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Larvicidal Effect of Trypsin Modulating Oostatic Factor (TMOF) Formulations on *Aedes aegypti* Larvae in the Laboratory

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Abstract

Trypsin Modulating Oostatic factor (TMOF), a peptide hormone originally isolated from the ovaries of adult *Aedes aegypti*, is currently under commercial development as a new insecticide. This study was carried out to evaluate the various formulations of TMOF *viz* recombinant TMOF yeast cell paste form, TMOF yeast cell dried powder form, a combination of TMOF and *Bti* rice husk, TMOF and *Bti* wettable powder and TMOF and *Bti* mosquito fudge cubes formulated against *Aedes aegypti* larvae in the laboratory. The TMOF yeast cell paste and dried powder were found to cuase mortality in larvae from 24 hours exposure to 96 hours exposure. These products had a prolonged residual effect for four weeks of observation. The TMOF and dried yeast powder (Pichia 11 and 12) caused 100% larval mortality after 96 hours of treatment. The TMOF and *Bti* in rice husk, wettable powder and mosquito fudge cubes were effective in causing mortality within 1 hour of treatment and gave a prolonged residual effect (no larva survived) against all larval stages for four weeks after treatment. The mosquito fudge cubes showed high larvicidal activity for three months after treatment. The TMOF and yeast cell and TMOF and *Bti* formulations have the potential to be utilized for dengue vector control.

Keywords: Trypsin Modulating Oostatic Factor (TMOF) formulations, *Bacillus thuringiensis* (*Bti*), vector control, *Aedes aegypti*

Introduction

Despite advances in medical science and new drugs for treating mosquito-borne diseases, they remain important diseases in humans, with an estimated two billion people worldwide living in areas where these diseases are endemic [1]. Since World War II, disease control methods have relied heavily on broad-spectrum synthetic chemical insecticides; there are being phased out in many countries due to insecticide resistance in mosquito populations. Thus, there is an urgent need for new agents and strategies to control these diseases. Today, the advent of recombinant DNA technology is having an enormous impact on agriculture and medicine. The ability to manipulate and recombine genes using this technology may be applied to improving larvicides for vector control [2].

Control of vector mosquitoes using microbial agents such as *Bacillus thuringiensis* H-14 (*Bti*), is

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a relatively recent development in Malaysia [3,4]. The effectiveness of *Bti* in control of mosquitoes has been demonstrated [5]. The most widely used are Vectobac® and Teknar®, which are based on *B. thuringiensis* subsp *israelensis*. VectoLex® a product based on *B. sphaericus* (*Bs*), has come to market recently for control of mosquito vectors of viral diseases. These products have achieved moderate commercial success in developed countries, but their high cost deters use in many developing countries. Moreover, concerns have been raised about their long-term utility due to resistance, which has already been reported to *B. sphaericus* in field populations of *Culex* mosquitoes in several different countries [2].

The use of the peptide hormone, Trypsin modulating oostatic factor (TMOF), as a pesticide, represents a novel biorational approach to insect control using a new mode of action different from that of *Bti* [6]. TMOF is an insect hormone originally isolated from the ovaries of *Aedes Aegypti* (Diptera: Culicidae) that regulates trypsin biosynthesis in the mosquito digestive system [7]. TMOF has been shown to inhibit the growth and development of mosquito larvae feeding on this peptide, resulting in death by starvation [8]. TMOF have been shown to inhibit trypsin biosynthesis in other medically important insects, including the housefly, *Musca domestica* L (Diptera: Muscidae), stable fly, *Stomoxys calcitrans* L (Diptera: Muscidae) and the cat flea, *Ctenocephalides felis* Bouche (Siphonaptera: Publicidae) [9]. TMOF can be easily engineered for high expression in recombinant bacteria [10]. This study presents the effects of TMOF fermented with yeast cells, TMOF and *Bti* wettable powder, the combination of TMOF and *Bti* in rice husk form and TMOF with *Bti* in the form of mosquito fudge cubes as larvicides against the dengue vector, *Ae. aegypti*.

Materials and methods

Colonization of mosquitoes

An established colony of insecticide susceptible *Ae. aegypti* susceptible mosquitoes which originated from the Institute for Medical Research (IMR) of Malaysia was reared at the insectarium of the Department of Biomedical Science, Universiti Kebangsaan Malaysia (National University of Malaysia). First instar larvae of this species were used for the test.

Larvicidal testing

For the bioassay of the toxicity of TMOF with yeast (Pichia 7, 11 and 12) against *Ae. aegypti* larvaeWHO standard procedures [11] were used. Twenty first instar larvae were introduced into each 500 ml beaker containing 200 ml distilled water. A minimum of three replicates was made for each concentration of TMOF with yeast tested. All TMOF with yeast and *Bti* samples were fermented and formulated at the Chemical Engineering Power Plant (CEPP), Universiti Teknologi Malaysia, Johor. They came inboth wet paste and dry powder.

Prior to testing, 160 ml distilled water was added to each beaker. Then, a stock solution of 20,000 ppm TMOF with yeast was prepared to make the desiredconcentration. Using the formula, $C_1V_1 = C_2V_2$, the correct amount of stock solution was added to each of the three replicate beakers and the mixture was stirred with a glass rod. For the negative and positive control beakers, a 20,000 ppm stock solution containing normal yeast (Nona Instant Yeast©) and another solution containing 10,000 ppm *Bti* were prepared to yield 400 ppm normal yeast and 1 ppm *Bti*. After each of the beakers was prepared with TMOF with yeast, *Bti* or normal yeast, 20 first instar *Ae. aegypti* larvae were transferred from a breeding tray into each beaker. Finally, distilled water was added to bring the volume of the beaker to 200 ml each. Observations were conducted after 1 hour and 24 hours. The mortality of the larvae was recorded until all test larvae died. The larval survival was recorded at 1 hour, 24 hours, 48 hours, 72 hours and 96 hours post-treatment. Dead larvae were replaced with live larvae and the test was conducted for four weeks to determine the residual effect of each formulation.

After a number of TMOF with yeast and *Bti* fermentations in the form of wet paste and dry powder were tested, Entogene-X Sdn Bhd with

the help of CEPP had formulated the possible end products in the form of rice husks and fudge to investigate the best medium in which the TMOF with *Bti* would be most effective. The rice husks were tested at 50 g, 100 g and 200 g in 200 ml distilled water by adding the rice husks to 160 ml distilled water and topping up the water to 200 ml just after transferring the 20 first instar *Ae. aegypti* larvae to the beakers. For the mosquito fudge cubes, each cube was added to a beaker and observed for larval survival at the same time intervals from 1 hour to 3 months.

Results

The efficacy of TMOF paste (Pitchia-P7), dried TMOF with yeast, TMOF with *Bti* wettable powder, TMOF with *Bti* rice husk (4% TMOF plus 4% *Bti*) and *Bti* only were evaluated against *Ae. aegypti* larvae (Fig 1). TMOF paste (Pichia-P7) caused mortality in 1st instar larvae at 24 hours, with the percent of larval survival of 55.7% at 24 hours and 37.5% at 96 hours. Pichia-P7 caused mortality (10.8% larval survival) until four weeks even without adding any more active ingredient. The dried TMOF with yeast gave a similar mortality pattern to Pichia-P7. The dried form of TMOF with yeast had a better effect than the paste form throughout the study. By the end of 4 weeks, the dried TMOF with yeast resulted in a 5.8% larval survival rate.

The combination of TMOF with *Bti* in wettable powder (WP) gave 12.5% larval survival 1 hour post-treatment. The TMOF with *Bti* rice husk (4% *Bti* plus 4% TMOF) formulation caused rapid mortality among 1st instar *Ae. aegypti* larvae 1 hour after exposure. At concentrations of 50 mg, 100 mg and 200 mg The TMOF with *Bti* rice husk (4% *Bti* plus 4% TMOF) resulted in larval survival rates of 24.1%, 22.5% and 16.7%, respectively. From 48 hours until 4 weeks no larvae survived in the groups treated with both formulations TMOF with *Bti* WP and rice husk. One ppm *Bti* gave a 15% larval survival at 1 hour; thereafter, no larvae survived until 2nd weeks after treatment. However, at the 3rd and 4th weeks after treatment onset, the larvae survival rates were 0.8% and 18.3%, respectively.

Fig 2 describs the larvicidal activity of Pichia 11 and 12 from one hour to 120 hours after treatment. Pichia 11 and 12 had no mortality

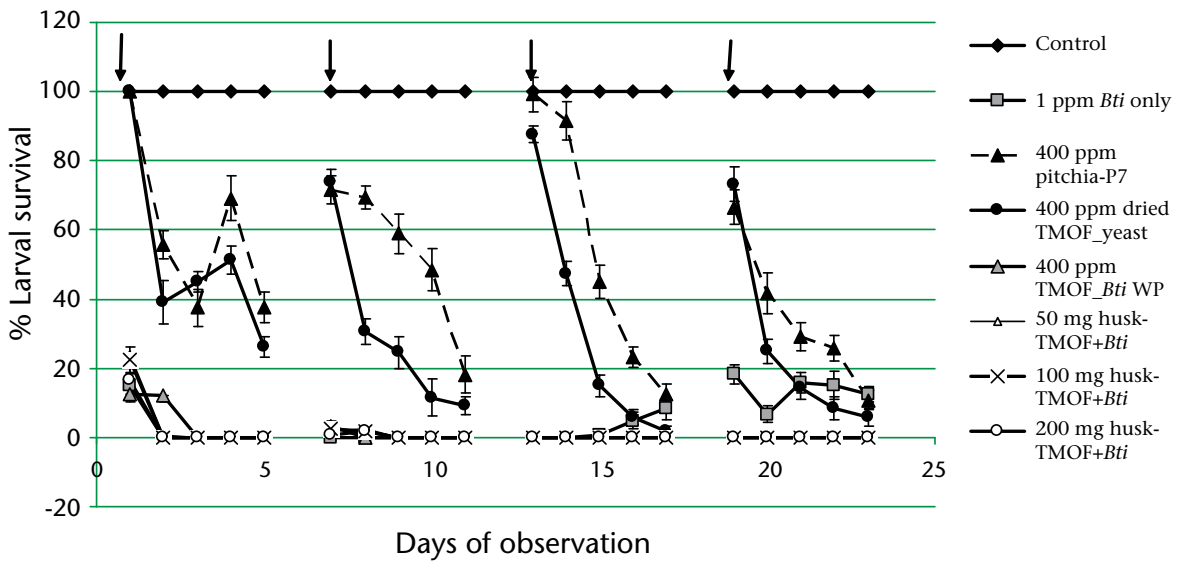

Fig 1 Larvicidal activity of TMOF with yeast cell paste and dried form compared with TMOF and *Bti* rice husk and wettable powder from the 1st to the 4th week of treatment. ↓ Indicates when the 1st instar larvae were added

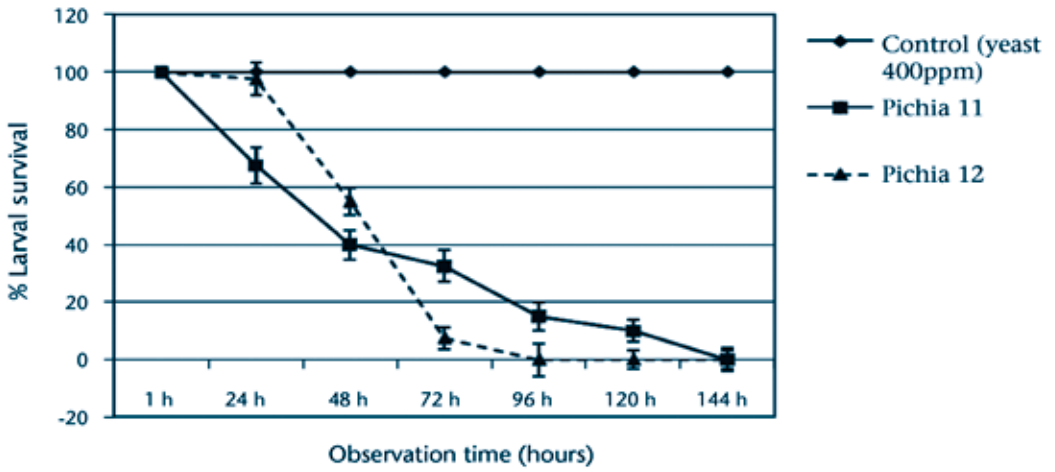

Fig 2 Larvicidal activity of TMOF with yeast cell dried form (Pichia 11 and Pichia 12) against 1st instar larvae from the 1st hour until the 144th hour of treatment.

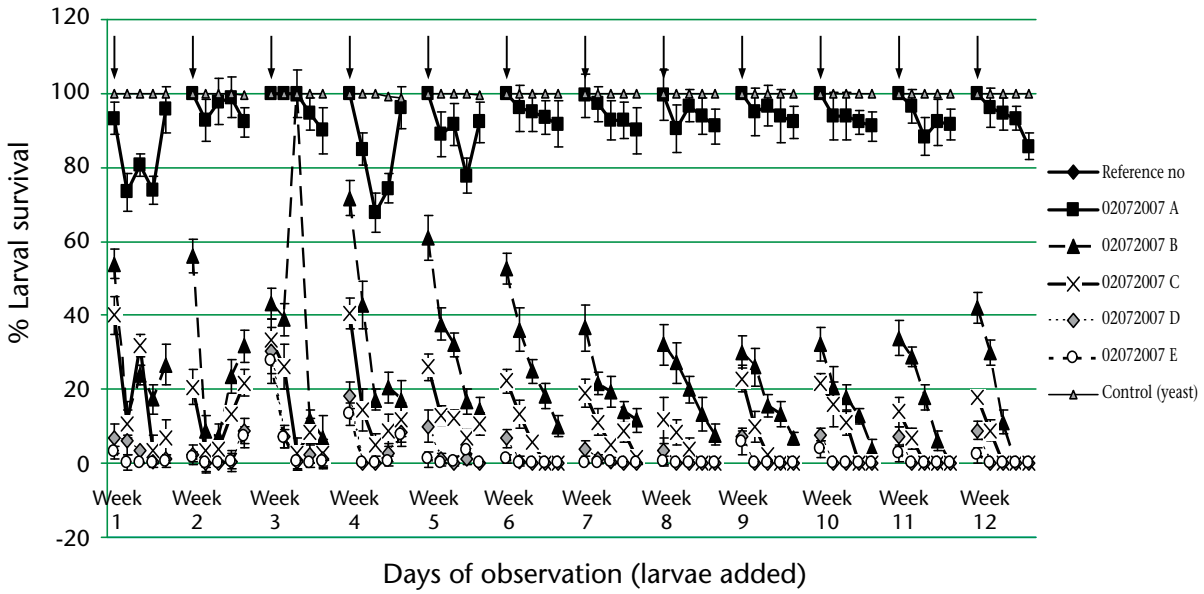

Fig 3 Larvicidal activity of mosquito fudge cubes for three months of treatment against *Ae. aegypti* larvae. ↓ Indicates when 1st instar larvae added.

by one hour of treatment, and at 24 hours there was 70% and 97.5% larval survival, respectively. However, Pichia 11 and 12 caused complete mortality at 96 and 120 hours, respectively. The larvicidal activity of the mosquito fudge cubes for three months is shown in Fig 3. The five different types were labeled A, B, C, D and E.

A was the control, without TMOF or *Bti* added, while B, C, D and E consisted of TMOF and *Bti*. Cube A caused a low mortality against the larvae

from the 1st week to the 12th week of treatment. Cubes B through E caused high mortality until the 12th week; cube E had the best larvicidal activity. Pichia 11 and 12 were the dried TMOF with yeast. Table 1 shows the efficacy of TMOF with *Bti* rice husk at different ratios of TMOF plus *Bti* (*viz*: 4% TMOF plus 4% *Bti*, 2% TMOF plus 2% *Bti*, 1% TMOF plus 1% *Bti*, 3% TMOF plus 1% *Bti* and 1% TMOF plus 3% *Bti*). These formulations also caused high mortality (100%) among all larval stages until 96 hours post-treatment. No significant differences in percent mortality were seen among the different concentrations tested ($p > 0.05$).

Table 1 Mortality caused by TMOF with *Bti* rice husk at different concentrations from one hour to 96 hours treatment against *Ae. aegypti* larvae.

	Concentrations (mg)	% Mortality					
		1 h	24 h	48 h	72 h	96 h	120 h
4% TMOF; 4% *Bti*	50	85.9	100	100	100	100	100
	100	87.5	100	100	100	100	100
	200	94.3	100	100	100	100	100
3% TMOF; 1% *Bti*	50	100	100	100	100	100	100
	100	95.0	100	100	100	100	100
	200	97.5	100	100	100	100	100
2% TMOF; 2% *Bti*	50	100	100	100	100	100	100
	100	100	100	100	100	100	100
	200	100	100	100	100	100	100
1% TMOF; 3% *Bti*	50	95	100	100	100	100	100
	100	100	100	100	100	100	100
	200	97.5	100	100	100	100	100
1% TMOF; 1% *Bti*	50	97.5	100	100	100	100	100
	100	97.5	100	100	100	100	100
	200	100	100	100	100	100	100

Discussion

Insects, including blood-sucking pests, must consume and digest a proteinaceous meal to acquire sufficient essential amino acids for growth, development and the production of mature eggs. Mosquitoes and houseflies produce oostatic hormones that inhibit egg development by inhibiting digestion of the protein meal, thereby limiting the availability of the essential amino acids necessary for egg development. They produce TMOF in the follicular epithelium of the ovary 12-35 hours after a blood meal. TMOF is then released into the hemolymph and binds to a specific receptor on midgut epithelial cells signaling the termination of trypsin biosynthesis. 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 [12]. Thus, controlling insects by interfering with their digestive enzymes, using trypsin inhibitors has been used to try to control insects [13-15]. Our

study showed TMOF fermented in yeast cells has a larvicidal effect against *Ae. aegypti*.

Recombination TMOF protein and its analogs using yeast cells to express the protein and release it into the larval medium will cause larval mortality by preventing normal food digestion essential for growth and development [16]. One study reported 38-83% of larvae died after being fed with TMOF cloned in yeast cells [17]. 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 TMOF does not cause rapid mortality among *Ae. aegypti* larvae. However, by 24 hours, Pichia-P7 led to only 55.7% larval survival and dried TMOF with yeast led to only 40% larval survival. 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, then the gene inside the yeast is expressed 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 [10]. TMOF with yeast may cause mortality amongst all larval stages from the 1st instar to the 4th instar and still cause mortality even four weeks after exposure without any added active ingredient. TMOF caused an LC_{50} value of 0.45 mM against *Cx. pipiens* five days after exposure [18].

TMOF with *Bti* was formulated from rice husk, TMOF with *Bti* wettable powder and mosquito fudge are possible end products for commercial used. 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 up until 4 weeks after exposure at each concentration tested. 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. Our results showed 1 ppm *Bti* gave complete mortality for the first 25 days, and after day 25 the larval survival increased to 8-20%. Previous studies reported *Bti* caused mortality quickly but had a shorter duration of activity, especially under natural conditions [19,20]. *Bti* toxin is composed of two proteins that co-crystallize into a single parasporal body [21]. After ingestion by a mosquito larva, the proteins are cleaved by proteases, yielding peptides that form active toxin [21,22]. These proteins bind to a receptor, a glucosidase in the midgut microvilli [23] and cause lysis of midgut cells after internalization [24].

This study showed all concentrations of TMOF with *Bti* rice husk tested (4% TMOF plus 4% *Bti*, 3% TMOF plus 1% *Bti*, 1% TMOF plus 3% *Bti*, 2% TMOF plus 2% *Bti* and 1% TMOF plus 1% *Bti*) caused high mortality (95-97%) within one hour of treatment and caused complete mortality (100%) until 96 hours after treatment. From this data, we can conclude even at lower ratios of TMOF and *Bti* (1%:1%), the effectiveness was as good as higher concentrations. When compared to the previous sample of TMOF with dried yeast powder in Pichia 11 and 12, it gave better results causing complete mortality by 96 hours of treatment.

The mosquito fudge cubes labeled B, C, D and E caused high mortality against *Ae. aegypti* larvae from the 1st to the 12th weekafter treatment. Cube E caused the highestmortality, resulting in 0% larval survival during the 1st through the 12th week after treatment. Cube A (control without active ingredient) also caused mortality (70% to 98% larval survival). This suggests the fudge cubes themself have killing properties. These cubes were formulated to attract larvae to ingest the TMOF causing starvation and death. The fudge cubes caused rapid killing within one hour of treatment.

TMOF with dried yeast in powdered form (Pichia 11 and 12) were toxic against larvae of *Ae. aegypti* and may be developed into commercial product. The combination of TMOF and *Bti* improved the larvicidal activity of TMOF resulting in rapid mortality and a prolonged residual effect. Further studies of the larvicidal activity of TMOF with yeast, TMOF with *Bti* rice husk and mosquito fudge cubes under field conditions needs to be carried out to determine the effect of TMOF with

yeast and TMOF with *Bti* against other mosquito species in the field.

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