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Residual effects of TMOF-Bti formulations against 1st instar *Aedes aegypti* Linnaeus larvae outside laboratory

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ABSTRACT

Objective: To evaluate the effectiveness and residual effects of trypsin modulating oostatic factor-*Bacillus thuringiensis israeliensis* (TMOF-Bti) formulations against *Aedes aegypti* (*Ae. aegypti*) (L.) larvae at UKM Campus Kuala Lumpur. **Methods:** Twenty first instar *Ae. aegypti* larvae were added in each bucket containing 4 L of water supplied with crushed dried leaf powder as their source of food. Combination of TMOF-Bti in rice husk formulation with the following weights *viz* 10, 25, 50 and 100 mg, respectively in duplicate was distributed in the buckets; while TMOF-Bti in wettable powder formulation each weighing *viz* 2, 5, 10 and 20 mg, respectively in duplicate was also placed in the buckets. The control buckets run in duplicate with 4 L of water and 20 first instar *Ae. aegypti* larvae. All buckets were covered with mosquito netting. Larval mortality was recorded after 24 hours and weekly for five weeks. A new batch of 20 1st instar larvae *Ae. aegypti* was introduced into each bucket weekly without additional TMOF-Bti rice husk formulation or wettable powder. The experiment was repeated for four times. **Results:** The result of the study showed that all formulations were very effective on the first two weeks by giving 100% larval mortality for all concentrations applied. The TMOF (2%) + Bti (2%) had a good residual effect until the end of 3rd week, TMOF (4%) + Bti (4%) until 4th week, wettable powder TMOF (20%) + Bti (20%) until the third week. **Conclusions:** From the results it can be concluded that the TMOF-Bti formulations can be utilized in dengue vector control.

1. Introduction

Dengue is the fastest emerging arboviral infection. The maximum burden is borne by countries of the Asia Pacific Region. Among the estimated 2.5 billion people at risk globally, about 1.8 billion reside in Asia Pacific countries. Its epidemiology is rapidly evolving, with increased frequency of outbreaks and expansion to new geographical areas that were previously unaffected[1]. Indiscriminate use of chemical insecticides can also select for pest populations with insecticide resistance[2]. Resistance is usually due to insecticide detoxification by mutant enzymes (isozymes) caused by resistant gene alleles, but some resistance may also be conferred by reduced toxicant uptake[3]. A study by WHO[4] suggested that temephos resistance against *Aedes aegypti* (*Ae. aegypti*) (L.) increased with time. Currently temephos has been widely used for dengue vector control as larvicide. Temephos has been in use for control of mosquito larvae (*Ae. aegypti*, *Culex* spp. and *Anopheles* spp.) in portable water since the early 1970s. It has been useful in the control of dengue and dengue haemorrhagic fever, malaria and filariasis[5]. In Malaysia, temephos (Abate®) is recommended as a larvicide by Ministry of Health and widely used since 1973[6].

Bacillus thuringiensis (*B. thuringiensis*) are gram-positive spore-forming bacteria with entomopathogenic properties. *B. thuringiensis* produce insecticidal proteins during the sporulation phase as parasporal crystals. These crystals are predominantly composed of one or more proteins (Cry and Cyt toxins), also called δ-endotoxins. Cry proteins are parasporal inclusion (crystal) proteins from *B. thuringiensis* that exhibit experimentally verifiable toxic effect to a target organism or have significant sequence similarity to a known Cry protein. Similarly, Cyt proteins are parasporal inclusion proteins from *B. thuringiensis* that exhibit

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hemolytic (cytolitic) activity or have obvious sequence similarity to a known Cyt protein. These toxins are highly specific to their target insect, are innocuous to humans, vertebrates and plants, and are completely biodegradable[7]. Studies on wettable granule formulation of *B. thuringiensis israeliensis* (Bti), VectoBac WG (Bti strain AM65-52) against dengue vectors, *Ae. aegypti* and *Aedes albopictus*, in dengue endemic site in the state of Selangor, Malaysia indicated that a significant reduction in *Aedes* sp. populations was evident 4 weeks after initiating the first Bti treatment[8].

Trypsin-modulating oostatic factor (TMOF), a mosquito decapeptide, terminates trypsin biosynthesis in the mosquito gut. The hormone is secreted from the ovary, starting 18 hours after the blood meal, circulates in the hemolymph, binds to a gut receptor and stops trypsin biosynthesis by exerting a translational control on trypsin mRNA[9]. TMOF binds a gut receptor and induces phosphorylation of a gut protein, which is cleaved by a protease and released from the gut[10]. The organic TMOF analogues were tested against herbivorous pest lepidopterans and larval *Ae. aegypti*. The compounds administered to microtiter plates or to leaf disks caused mortality to mosquito larvae and the diamondback moth, *Plutella xylostella*, larvae 3-6 days after treatment. The surviving diamondback moth larvae were sluggish, immobile and stopped feeding[11]. A research conducted using *Ae. aegypti* 1st instar larvae in the laboratory was found to be able to improve the activity of these product by giving quick mortality effect within 1 hour treatment and prolong their residual effect (no larvae survived) against all larval stages until four weeks of treatment[12]. TMOF-Bti are the combination of TMOF and *B. thuringiensis israeliensis* which showed increased efficacy in killing mosquitoe larvae[13]. The present study was aimed to determine the outdoor residual effect of TMOF-Bti in rice husk and wettable powder formulations at different weights in the area of UKM campus against 1st instar *Ae. aegypti* larvae.

2. Materials and methods

An established colony of *Ae. aegypti* (susceptible strain) which originated from Institute for Medical Research (IMR) of Malaysia were reared at the insectarium of the Biomedical Science Programme, Universiti Kebangsaan Malaysia (National University of Malaysia). Only the first instar *Ae. aegypti* larvae were used as test species.

Materials used in this trial were 6 litre buckets and mosquito netting. Combinations of TMOF-Bti in rice husk formulations (2% TMOF + 2% Bti; 4% TMOF + 4% Bti) and wettable powder formulations (20% TMOF + 20% Bti) were produced by EntogeneX Industries Sdn. Bhd. of Malaysia. The larval food was crushed dry leaf powder. *Ae. aegypti* larvae used in this trial were reared from insectarium in Faculty of Health Sciences, UKM. Twenty first instar larvae *Ae. aegypti* were placed in each plastic bucket containing 4 L water. TMOF-Bti combinations in rice husk formulation in each of the following weights *viz* 10, 25, 50 and 100 mg, respectively were placed in 4 L of water in each bucket; while TMOF-Bti in wettabe powder formulations each measured in following weights *viz* 2, 5, 10 and 20 mg were each placed in similar buckets, respectively. Each measurement run as duplicates. Crushed dried leaf powders were supplied as source of food for the larvae. All buckets were covered by mosquito netting. Larval mortality was recorded after 24 hours and weekly. A new batch of 20 1st instar larvae *Ae. aegypti* was introduced into the buckets weekly without additional TMOF-Bti.

3. Results

Figure 1 indicated the residual effect of rice husk formulation (TMOF 2% + Bti 2%) against 1st instar *Ae. aegypti*. For the first week after application of rice husk in various weights, 100% larval mortality occurred 24 hours and one week in the first week of exposure for all concentrations. Control indicated 4% larval mortality after 24 hours and 10% larval mortality after one week, respectively. After the first addition of 20 1st instar larvae into the buckets for all weights of rice husk formulation in the beginning of the second week, the larval mortality decreased to 67.75% for 10 mg, 72.5% for 25 mg, 73.7% for 50 mg, 78.1% for 100 mg, respectively. At the end of the second week, the larval mortality increased to 100% for (25-100 mg) concentrations and 90% mortality for 10 mg concentration. The control indicated 6% mortality after 24 hours in the second week and 8% mortality at the end of the second week. The residual effect was still effective for all concentrations (10-100 mg) showing 79%-98% mortality at the end of the third week with the control showing 5% mortality at the end of the third week. At the end of the fourth week, the residual effect showed 73%-95% larval mortality, at the end of fifth week, the residual effect showed 63%-91% larval mortality, respectively. Thus, for concentration of 100 mg, the (TMOF 2% + Bti 2%) had a residual effect of more than 90% even after 5 weeks of exposure, 50 mg concentration, the residual effect was up to 83% larval mortality. Thus, the (TMOF 2% + Bti 2%) had a good residual effect until the end of the third week.

Figure 2 indicated the (TMOF 4% + Bti 4%) residual efficacy against 1st instar *Ae. aegypti* larvae. After the end of one week to all concentrations (10-100 mg), 100% larval mortality occurred and 11% larval mortality for the control. At the end of the second week the residual effect indicated 95%-100% larval mortality with 10% larval mortality for the control. At the end of the third week, the larval mortality indicated 94%-98% for all concentrations and the control with 7% larval mortality. At the end of the fourth week, the larval mortality indicated the residual effect of 100 mg at 74%-100% for (TMOF 4% + Bti 4%). At the end of fifth week of exposure, it still showed a high level mortality of 96% and 76% for the

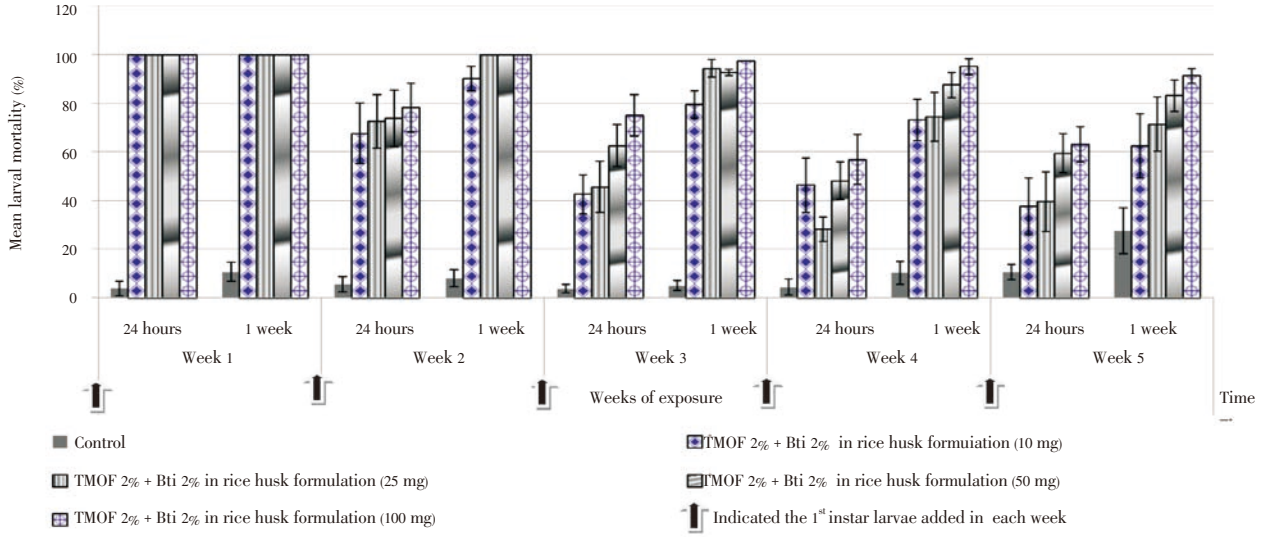

Figure 1. Weekly residual effect of rice husk formulation (TMOF 2% + Bti 2%) on 1st instar *Ae. aegypti*.

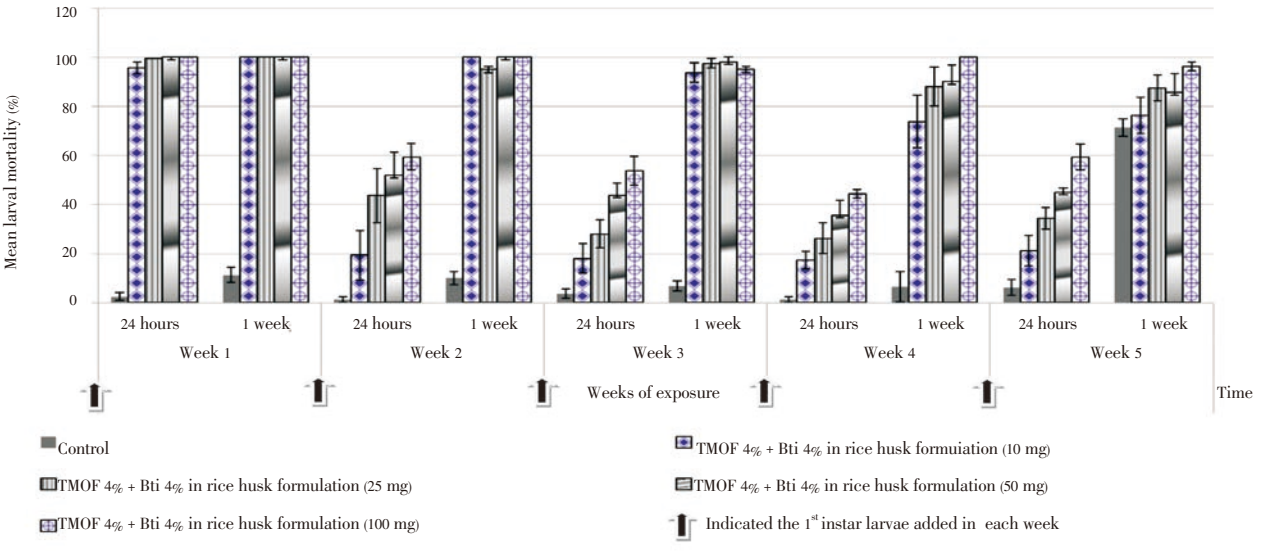

Figure 2. Weekly residual effect of rice husk formulation (TMOF 4% + Bti 4%) on 1st instar *Ae. aegypti*.

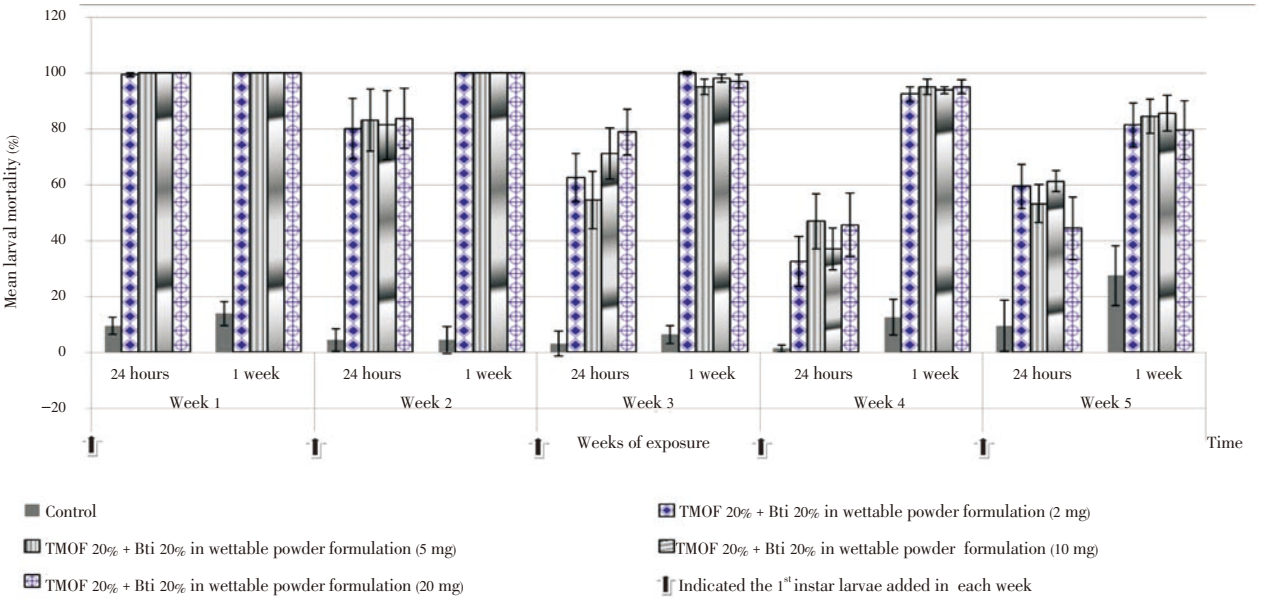

Figure 3. Weekly residual effect of wettable powder formulation (TMOF 20% + Bti 20%) against 1st instar *Ae. aegypti*.

10 mg concentration, respectively. However, there was high mortality of 70% for the control. Thus, the (TMOF 4% + Bti 4%) had a good residual effect until at the end of the fourth week.

Figure 3 indicated the residual efficacy of the wettable powder formulation (TMOF 20% + Bti 20%) against 1st instar *Ae. aegypti* larvae outside laboratory. For all concentrations of (2-20 mg), 100% larval mortality occurred at the end of second week exposure. At the end of the third week larval mortality indicated 95%-100% with 6% for the control. At the end of the fourth week, larval mortality occurred between 92%-95% for the concentrations between 2-20 mg with 12% larval mortality for the control. The residual effect decreased in the fifth week with larval mortality in the range of 79%-85% with 27% for the control. Thus, (TMOF 20% + Bti 20%) had a residual effect until the end of the fourth week.

Kruskal-Wallis test on rice husk formulation (2% TMOF + 2% Bti), rice husk formulation (4% TMOF + 4% Bti), wettable powder (20% TMOF + 20% Bti) indicated P=0.089, P=0.257, P=0.953, respectively. Thus, there was no significant difference on larval mortality across the four amount (10, 25, 50, 100 mg) of TMOF-Bti in rice husk and across the four amount (2, 5, 10, 20 mg) of wettable powder formulations tested. Kruskal-Wallis test on (2% TMOF + 2% Bti) rice husk formulation, (4% TMOF + 4% Bti) rice husk formulation, (20% TMOF + 20% Bti) wettable powder formulation indicated P=0.919 indicating there was no significant difference between the three concentrations of TMOF-Bti tested on larval mortality.

4. Discussion

Mosquito larvae also synthesize trypsin as their major protease and use the enzyme to digest decaying organic material or small organisms like algae that are found in ponds and marshes[14]. The mechanism of action of TMOF against larvae may need more than one hour to cause mortality. The process begins when mosquito larvae consume heat killed yeast containing TMOF and a sufficient amount of the hormone crosses the digestive system into the hemolymph stopping protein digestion and development. The larvae eventually die from starvation or other causes resulting from delayed development[15]. Norashiqin *et al* reported that TMOF with Bti rice husk and wettable powder caused high mortality even within one hour after exposure and resulted in complete mortality (0% larval survival) from 24 hours upto 4 weeks after exposure at each concentration tested[12]. Our result indicated that TMOF-Bti in rice husk and wettable powder formulations were able to maintain their residual effect effectively for 3-4 weeks after the first application and placing additional 20 first instar mosquito larvae weekly in each bucket. The mortality effect of TMOF with Bti may be influenced by the rapid killing effect of Bti and prolonged killing effect of TMOF due to the residual effect.

Starved first instar *Ae. aegypti* larvae were 35-fold more sensitive to Bti toxins than fed larvae. TMOF enhances the effect of Bti toxins in organic rich environments that are abundant in nature[13]. From a laboratory study it showed that all concentrations of TMOF with Bti rice husk tested (4% TMOF + 4% Bti, 3% TMOF + 1% Bti, 1% TMOF + 3% Bti, 2% TMOF + 2% Bti and 1% TMOF + 1% Bti) caused high mortality (95%-97%) within one hour of treatment and caused complete mortality (100%) until 96 hours after treatment[12]. They concluded that even at lower ratios of TMOF and Bti (1%: 1%), the effectiveness was as good as at higher concentrations. The present study indicated that even at a low concentration of (TMOF 2% + Bti 2%), the effectiveness was as good as in the higher concentration where they were able to cause larval mortality of 100% in each bucket in only 24 hours and one week duration time. Statistical analysis also indicated there was no significant difference (P=0.919) between all the concentrations used in this study.

A study in the laboratory has shown that 400 ppm of *Pichia*-TMOF and 300 ppm *Pichia*-TMOF were able to cause 100% and 67% cumulative mortality on *Ae. aegypti* larvae on the eighth day, respectively, while 200, 100 and 50 ppm concentrations showed obvious stunted effect on *Ae. aegypti* larvae[16]. While another study reported 38%-83% of larvae died after being fed with TMOF cloned in yeast cells. They also demonstrated the peptide's larvicidal properties in a field test. TMOF with yeast paste (*Pichia*-P7) and TMOF with dried yeast caused no mortality within one hour of exposure. This indicates that TMOF does not cause rapid mortality among *Ae. aegypti* larvae[17]. From the present study, the larval mortality decreased in the second week of the study for the first 24 hours, after one week, the larval mortality increased to 100%. This explains the mechanism of action of TMOF against mosquito larvae whereby it killed the larvae slowly by inducing hunger to the larvae and eventually lead to the larval death.

The combination of TMOF-Bti was able to overcome the problem which occurred when Bti alone was used as dengue vector control whereby Bti could not function well as larvicide in a natural environment which is rich with food source. So, the use of both toxins in early developmental stages of larvae is expected to enhance the effect of low toxin doses and reduce the amount of Bti required in sewage treatment ponds in which it is not effective alone[13]. The activation of the δ-endotoxins in Bti may be accomplished by pre-existing endogenous larval gut trypsin before its level in the midgut is reduced by TMOF. In addition, larval starvation, which occurs later on, will slow down repair of sub-lethal damaged gut epithelium by δ-endotoxins enhancing gut absorption of TMOF into the hemolymph[18]. Starved larvae may also eat more recombinant yeast cells bearing toxins thus enhancing larval demise[13]. Toxicity of Cry1C against advanced larval stages (third and fifth instars) of *Spodoptera littoralis* is, indeed, reduced due to high levels of protease activity in the gut which rapidly degrades the

ingested toxin[19]. The study on larvicidal effect of TMOF-Bti on *Ae. aegypti* larvae by Norashiqin *et al*[12] in the laboratory did not provide food source for the larvae in the test. The larvae were left in hunger prior to the test. Compared to the present study conducted outside the laboratory, food were supplied to the larvae for their survival, however, with food supplied, the result showed that TMOF-Bti still caused mortality to the larvae effectively.

In the study in the laboratory on the larvicidal effect of TMOF on *Ae. aegypti* by using three different weights of TMOF + Bti in rice husk formulation *viz* 50, 100 and 200 mg in different concentrations *viz* (4% TMOF + 4% Bti, 3% TMOF + 1% Bti, 1% TMOF + 3% Bti, 2% TMOF + 2% Bti and 1% TMOF + 1% Bti)[12], they found even using the lowest amount of TMOF-Bti rice husk, it was still able to cause 100% larval mortality in 24 hours using different concentrations of TMOF-Bti. This proved that TMOF-Bti can be effectively utilized to control *Ae. aegypti* larvae even with low amount of TMOF-Bti at 10 mg for rice husk formulation and 2 mg for wettable powder formulation. We conclude that TMOF-Bti in rice husk and wettable powder formulations were able to maintain their residual effects upto 3-4 weeks effectively. Thus it could be utilized for usage to control dengue vectors outside the laboratory.

Conflict of interest statement

We declare that we have no conflict of interest.

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