquatic habitats using suitable plastic dippers. The specimens were transported to the Entomology Laboratory in plastic containers filled with water from the original collection site to minimize stress on the insects during transport.

In the laboratory, the fourth-instar larvae were separated from the pupae using fine plastic pipettes. Each group was placed in a 250 mL plastic container containing distilled water. The larvae were fed ground fish food to support their growth and development. The pupae were maintained in the same plastic containers and transferred into mosquito rearing cages in the laboratory until they emerged as adults (Figure 1). Following adult emergence, they were kept inside the cages to mate and oviposit, thereby re-initiating the life cycle to obtain successive generations for various bioassays. The mosquitoes were reared under constant laboratory conditions at a temperature of 27 ± 2°C, a relative humidity of 70 ± 5%, and a photoperiod of 12:12 h (light: dark).



Figure 1. Laboratory mosquito rearing cages.

Bacterial activation, culture conditions and extraction

The Bt used in this study was a local isolate of *B. thuringiensis* subsp. *israelensis* (Bti). The bacteria were first isolated from a soil sample, cultured, and morphologically identified in the laboratory. The isolate was cultured on Nutrient Agar plates and incubated at 37°C for 48 h to obtain well-isolated colonies to activate the bacteria. The active bacterial growth was then transferred to an Eppendorf tube with liquid Nutrient Broth and mixed well with a micropipette. This suspension was then placed in a sterile Erlenmeyer flask with Nutrient Broth. The flask was then incubated in a shaking incubator at 37°C and 110 rpm for 48 h for optimal growth. The whole-culture liquid broth was not purified and was used directly as the crude extract for all subsequent bioassays and was referred to as the original stock suspension (Figure 2).

Preparation of serial dilutions and bacterial quantification

A series of decimal dilutions of the Bti crude extract was prepared in sterile peptone water to assess the bio-efficacy of the crude extract. Peptone water was made, divided into 9 mL portions in glass test tubes and autoclaved at 121°C for 20 min. The original whole

culture stock suspension was serially diluted to prepare the exact test concentrations for the bioassays. Specifically, 1 mL of the bacterial stock was thoroughly mixed with 9 mL of sterile peptone water to get the 10^{-1} dilution. Then 1 mL of this 10^{-1} suspension was transferred to another tube of peptone water to make the 10^{-2} dilution. Finally, 1 mL of the 10^{-2} dilution was placed in a third tube to make the 10^{-3} dilution. The plate count method was used to quantify the bacterial density and confirm the viability. To sum up, 100 µL of the 10^{-3} dilution was spread on Nutrient Agar, triple-plated, and incubated at 37°C for 24 h. On the 10^{-3} plates, 280 colonies were counted. The original stock suspension was estimated at 2.8×10^5 CFU/mL using the standard formula for calculating the number of colony-forming units. Thus, the actual concentrations of exposure in the subsequent bioassays were carefully determined as 2.8×10^4 CFU/mL for the 10^{-1} dilution, 2.8×10^3 CFU/mL for the 10^{-2} dilution, and 2.8×10^2 CFU/mL for the 10^{-3} dilution.

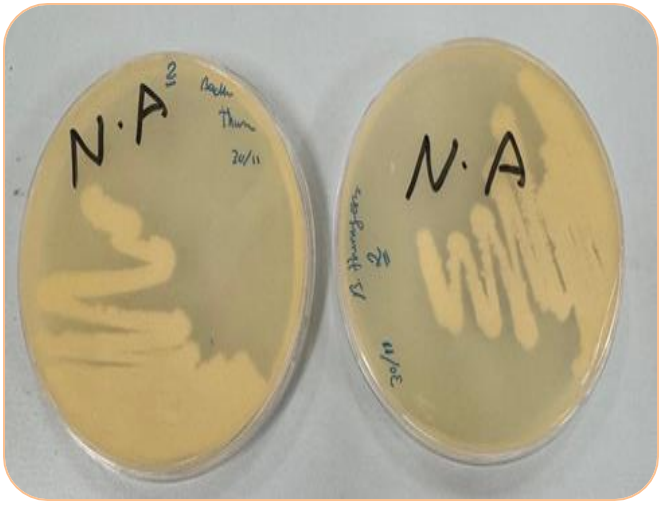


Figure 2. Activation of *B. thuringiensis* on Nutrient Agar.

Bioassay and evaluation of biological efficacy

The biological activity of the *B. thuringiensis* extract was tested against three different developmental stages of the *C. quinquefasciatus* mosquito, namely the egg stage, fourth larval instar, and pupal stage. These assessments were done using three dilutions of the bacterial extract (10^{-1}, 10^{-2}, and 10^{-3}). One egg raft (about 100-150 eggs) was added to each dilution for the treatments. Likewise, 20 fourth-instar larvae and 20 pupae were subjected to each replicate. The effect of the bacterial extract was evaluated at all stages with three replicates for each dilution to have a comprehensive evaluation of the effect of the bacterial extract throughout the mosquito life cycle. In all bioassays, an untreated control group was maintained in distilled water (no bacterial extract or peptone vehicle) under the same conditions to determine the natural mortality.

Statistical analysis

A completely randomized design (CRD) was used for the bioassays. The experimental unit consisted of 20 larvae/pupae or an egg raft for the ovicidal assay in each plastic container. All treatments, including the untreated control, were replicated three times. Data were analyzed using GenStat for Windows (VSN International, 2009) and SPSS Statistics (Version 26.0). The assumptions of Analysis of Variance (ANOVA) were checked prior to conducting parametric tests: the Shapiro-Wilk test was used to test the normality of the data distribution, and Levene's test was used to test the homogeneity of variances. The biological parameters were analyzed using one-way ANOVA because the assumptions were fulfilled with different concentrations of bacterial extract. The Fisher's Least Significant Difference (LSD) test was used to compare means at $P \leq 0.05$ significance level. Moreover, mortality data of fourth instar larvae and pupae were analyzed using Probit regression analysis to determine the median lethal concentration (LC_{50}) and 90% lethal concentration (LC_{90}) values with their 95% confidence intervals, slopes, and Pearson chi-square values to confirm the model fit.

Results

Ovicidal activity and egg hatching rate

The direct toxicity of the *B. thuringiensis* extract on the egg stage of *C. quinquefasciatus* was found to be highly significant and dose-dependent. The highest concentration (10^{-1}) caused a 100% mortality rate and 0.00% hatching success, completely inhibiting embryogenesis and hatching. This potent effect was statistically similar to that of the 10^{-2} concentration, with a 97.67% mortality rate and a very low hatching rate of 2.33%. The ovicidal activity was significantly reduced at the lowest concentration of 10^{-3} with an 8.67% hatching rate and 91.33% mortality rate. The untreated control group, however, showed normal development with 0.00% mortality and a complete 100% hatching in control groups (Table 1; Figure 3). The analysis of variance revealed that all tested groups were highly significantly different (LSD = 2.772, $P \leq 0.05$).

Table 1. The effect of different concentrations of *B. thuringiensis* extract on the mortality and hatching rates of *C. quinquefasciatus* eggs.

Concentration	Egg Mortality (%) ± SE	Egg Hatching (%) ± SE
10^{-1}	100.00 ± 0.00 [a]	0.00 ± 0.00 [c]
10^{-2}	97.67 ± 1.45 [a]	2.33 ± 1.45 [c]
10^{-3}	91.33 ± 0.88 [b]	8.67 ± 0.88 [b]
Control	0.00 ± 0.00 [c]	100.00 ± 0.00 [a]
L.S.D. (P ≤ 0.05)	2.77	2.77

Means within a column followed by different letters are significantly different (P ≤ 0.05).

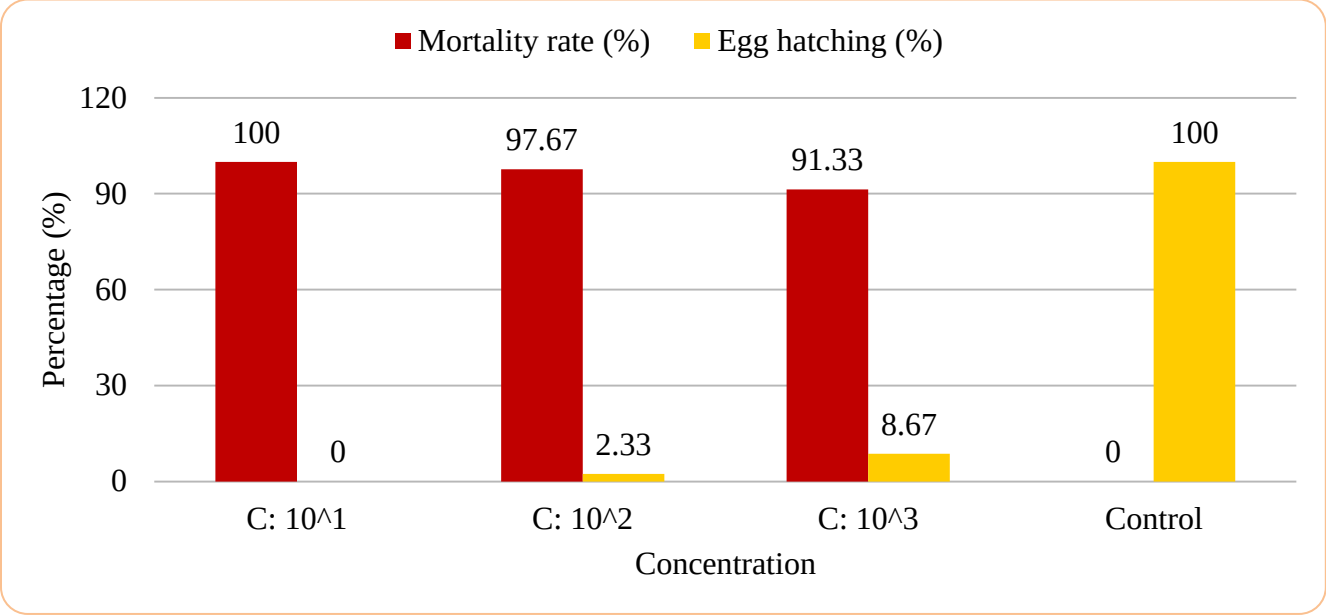


Figure 3. Ovicidal activity and egg hatching rates of *C. quinquefasciatus* exposed to serial dilutions of *B. thuringiensis* extract and compared to the untreated control.

Larvicidal efficacy and subsequent developmental impacts

The bioassay on the fourth instar larvae of *C. quinquefasciatus* showed that the extract of *B. thuringiensis* was highly toxic to the larvae and had significant delayed activity on the later stages. The larvicidal activity was dose-dependent in the extract. Larval mortality was the highest in the highest concentration (10^{-1}) with 70.00%. This was significantly higher than the mortality observed at the 10^{-2} concentration (56.67 %) and the 10^{-3} concentration (43.33 %). The untreated control group had no mortality (0.00 %). The analysis of variance showed that all tested concentrations were highly significant.

The bacterial extract showed high delayed toxicity to the larvae that survived the first treatment and were able to pupate. The pupal mortality was always high, and there was no significant statistical difference among the three extract concentrations. The mortality reached 68.33% at the 10^{-2} concentration, followed closely by 67.00% at the 10^{-1} concentration, and 62.33 ± 8.56% at the 10^{-3} concentration. The pupal mortality of these groups was significantly higher than that of the control with 0.00% pupal mortality.

The cumulative stress from the larval exposure resulted in a drastic and significant reduction in adult emergence. The emergence rates were severely inhibited at the higher concentrations, recording only 10.00% at the 10^{-1} concentration and 13.33% at the 10^{-2} concentration (both grouped no significant difference between them). The emergence rate slightly increased to 23.33% at the lowest concentration of 10^{-3}. In contrast, the control group exhibited a complete emergence success rate of 100% (Table 2; Figure 4).

Table 2. The effect of the concentrations of *B. thuringiensis* extract on the direct larval mortality, delayed pupal mortality and adult emergence of *C. quinquefasciatus* (treated as fourth instar larvae).

Concentration	Larval Mortality (%) ± SE	Pupal Mortality (%) ± SE	Adult Emergence (%) ± SE
10^{-1}	70.00 ± 2.89 [a]	67.00 ± 8.89 [a]	10.00 ± 2.89 [c]
10^{-2}	56.67 ± 1.67 [b]	68.33 ± 4.48 [a]	13.33 ± 1.67 [c]
10^{-3}	43.33 ± 7.26 [c]	62.33 ± 8.56 [a]	23.33 ± 4.41 [b]
Control	0.00 ± 0.00 [d]	0.00 ± 0.00 [b]	100.00 ± 0.00 [a]
L.S.D. (P ≤ 0.05)	13.03	21.42	9.01

Means within a column followed by different letters are significantly different (P ≤ 0.05).

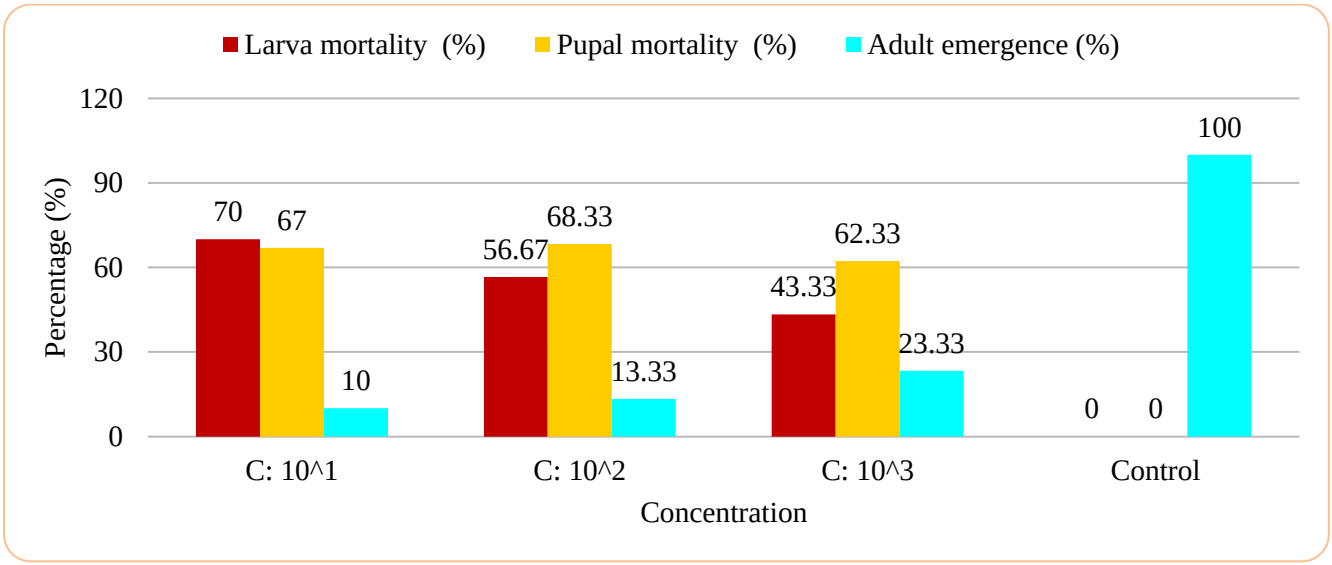


Figure 4. Percentages of direct larval mortality, delayed pupal mortality, and successful adult emergence of *C. quinquefasciatus* after exposure of fourth-instar larvae to serial dilutions of Bt extract.

Direct pupicidal efficacy and impact on adult emergence

The direct toxicity of the extract of *B. thuringiensis* was tested against the pupae of *C. quinquefasciatus* in the final bioassay. As with the previous stages of development, pupal mortality showed a marked dose response. The highest concentration (10^{-1}) had the highest pupal mortality (80.00%). As the concentration decreased, mortality decreased to 70.00% at the 10^{-2} concentration and to 60.00 at the 10^{-3} concentration. The mortality rates of all treated groups were significantly higher than that of the untreated group, which had 0.00% mortality (LSD = 11.53, P ≤ 0.05). The emergence of adult mosquitoes from the pupae treated with the extracts was thus inversely proportional to the concentrations of the extracts applied. The lowest emergence success was recorded at the 10^{-1} concentration with only 20.00% of the pupae emerging as adults. The emergence rate gradually improved at lower doses, recording 30.00% at the 10^{-2} concentration and 40.00% at the 10^{-3} concentration. The control group, on the other hand, had a 100% emergence rate. The emergence rate of different treatments varied significantly (LSD = 11.53, P ≤ 0.05) (Table 3; Figure 5).

The data on mortality of fourth-instar larvae and pupae were subjected to Probit regression analysis to quantify the dose-response relationship (Table 4). The analysis showed that the extract was highly toxic with median lethal concentration (LC^{50}) values of 3.13×10^{-3} for fourth instar larvae and 1.44×10^{-4} for pupae. Furthermore, the 90% lethal concentration (LC_{90}) values were extrapolated to be 15.82 for larvae and 3.394 for pupae. Both Probit models fitted the observed concentration-mortality data very well, with low Pearson chi-square values of 0.00266 and 0.01208, and non-significant goodness-of-fit tests (P > 0.05), respectively, demonstrating the high degree of goodness of fit of the models to the observed data, which confirms the efficacy of the Bti crude extract in a dose-dependent manner.

Table 3. The effect of different concentrations of *B. thuringiensis* extract on the direct pupal mortality and subsequent emergence of adults of *C. quinquefasciatus* (treated directly as pupae).

Concentration	Pupal Mortality (%) ± SE	Adult Emergence (%) ± SE
10^{-1}	80.00 ± 2.89 [a]	20.00 ± 2.89 [c]
10^{-2}	70.00 ± 2.89 [ab]	30.00 ± 2.89 [bc]
10^{-3}	60.00 ± 5.77 [b]	40.00 ± 5.77 [b]
Control	0.00 ± 0.00 [c]	100.00 ± 0.00 [a]
L.S.D. (P ≤ 0.05)	11.53	11.53

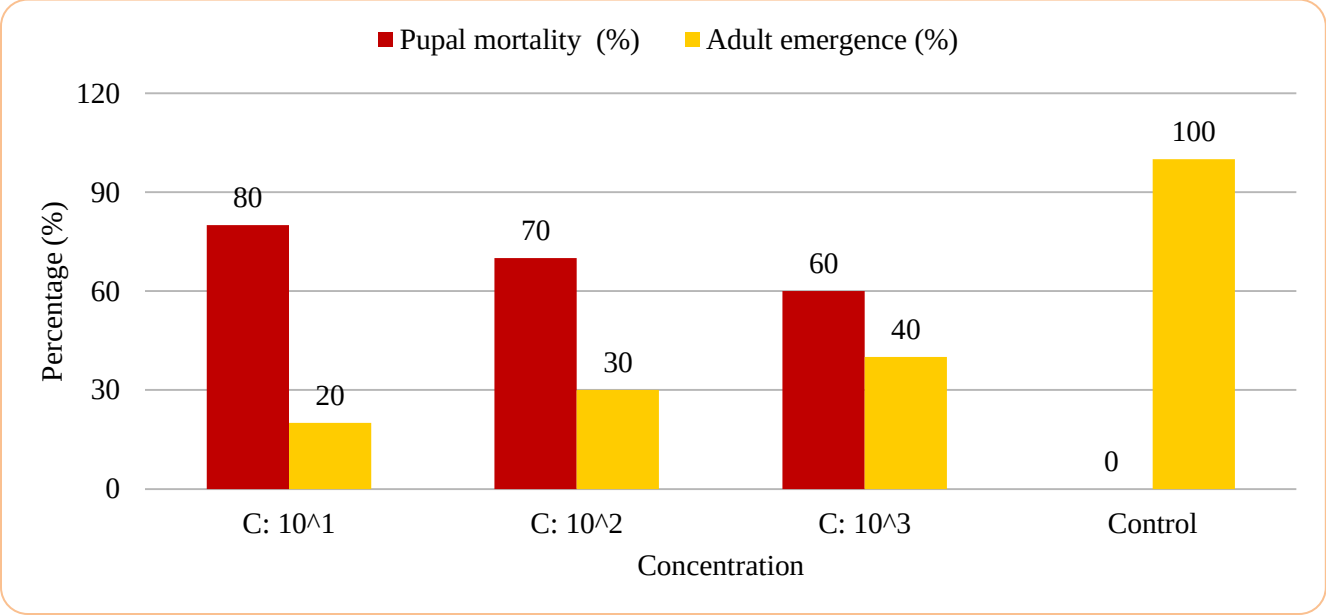


Figure 5. Effectiveness of *B. thuringiensis* extract against *C. quinquefasciatus* pupae and subsequent emergence of adults when pupae were exposed to serial dilutions of the extract.

Table 4. Toxicity of *B. thuringiensis* crude extract against fourth-instar larvae and pupae of *C. quinquefasciatus* using probit regression analysis.

Developmental stage	LC_{50}	95% CI for LC_{50}	LC_{90}	95% CI for LC_{90}	Slope ± SE	Pearson χ^2 (df = 1)	P-value
Fourth-instar larvae	3.13×10^{-3}	7.40×10^{-4}–1.32×10^{-2}	15.82	9.58×10^{-2}–2.61×10^{3}	0.346 ± 0.118	0.00266	0.959
Pupae	1.44×10^{-4}	3.69×10^{-6}–5.65×10^{-3}	3.394	1.95×10^{-2}–5.92×10^{2}	0.293 ± 0.123	0.01208	0.912

Note: LC values are reported as log10 transformations of the tested dilution factors. The high P-values (P > 0.05) suggest a good fit of the Probit model.

Discussion

The present study offers strong evidence of the bio-efficacy of the crude extract of *B. thuringiensis* (Bt) against various stages of the development of *C. quinquefasciatus*. The Integrated Vector Management (IVM) approach is shifting to eco-friendly microbial products due to the increasing difficulty of vector control with conventional synthetic insecticides (such as pyrethroids and organophosphates) that are being encountered worldwide (Abbasi, 2025). The results obtained show that the tested Bt extract is an effective larvicide, as well as having excellent ovicidal, pupicidal, and delayed developmental disruption properties.

One of the most important results of this study was the strong ovicidal effect, as egg hatching was completely inhibited at the highest extract concentration (10^{-1}).

Traditionally, Bt is referred to as a stomach poison that works via parasporal crystals in the actively feeding larval stages (Sunarti, 2025). Therefore, the strong ovicidal activity seen here is atypical for classical Bt activity. We used a crude bacterial extract (whole-culture suspension) instead of a purified spore-crystal formulation, however, we believe that the extract may contain a mixture of extracellular secondary metabolites and secreted lytic enzymes (chitinases and proteases). These bioactive compounds have been reported to degrade or penetrate the complex structure of mosquito eggs, (Katak et al., 2023; Castro et al., 2025; Prashar et al., 2025). However, since these compounds were not chemically characterized in our study, this mechanism remains a proposed hypothesis. When fourth-instar larvae were exposed to the extract, significant dose-dependent mortality was observed (up to 70%). Physiologically, this acute toxicity is mainly due to the ingestion of the δ-endotoxins (Cry and Cyt proteins) (Badr et al., 2026). In the extremely alkaline midgut of the larva, these protoxins are solubilized and proteolytically cleaved into active toxic fragments. The activated fragments then interact with specific glycoprotein receptors (aminopeptidase N (APN), alkaline phosphatase (ALP), cadherins) located on the membrane of the brush border of the midgut (Li et al., 2024). This receptor binding promotes toxin oligomerisation and pore formation, resulting in osmotic imbalance, midgut epithelial lysis, septicemia, and death of the larvae (Endo, 2022). Our study recorded not only immediate (acute) effects, but also strong delayed (sub-lethal) effects in the surviving larval cohorts. Larvae that were able to survive the first chemical stress had a very high pupal mortality (up to 68.33%) and a significantly reduced adult emergence rate (as low as 10%). The energetic costs of sub-lethal intoxication can be blamed for this cascading failure in metamorphosis. Surviving larvae use a significant portion of their energy reserves to heal damaged midgut tissues and to neutralize bacterial metabolites (Muita and Baxter, 2023). Thus, the severe structural damage to the midgut compromises the ability to absorb nutrients and results in chronic starvation. This energy deficiency results in a lack of physiological reserves required for pupation and emergence of adults, which is a very energy-consuming process (Hong et al., 2025). These results highlight the actual epidemiological importance of the extract as it is not toxic to larvae immediately, but it has a significant impact on the reproductive capacity and vectorial capacity of the vector (da Silva Sá et al., 2023).

The most interesting result of this bioassay is the high direct effect on the pupae of *C. quinquefasciatus* (up to 80%). The high direct toxicity against the non-feeding pupal stage of *C. quinquefasciatus* (up to 80%) is also unusual. The ingestion-based mechanism of action of Bt toxins is not applicable to pupae and we hypothesize that the crude extract is contact toxic (Chattopadhyay et al., 2004). The secondary metabolites may have direct effect on the pupal integument, or they may interact with the surface tension of the water, preventing the pupal respiratory trumpets from functioning and resulting in hypoxia (Melo et al., 2016). This multi-target approach provides the crude extract with a great operational benefit over conventional commercial products because it can kill overlapping mosquito generations in the same breeding site (larvae and pupae).

This study has proven the high bio-efficacy of Bti crude extract, but it has some drawbacks. First, the crude extract was not biochemically characterized, so the proposed ovicidal and pupicidal mechanisms are speculative. HPLC and Mass spectrometry should be used in future studies to isolate the specific bioactive compounds. Second, the lack of a positive control (such as a commercial Bt formulation) makes it impossible to directly compare the relative efficacy of the intervention with other interventions on the market. Methodologically, a sterile vehicle control (peptone water) was not included; however, the high mortality rate in a dose-dependent manner strongly suggests the efficacy of the bacteria and not the toxicity of the vehicle. Lastly, in bioassays using whole-culture suspensions, non-specific secondary physical effects like localized oxygen depletion (hypoxia) or physical blockage by microbial overgrowth cannot be completely excluded as factors.

In conclusion, the crude extract of Bt shows high multi-stage toxicity against *C. quinquefasciatus* in controlled laboratory conditions. It has a dual mode of action, both as a midgut toxin to feeding larvae and as a potential contact/physiological disruptor to non-feeding eggs and pupae, which demonstrates its potential bio-efficacy. These initial laboratory results indicate that these bacterial extracts may be a useful biological alternative to synthetic chemicals for controlling insecticide-resistant mosquito populations, but claims of their ultimate environmental safety and field applicability are

premature at this point. Before this extract can be recommended for use in operational mosquito control programs, comprehensive future studies are needed, including extensive field-scale trials and rigorous safety testing on non-target aquatic organisms.

Authors' Contributions

TAT and SAK conceptualized and designed the experiment. TAT conducted the field investigations and performed the experimental work. SAK provided overall supervision and ensured methodological and analytical consistency throughout the study. MAH contributed as a co-supervisor, providing critical guidance on experimental design, data interpretation, and research refinement at various stages of the project. TAT and SAK prepared the initial manuscript draft, including structuring the content, compiling the results, and integrating preliminary interpretations. All authors critically reviewed and revised the manuscript, contributed to its intellectual refinement, verified the data and analyses, and approved the final version for submission.

Research Funding

This research did not receive any grant from funding agencies.

Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 3: Good Health and Well-being
SDG 12: Responsible Consumption and Production
SDG 15: Life on Land

Policy Addressed

The study emphasizes Integrated Vector Management, sustainable biological control, and evidence-based biopesticide evaluation, while highlighting the need for further field validation and environmental safety assessments before practical implementation.

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