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# Synergistic larvicidal action of *Citrus limon* (L.) Osbeck (Rutaceae) and *Bacillus thuringiensis* Berliner 1915 (Bacillaceae) against the dengue vector *Aedes aegypti* Linnaeus 1762 (Diptera: Culicidae)

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## Abstract

Botanical and microbial insecticides are promising alternatives to synthetic pesticides for mosquito control because of lower toxicity to non-target organisms and their innate biodegradation ability. The present study was aimed to determine the synergistic larvicidal action of *Citrus limon* and *Bacillus thuringiensis* on the dengue vector *Aedes aegypti*. The crude methanolic leaf extract of *Citrus limon* and *Bacillus thuringiensis* were tested separately on the third instar larvae of *Aedes aegypti* at concentrations of 100, 200, 300, 400 and 500 mg/L and at 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L respectively. Larval mortality was observed after 24 and 48 hours and the corresponding $LC_{50}$ values were 285.1 and 219.5 mg/L for *Citrus limon* and 1.9 and 1.4 mg/L for *Bacillus thuringiensis* respectively. The synergistic larvicidal action showed high mortality and its $LC_{50}$ values were 158.5 and 109.9 mg/L after 24 and 48 hours of exposure respectively. This synergistic interaction was due to the phytocompounds of *Citrus limon* and toxins from *Bacillus thuringiensis* which showed toxicity on the larvae of the dengue vector, *Aedes aegypti*. Hence, the exploitation of plant chemicals and microbial pesticides can be suggested for use in mosquito vector control program for the control of mosquito-transmitted diseases.

**Keywords:** *Citrus limon; Bacillus thuringiensis; Aedes aegypti*; larvicidal; Synergistic action

## 1. Introduction

Mosquitoes present an immense threat to millions of people worldwide, since they act as vectors for malaria, Japanese Encephalitis, filarial and yellow fever, dengue, chikungunya and Zika virus fever [1, 2]. The misuse and overuse of synthetic pesticides have led to the vast destruction of beneficial organisms along with detrimental effects on the environment. The war against mosquitoes by method for concoction pesticides has fizzled because of the resistance created by mosquitoes [3]. Botanicals and microbial pesticides are promising alternatives for mosquito control because of lower toxicity to non-target organisms and their innate biodegradation ability. A number of plants and microbes have been reported as selectable subjects with little or no harmful effect on non-target organisms and the environment. Phytochemicals derived from plant sources act as larvicides and researches have proved the effectiveness of plant derived secondary compounds, *viz*., alkaloids [4, 5], flavonoids [6, 7], saponins [8, 9], steroids [10], and tannins [11] as mosquito larvicides. Besides these, microbial insecticides is also an important component of the integrated vector control strategy, wherein *Bacillus thuringiensis* a bacterium which produces bacteriocin compounds of insecticidal properties is a well-known bacterial agent which have been used successfully for mosquito control [12].

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*Citrus* species well known for its economic importance has been identified for its larvicidal activities. The potential use of extracts from *Citrus* species as a mosquito larvicide has been studied, *viz*., *Citrus aurantium* (sour orange), *Citrus grandis* (pomelo), *Citrus aurantifolia* (key lime) [13], *Citrus limon* (lemon), *Citrus sinensis* (red blood orange), *Citrus paradisi* (grapefruit), *Citrus reticulata* (mandarin orange) [14], *Citrus mitis* (calamondin), *Citrus jambhiri* (rough lemon) [15] and *Citrus limetta* (sweet lime) [16] against *Aedes aegypti*, and *Citrus limon*, *Citrus paradisi* and *Citrus sinensis* against *Culex pipiens* [17]. Microbial insecticides on the other hand, due to their selective toxicity and ready decomposability in the ecosystem, are being considered as alternatives to chemical insecticides as through their metabolites they act as potential mosquito larvicides. The genus *Bacillus* is extensively studied for mosquitocidal properties. *Bacillus thuringiensis*, *Bacillus sphaericus* have been used for mosquito control [18-20], *Bacillus alvei*, *Bacillus brevis* [21], *Bacillus circulans* [22] and *Bacillus subtilis* [23] are reported to produce mosquitocidal toxins. Thus, this study was aimed to determine the synergistic larvicidal action of *Citrus limon* and *Bacillus thuringiensis* on the dengue vector *Aedes aegypti*.

## 2. Material and methods

### 2.1. Plant collection and preparation of phytoextracts

Mature and healthy leaves of *Citrus limon* were collected from Nagercoil, Kanyakumari district, Tamil Nadu, India. Taxonomical identity of the plant was confirmed at Department of Botany and Research Centre, Nagercoil, Kanyakumari district, Tamil Nadu, India. The leaves were then brought to the laboratory, washed in dechlorinated water, shade dried and powdered with the aid of an electric blender separately. The powdered plant parts (250 g) were extracted with methanol (750 mL) in a Soxhlet apparatus [24]. The crude solvent leaf extract thus obtained were then stored in air tight sterilized amber coloured bottles at 4°C for bioassay.

### 2.2. Phytochemical screening

The active phytochemical substances in *Citrus limon* leaf extracts were qualitatively determined using the various methods described by Harborne [25] for alkaloids and phenolics, Van Burden and Robinson [26] for tannins, Obadoni and Ochuko [27] for saponins, Boham and Kocipai [28] for flavonoids, and Okwu and Okwu [29] for vitamins.

### 2.3. Microbial collection and preparation

*Bacillus thuringiensis* was obtained from Inbiotics laboratory, Nagercoil, Tamil Nadu, India. The required quantity of this microbe was thoroughly mixed with distilled water for bioassay.

### 2.4. Test mosquitoes

The eggs of *Aedes aegypti* were obtained from Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, India which were free of exposure to insecticides. Cyclic generations of this vector mosquito was maintained separately in mosquito cages (2'x2'x2') in an insectary with a mean room temperature of 27 ±2 °C and a relative humidity of 70-80 %. The adult mosquitoes were fed on ten per cent glucose solution in water. The eggs laid in ovitraps placed inside the mosquito cages were then transferred to enamel larval trays maintained in the larval rearing chamber. The larvae were fed with larval food (dog biscuits and yeast in the ratio 3:1). The larvae on becoming pupae were collected, transferred to plastic bowls and kept inside another mosquito cage for adult emergence.

### 2.5. Larvicidal bioassay

Larvicidal bioassay was carried out as per the guidelines of World Health Organization with minor modifications [30]. Larvicidal activity at test concentrations of 100, 200, 300, 400 and 500 mg/L for the plant and at 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L for the microbial extract and in addition, their synergistic activity was also assessed. The required test concentrations and quantity of test solution was prepared by serially diluting one per cent stock solution of the crude extract. Third instar of larvae compared to first and second instar have a larger body size and more adaptive to the environment while fourth instar in approximately 48 hours will turn into a pupa which formed the basis for selection of the third instar larva as the research sample. Twenty five early third instar larvae from laboratory colonized *Aedes aegypti* of $F_1$ generation were introduced into glass beakers (250 mL) containing 200 mL of distilled water and test concentration. Untreated control (distilled water) and treated control (Tween 80 added + distilled water) were maintained separately and run simultaneously. Mortality was observed 24 and 48 hours after treatment. Moribund larvae were scored dead when they showed no signs of movement when probed by a needle at their respiratory siphon. A total of five replicates per trial and a total of three trials for each concentration were carried out.

### 2.6. Statistical analysis

The per cent larval mortality was calculated using the formula (1) and corrections for control mortality (5-20%) when necessary was done using formula (2) of Abbott's [31]. Statistical analysis of all mortality data of larvicidal activity were subjected to probit analysis. ANOVA and regression analysis tests were used to determine if the mortality in treated bioassays significantly differed from that of the controls and at which doses in particular and the differences were considered as significant at $P \leq 0.05$ level. All statistics were conducted in IBM SPSS Statistics v22 with significance set at 95 % confidence [32].

$$Per\ cent\ larval\ mortality\ (1) = \frac{Number\ of\ dead\ larvae}{Number\ of\ larvae\ introduced} \times 100$$

$$\text{Corrected percentage of control mortality (2)} = \frac{1 - n\ in\ T\ after\ treatment}{n\ in\ C\ after\ treatment} \times 100$$

Where, *n* is the number of larvae, T: treated and C: control.

## 3. Results

The preliminary qualitative phytochemical analysis of *Citrus limon* methanolic leaf extract tested positive for flavonoids, flavones, limonene, folic acid, tannins, vitamins A, B1 and C. No larval mortality was observed in treated and untreated control. The mean and percentage mortality of *Aedes aegypti* larva treated with the various concentration of *Citrus limon* extract after 24 and 48 hours are presented in Figure 1 and its $LC_{50}$ values after 24 and 48 hours are 285.1 and 219.5 mg/L respectively (Table 1). The mean and percentage mortality of *Aedes aegypti* larva treated with the various concentration of *Bacillus thuringiensis* extract after 24 and 48 hours are presented in Figure 2 and its $LC_{50}$ values are 1.9 and 1.4 mg/L after 24 and 48 hours respectively (Table 1). The synergistic effect of the plant and microbe exerted a marked inhibitory effect on *Aedes aegypti* larvae which was visibly noted by its movement. The mean and percentage mortality of *Aedes aegypti* larva caused due to the synergistic activity after 24 and 48 hours are presented in Figure 3 and its corresponding $LC_{50}$ values are 158.5 and 109.9 mg/L respectively (Table 1). Regression analysis relationship between the plant extract, microbial and their synergistic activity was highly significant which indicated marked effect on the larva of *Aedes aegypti*. Further, ANOVA revealed the relationship between larval mortality in reference with concentration of *Citrus limon* and *Bacillus thuringiensis* as well as their synergistic extract which indicated that the increasing concentrations had a significant effect on *Aedes aegypti* (Table 1).

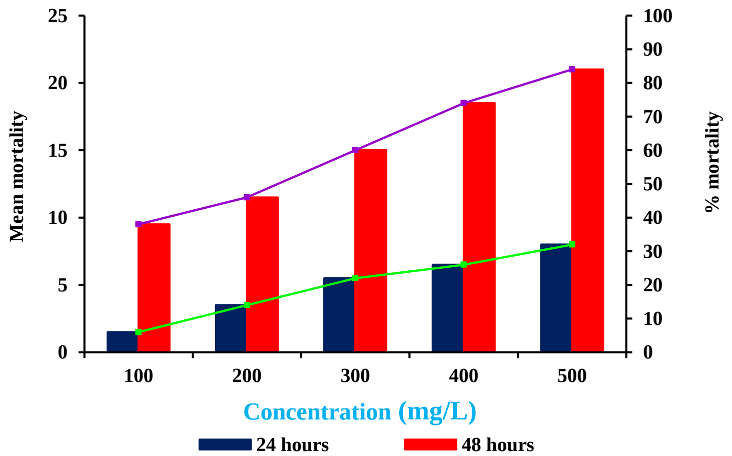


**Figure 1** Mean and per cent larval mortality of *Aedes aegypti* by *Citrus limon*

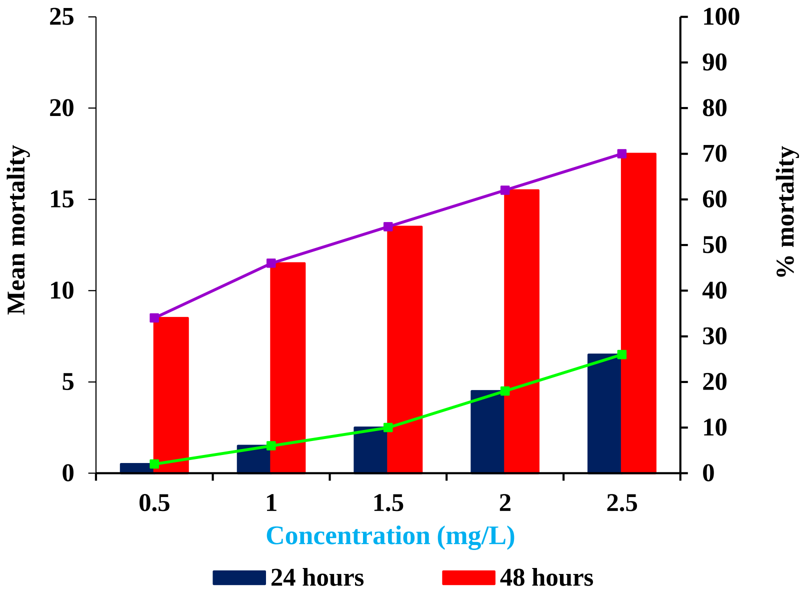


**Figure 2** Mean and per cent larval mortality of *Aedes aegypti* by *Bacillus thuringiensis*

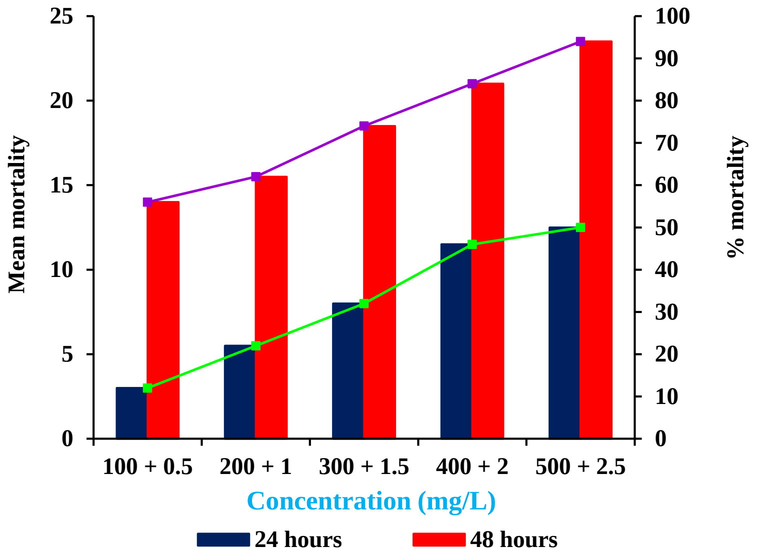


**Figure 3** Mean and per cent larval mortality of *Aedes aegypti* by synergistic effect

**Table 1** Statistical inferences on the mortality of *Aedes aegypti* larvae

| Particulars | Hours | $LC_{50}$ (mg/L) | Regression | $R^2$ | F value | P value |
|---|---|---|---|---|---|---|
| *Citrus limon* | 24 | 285.1 | y = 0.16x + 0.2 | 0.9846 | 192.0 | 0.000814 |
| | 48 | 219.5 | y = 0.13x + 1.1 | 0.9923 | 385.71 | 0.000288 |
| *Bacillus thuringiensis* | 24 | 1.9 | y = 0.3x + 6.1 | 0.9923 | 96.43 | 0.002245 |
| | 48 | 1.4 | y = 0.22x + 6.7 | 0.9918 | 363.0 | 0.000316 |
| *Citrus limon* + *Bacillus thuringiensis* | 24 | 158.5 | y = 0.25x + 0.6 | 0.9812 | 156.25 | 0.001104 |
| | 48 | 109.9 | y = 0.25x + 1.2 | 0.9921 | 379.11 | 0.000296 |

## 4. Discussion

The larval stages of mosquitoes are attractive targets for pesticides since they breed in water, which makes it easy to deal with them in this habitat. The use of conventional pesticides in the water sources, however, introduces many risks to the environment. Natural pesticides, especially those derived from plants, are more promising in this aspect. The genus *Citrus* has the potential to kill the larvae of *Aedes aegypti* and *Aedes albopictus* [14, 15, 33, 34]. Shrankhla and Kumar [35] indicated the *Citrus limon* peel waste extract to exhibit 75 to 100 % mortality on the larvae of *Culex quinquefasciatus*. Akram *et al*. [15] reported *Citrus limon* essential oil to be effective against the fourth instar larvae of *Aedes albopictus* with a $LC_{50}$ of 137.258 ppm. Similarly, Din *et al*. [14] found its essential oil from fruit and peel extract to be toxic with $LC_{50}$ of 468.69 and 395.59 ppm respectively. Supenah *et al*. [36] tested the effect of *Citrus limon* juice against the third instar of *Aedes aegypti* at 5, 10, 15, 20 and 25 % concentration and the average larval mortality after 48 hours of exposure was 100 % from 10 % concentration and 62 % larval mortality for 5 % concentration. Though this study exhibited 100% larval mortality of *Aedes aegypti*, the concentrations were very high when compared to the present study. Phytochemicals produce impressive results and have proven efficiency in mosquito larval mortality. The variations in the composition of active ingredients (phenolics, saponins, flavonoids, alkaloids and tannins) observed among the extracts from the same species of *Citrus* fruit, reveals that the amount of active ingredients present in the different parts of the same species of *Citrus* fruits vary [37]. *Citrus* species are commonly known to contain flavonoids, coumarins and carotenoids and its bioactivities due to these bioactive compounds are affected by climate and geographical distribution [38]. Mallick *et al*. [39] found that alkaloids, terpenoids, steroids and flavonoids in *Citrus maxima* extract caused mortality to *Culex quinquefasciatus* larvae. The phytochemical contents in *Citrus limon* leaf extract include flavonoids and tannins which are reported to act as larvicides against *Aedes aegypti*. Flavonoids are poisonous phytocompounds and they enter the mouth of insect larva/ natural holes in the body of insects and cause immersion in the nerves. Further, they also cause protein clotting and protein denaturation which causes the permeability of the cell wall in the digestive tract to decrease which leads to disrupted nutrient transport leading to death of mosquito larvae. On the other hand, tannins, act as stomach poisons by reducing the activity of digestive enzymes and absorption of food [40]. Interestingly apart from the flavonoids and tannins, limonoids in *Citrus* species [41] exhibit a wide range of biological activities including insecticidal [42] and mosquitocidal [33, 43] especially larvicidal as they arrest the metabolic activities of the larvae [44]. Limonoids in *Citrus limon* are digestive larvicides and inhibit skin changes in larvae as they enter the body of mosquito larva and on entering the digestive organs, they disrupt the body's metabolism which results in a lack of energy for life activities which causes the larvae to spasm and eventually die [45]. All these features were observed in the larvae of *Aedes aegypti* treated to *Citrus limon* extracts in the present study.

*Bacillus thuringiensis* is pondered as the reference larvicidal agent for mosquito control operations at lethal doses as no resistance mechanism has been reported [12]. These toxins remain harmless to other insects, fish and animals and its essential toxin could act in a synergistic manner as no mechanism of resistance has been detected against mosquitoes [46]. *Bacillus thuringiensis* toxin possesses the capacity to breakdown the larval mid gut epithelium, after ingestion of the spore quickly dissolves in the lumen of the anterior stomach of larvae [47]. The toxins on reaching the midgut of the larvae disrupts the osmotic balance of the cells by forming pores in the cell membrane causing lysis of cell. The gut becomes paralyzed forcing the larvae to stop feeding and as a result, the larvae die within few hours of ingestion due to lethal septicaemia [48, 49]. This may be due to the amount of toxin ingested, degree of larval midgut damage and the mode of action of the some of the components of binary toxins which synergically bind to specific receptors in larval midgut leading to formation of pores in the epithelial cell membrane which induce the death of larvae [50]. Further, Poopathi and Abidha [12] have reported in detail that these toxins in the insect digestive fluid travels across the peritrophic matrix and binds to specific receptors called cadherins on the brush border membrane of the gut cells resulting in activation of an oncotic cell death and concentrate on regions of the cell membrane called lipid rafts resulting in pore formation, osmotic cell shock, and ultimately larval death. However, it is to be noted that the binding of the toxin protein to the gastric caecum and posterior mid-gut has been observed in *Culex pipiens* as *Aedes aegypti* are resistant species [51].

The synergistic factor has been worked out and higher synergism was found in the present study on the larval instars. Synergism, combinational mode of action and counter-resistance effect are unique evolutionary traits of *Bacillus thuringiensis* which makes it the ideal biocontrol agent and explains why it is still the most efficient tool for mosquito control. As *Bacillus thuringiensis* produce a cocktail of toxins, and the mode of action is likely to involve the same mechanisms if not the same binding receptors, cross-resistance might evolve rapidly if botanical insecticides are used in combination with them. Ludlum *et al.* [52] have reported that aromatic compounds and plant allelochemicals increase the *Bacillus thuringiensis* activity. The addition of *Bacillus thuringiensis* with the plant extract have an adverse effect upon larval mortality and the same was observed in the present study too. The combined effect of *Bacillus thuringiensis* along with *Citrus limon* increased the mortality rate rather than the plant or microbe alone. During synergism, *Bacillus* *thuringiensis* and *Citrus limon* decreased the time to kill which increased the percentage of larval mortality when compared with treatment containing only *Bacillus thuringiensis*. The synergistic application of plant extracts with microbes produced a high mortality of the target organism due to increased toxicity. Their larvicidal activity is due to large amounts of proteins produced during sporulation and transformed into toxins under specific conditions after ingestion by mosquito larvae which is determined by both the structure of the proteins produced by the bacterium strain and the presence of proteolytic enzymes, and receptor in the midgut of mosquito larvae [53]. These factors would have played the key role in bringing about mortality in *Aedes aegypti* larvae in the present study. Moreover, the active phytocompounds in *Citrus limon* extract would have interacted with the toxins of *Bacillus thuringiensis* which could have certainly increased the toxicity against mosquito larvae.

## 5. Conclusion

In view of the above, the synergistic interaction of plant compounds from *Citrus limon* and toxins from *Bacillus thuringiensis* showed toxicity on the larvae of the dengue vector, *Aedes aegypti*. This study should thus promote the use of these natural biocides in the implementation of national policies for effective control of dengue vectors. Henceforth, the exploitation of plant chemicals and microbial pesticides can be suggested for use in mosquito vector control program for the control of mosquito-transmitted diseases. Moreover, adopting this kind of strategy would enable the use of pesticides that are safe for the environment in the future.

## Compliance with ethical standards

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### *Disclosure of conflict of interest*

The authors declare that there is no conflict of interest.

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