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Laboratory Evaluation of Temephos, Grass Infusion, and *Piper aduncum* Extracts against the Ovipository Responses of *Aedes aegypti*

Ling Siew Ling, Sallehudin Sulaiman, Hidayatulfathi Othman

Biomedical Science Programme, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz 50300 Kuala Lumpur, Malaysia

Abstract

The objective of this study was to evaluate the effect of Temephos, grass infusion, and *Piper aduncum* extracts, against the ovipository activity of *Aedes aegypti* (L) in the laboratory. Three concentrations of Temephos and *P. aduncum* extracts (0.1 ppm, 0.5 ppm, and 1.0 ppm), and grass infusion (10%, 25%, and 50%) were used. *Ae. aegypti* laid significantly more eggs in ovitraps treated with grass infusion ($p < 0.05$) than in ovitraps with distilled water, and in ovitraps with *P. aduncum* extract. Oviposition Activity Index (OAI) values for the grass infusion were +0.60, +0.65, +0.62, for Temephos +0.28, -0.10, +0.47, and for *P. aduncum* +0.21, +0.20, +0.07, respectively. There were no significant differences between the percentages of hatchable eggs with treatments at three concentrations ($p > 0.05$, $p = 0.84, 0.86, 0.66$), and among three concentrations of grass infusion, Temephos and *P. aduncum* extracts ($p > 0.05$, $p = 0.15, 0.22, 0.68$), respectively; this indicated that there was no ovicidal effect on *Ae. aegypti* eggs. The residual effects of 10% grass infusion remained good for 50 days, 50% grass infusion for 40 days, *P. aduncum* 0.1 ppm until 10 days, 1.0 ppm for 70 days, while Temephos 0.1 ppm was effective for 20 days, and Temephos 1.0 ppm 50 days. In conclusion, extracts of *P. aduncum* 1.0 ppm were repellent against the oviposition of *Ae. Aegypti*, while the grass infusion was attractant.

Keywords: *Aedes aegypti*, *Piper aduncum*, oviposition, ovitraps, Temephos

Introduction

Aedes aegypti (L) is involved in the transmission of dengue fever and dengue hemorrhagic fever; effective drugs or vaccines are unavailable. The control of *Ae. aegypti* is mainly achieved by eliminating oviposition sites. Mosquito habitats can be eliminated by emptying and cleaning them frequently, by removing developing stages using insecticides or biological-control agents, by killing adult mosquitoes using insecticide, or by combinations of these methods [1]. The selection of oviposition site is a crucial event for gravid mosquitoes, to enable them to sustain the next generation. Site selection is mediated by visual, olfactory, tactile, and chemo-tactile cues [2].

Organic mixtures, such as grass infusions, have been used in ovitraps to monitor the oviposition activity of *Ae. aegypti* [3]; the attractiveness of the organic mixture depends on the type(s) [4] and concentration of organic material [5]. Essential oils and extracts of *Piper aduncum* have been used as repellents and in aerosol sprays, and show potential

Correspondence:
Ling Siew Ling,
E-mail: <osiewo88@yahoo.com>

utility in controlling dengue vectors and dengue hemorrhagic fever [6,7]. The aim of this study was to investigate the effect of Temephos, grass infusion, and extract of *P. aduncum,* on the ovipository activity of gravid *Ae. aegypti* mosquitoes in the laboratory.

Materials and methods

Extraction of *Piper aduncum*

P. aduncum leaves were collected from Gombak (latitude: 3° 17′ 60 N; longitude: 101° 42′ E), 25 km from Kuala Lumpur City. The leaves were washed and shade-dried at room temperature. The dried leaves were ground into small pieces. Extraction of *P. aduncum* was completed by using hydro-distillation in a Clavenger-type apparatus for eight hours. The oil layer of the extraction was separated, dried over anhydrous sodium sulfate, and stored in aluminum-foil-sealed glass ampoule and stored below 8°C, until used.

Stock solutions were prepared by dissolving one gram of plant extract in acetone (Fisher Scientific Malaysia, Pte, Ltd), Tween 80 (Fisher Scientific Malaysia, Pte, Ltd) and distilled water. Stock solutions were then diluted to the required concentration.

Insecticide

Temephos, or Abate®500E was obtained from BASF (Malaysia) Sdn Bhd.

Preparation of grass infusion based on modification of Pontes *et al* [8]

Zoysia matrella (manila grass) and *Axonopus compressus* (cow grass) were used. Dried grasses were collected from Tun Syed Nasir College, Universiti Kebangsaan Malaysia (latitude: 3° 10′ 22 N; longitude: 101° 43′ E). The grasses were identified by a botanist at the Herbarium Laboratory of the Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The grass infusion was prepared by steeping 12.5 g of dried grass in 1.5 liters of distilled water in a 5-liter mineral-water bottle for seven days at room temperature. The grasses were then sieved and the grass infusion was stored in a 1.5 liter mineral-water bottle and stored below 8°C until required for analysis.

Mosquitoes

The susceptible strain of *Ae. Aegypti,* which originated from the Institute for Medical Research, Kuala Lumpur, was colonized at the insectarium of the Department of Biomedical Science, Universiti Kebangsaan Malaysia. In this study, 3-5-day-old gravid *Ae. aegypti* females and males were used. The colonies were maintained and all the experiments were carried out in conditions of 29 ± 0.2°C and 61-62% relative humidity. Sucrose (10%) was provided to the mosquitoes. A guinea pig was used for blood feeding, and the mosquitoes were made to fast for one day before feeding. The guinea pig's hair was shaved and the animal was then put into a cage to feed the mosquitoes for one hour. For residual testing against oviposition, a new batch of mosquitoes was introduced into the cage every 10 days.

Laboratory bioassays for oviposition test based on modification of Sharma *et al* [9]

The study was conducted using 36 x 36 x 36 cm cages with a circular opening fitted with cloth sleeves. Six cages were used and each cage contained 4 ovitraps, which contained distilled water as control, and ovitraps treated with solution of Temephos, grass infusion, and extracts of *P. aduncum*. Different concentrations of Temephos, grass infusion and *P. aduncum* extract were prepared from the stock solutions. Twenty 3-5-day-old gravid female *Ae. aegypti* and 20 3-5-day-old male mosquitoes were then placed in the cage for 10 days to ensure all eggs were laid. The mean total eggs laid and Oviposition Activity Index (OAI) in each concentration from 6 replicates were calculated. OAI was calculated according to Kramer & Mulla [10] using the formula:

$$OAI = N_t - N_c / N_t + N_c$$

In this formula, N_t is the number of eggs in the test solution; N_c is the total number of eggs in the control solution. OAI ranges from -1 to +1, with 0 indicating a neutral response. Compounds with an OAI of +0.3 and above are considered attractant, while those with -0.3 and below are considered repellent. In this study, 6 replicates were conducted.

Ovicidal bioassay based on modification of Su & Mulla [11]

This method was used to observe the ovicidal activity of those substances on the eggs of *Ae. aegypti*. The number of eggs on paddles was then counted and rinsed in dechlorinated water for 10 days to ensure that all eggs hatched. The numbers of hatched larvae were counted and the percentage of eggs hatched calculated by the following formula: Hatching rate = (Number of hatched larvae / Total number of egg on the paddles) x 100%

Residual test against oviposition

The method used in the laboratory bioassays for the oviposition test was also used to test the residual effect of the treatment on oviposition of *Ae. aegypti*. The concentrations tested were 0.1 ppm & 1.0 ppm of Temephos and *P. aduncum* extracts, and 10% & 50% for grass infusion. The volume of solution in the ovitraps was 200 ml. The test solutions were added up to 200 ml for every 10 days after a new batch of mosquitoes was used. The total duration of this test was 70 days.

Statistical analysis

The mean number of eggs deposited in test and control ovitraps, and percentage egg hatchability were analyzed using one-way analysis of variance (ANOVA), Kruskal-Wallis test, and Mann-Whitney test. All analysis was performed with SPSS for Windows (version 17.0) and statistical significance was determined at $p < 0.05$.

Results

Laboratory bioassays for oviposition test

The effects of Temephos, grass infusion and *P. aduncum* extract against the oviposition activity of *Ae. aegypti* are summarized in Table 1. 25% grass infusion showed the highest mean number of eggs laid, 372.2 ± 77.2, and the three concentrations of grass infusion showed significantly higher eggs laid than the controls ($p < 0.05$). Moreover, 1.0 ppm of Temephos had the highest mean number of eggs laid in 3 concentrations (0.1 ppm, 0.5 ppm, and 1.0 ppm), and its value was 221 ± 92.2 eggs. 0.1 ppm and 0.5 ppm of Temephos showed significantly less than 10% and 50% grass infusion, respectively ($p < 0.05$). In addition, 1.0 ppm of Temephos also showed significantly more than 0.5 ppm of Temephos ($p < 0.05$). While 0.1 ppm and 0.5 ppm of *P. aduncum* extracts had similar values of mean number of eggs, which were 120.5 ± 37.9 and 120.0 ± 24.8, respectively, all concentrations showed significantly less than all concentrations of grass infusion ($p < 0.05$). Furthermore, the OAI of grass infusion ranged from +0.60 to +0.65, Temephos ranged from -0.11 to +0.47 and *P. aduncum* extract ranged from +0.07 to +0.21. These results indicated that gravid *Ae. aegypti* preferred to lay their eggs in the treated ovitraps rather than the control ovitraps. Also, *P. aduncum* had potential repellent activity against *Ae. aegypti* oviposition, since all *P. aduncum* OAI values were < +0.3.

Ovicidal bioassay

The percentage egg hatchability of *Ae. aegypti* with grass infusion, Temephos, and extracts of *P. aduncum* are presented in Table 2. The percentage egg hatchability with grass infusion, Temephos and *P. aduncum* extracts in three concentrations ranged from 50.4% to 77.0%, and did not show significant differences with control ($p > 0.05$, $p = 0.84, 0.86, 0.66$) or among the three concentrations ($p > 0.05$, $p = 0.15, 0.22, 0.68$). This showed that there is no ovicidal activity of grass infusion, Temephos, and *P. aduncum* extracts to *Ae. aegypti* eggs.

Residual test of oviposition

OAI values and mean number of *Ae. aegypti* eggs laid were used in this test to determine residual effects of grass infusion, *P. aduncum* extracts and Temephos on the oviposition of *Ae. aegypti*. Based on Fig 1, the highest OAI values of 10% grass infusion, 50% grass infusion, *P. aduncum* extracts 0.1 ppm, *P. aduncum* extracts 1.0 ppm, Temephos 0.1 ppm, and Temephos 1.0 ppm were +0.28, +0.58, +0.25, +0.04, +0.15, and +0.09, respectively, while the lowest OAI values of those substances were -0.21, -0.28, -0.25, -0.38, -0.16, and -0.33, respectively.

Table 1 Total and mean number of eggs laid, and Oviposition Activity Index (OAI) of *Ae. aegypti* tested with grass infusion, Temephos, and *P. aduncum* at 3 concentrations for 10 days.

Concentration	Number of eggs laid	Mean number of eggs laid ± SEM	OAI
Control (distilled water)	476	79.3 ± 14.2	-
Grass infusion (%)			
10	1,881	313.5 ± 36.0[a]	0.60
25	2,233	372.2 ± 77.2[a]	0.65
50	2,004	334 ± 78.9[a]	0.62
Temephos (ppm)			
0.1	843	140.5 ± 24.7[b]	0.28
0.5	391	65.2 ± 7.8[c]	-0.10
1.0	1,326	221 ± 92.2[d]	0.47
Extract of *P. aduncum* (ppm)			
0.1	723	120.5 ± 37.9[b]	0.21
0.5	720	120 ± 24.8[c]	0.20
1.0	550	91.7 ± 18.2[e]	0.07

Significant differences ($p < 0.05$) with a) control, b) 10% grass infusion, c) 25% grass infusion, d) 0.5 ppm Temephos, e) 50% grass infusion. OAI implies oviposition activity index, it ranges from -1 to +1. ≥ +0.3 implies attractant, ≤ -0.3 implies repellent.

Table 2 Ovicidal activity of control, grass infusion, Temephos, and *P. aduncum* extract at 3 concentrations against *Ae. aegypti* for 10 days.

Concentration	% hatching ± SEM
Control (distilled water)	74.0 ± 6.9
Grass infusion (%)	66.4 ± 5.9
10	
25	76.7 ± 4.2
50	63.9 ± 3.5
Temephos (ppm)	63.6 ± 7.6
0.1	
0.5	77.0 ± 8.1
1.0	67.4 ± 10.9
Extract of *P. aduncum* (ppm)	67.0 ± 9.0
0.1	
0.5	75.2 ± 12.2
1.0	50.4 ± 17.2

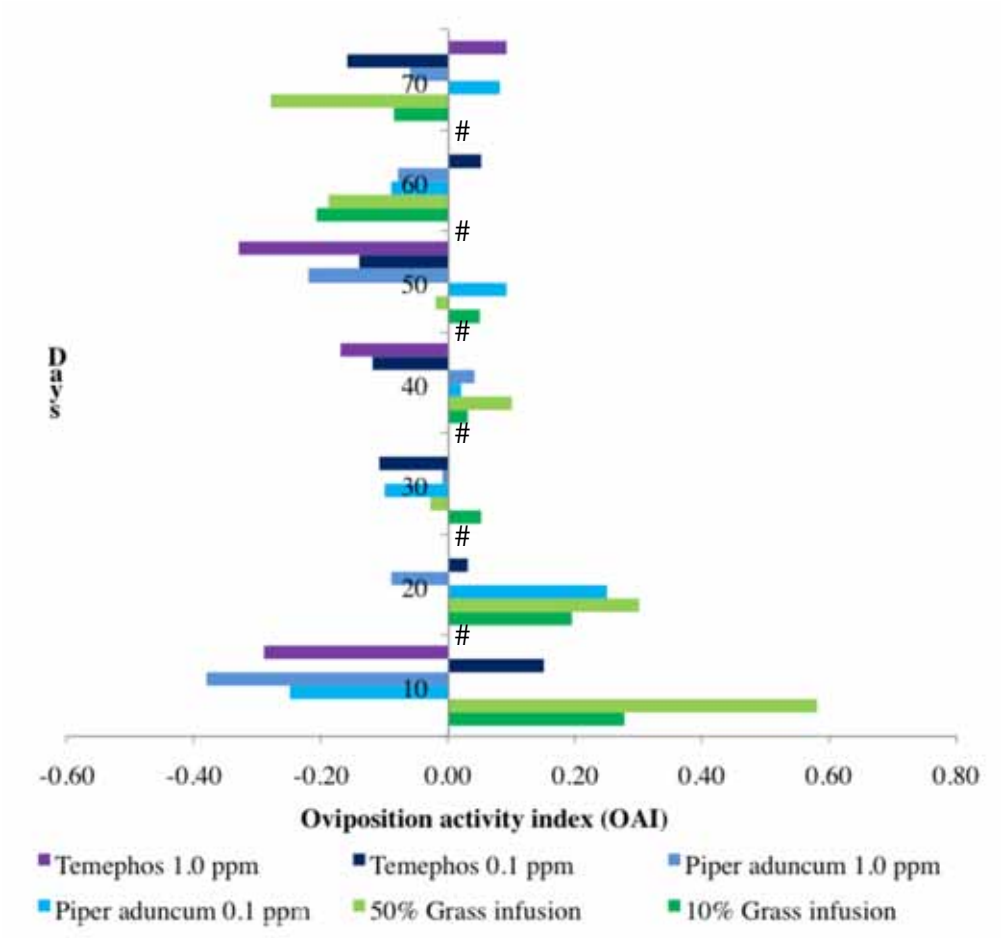

Fig 1 OAI values of three types of substances of *Ae. aegypti* in residual test for 70 days. OAI implies oviposition activity index, and ranges from -1 to +1. ≥ +0.3 implies attractant, ≤ -0.3 implies repellent. # implies new batch of mosquitoes was used.

According to Fig 2, the mean number of eggs laid by *Ae. aegypti* in control (distilled water) for all six substances (10%, 50% grass infusions, *P. aduncum* 0.1 ppm, 1.0 ppm, and Temephos 0.1 ppm and 1.0 ppm) for 70 days were irregular. The highest mean number of eggs laid by *Ae. aegypti* for those substances were 142, 198, 423, 119, 136, 152, and 138, respectively, while the lowest values for those substances were 59, 44, 46, 57, 50, 50, and 46, respectively. The results showed that 10% grass infusion had good residual effect till 50 days while 50% grass infusion till 20 days. *P. aduncum* extracts 0.1 ppm did not have good residual effect till 10 days while extracts of *P. aduncum* 1.0 ppm had good residual effect till 70 days. The residual effect of Temephos 0.1 ppm was till 20 days while Temephos 1.0 ppm could stand for 60 days.

Discussion

This study revealed that grass infusion, Temephos, and *P. aduncum* extracts had effects on the oviposition activity of gravid *Ae. aegypti*. 10%, 25% and 50% grass infusion attracted *Ae. aegypti* to lay their eggs in the paddles, as was shown in the study by Chadee *et al* [12]. Their study found that grass infusion was not a repellent to oviposition activity of *Ae. aegypti*, and has been recommended to increase the probability of oviposition [13]. Attractiveness of grass infusion

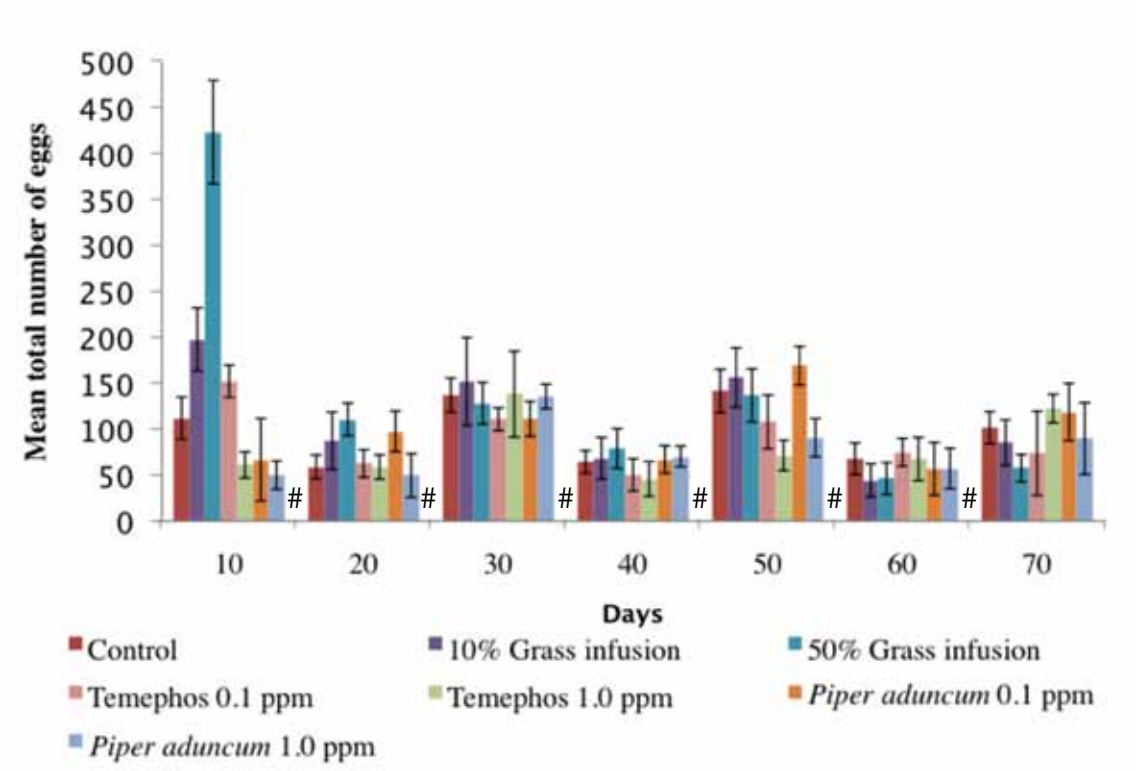

Fig 2 Mean number of *Ae. aegypti* eggs laid for 70 days. # implies new batch of mosquitoes was used.

may be affected by quality and relative quantity of grass and water, fermentation period, temperature during the fermentation process, dilutions and other factors [14]. Therefore, the ratio of dry grass to distilled water, temperature, and the fermentation period were controlled in order to have bias in this study. From previous studies, bacteria *Aerobacter aerogenes* [15] had been isolated from the grass infusion; they could produce the chemical which can attract *Ae. aegypti* and *Culex quinquefasciatus* to lay their eggs. Many years later, Millar *et al* [16] identified five chemical compounds in grass infusion: phenol, 4-methylphenol, indole, 4-ethylphenol and 3-methylphenol. These natural chemical compounds can affect the behavior of oviposition of mosquitoes [17], consequently, grass infusion used in this study may contain the bacteria which can produce these chemicals.

In this study, the three concentrations of Temephos (0.1 pm, 0.5 ppm and 1.0 ppm) did not show significant differences to control (distilled water) ($p > 0.05$, $p = 0.119, 0.937, 0.485$). According to Canyon & Speare [18], the number of eggs laid in Temephos, Malathion, Permethrin and Methoprene were more than those in the control. Temephos at 1.0 ppm may not repel *Ae. aegypti* from laying their eggs [19], thus low dose concentrations at 0.1 ppm to 1.0 ppm can attract *Ae. aegypti* to lay their eggs and thereby control the larval stage of *Ae. aegypti*. The results would affect the outbreak of dengue in those areas where Abate is used to control the larval stage of *Ae. aegypti*.

Extracts of *P. aduncum* had been used as a repellent against the *Ae. aegypti* adults [6] and also used in aerosol sprays against *Ae. aegypti* and *Ae. albopictus* [7]. From this study, results revealed that the number of eggs laid in *P. aduncum* extract at three concentrations (0.1 ppm, 0.5 ppm and 1.0 ppm) were significantly lower than grass infusion ($p < 0.05$) and OAI values were all less than +0.3. This indicated that *P. aduncum* had repellent effect on oviposition of *Ae. aegypti,* a factor which had not been tested before.

The percentage of *Ae. aegypti* egg hatchability is quite high, showing that substances such as grass infusion, Temephos and *P. aduncum* extract did not have ovicidal activity to the eggs. When hatched larvae died, it was due to the residual insecticide or chemicals on the surface of the eggs [19].

Oviposition can be affected by other factors such as the behavior of "skip oviposition" which will affect mosquitoes laying their eggs in the extract [20]. In addition, the number of eggs laid in the ovitraps may be affected by the presence of chemicals, whether natural or synthetic, as indicated in the study where secondary metabolites produced by *Trichoderma viride* could attract *Cx. quinquefasciatus* to lay their eggs [17]. According to Surtees [21], contaminated water, water temperature, water depth and light intensity can affect the oviposition activity of *Ae. aegypti*. Also, the color of ovitraps can alter the oviposition activity, according to a study which showed that black color is the most preferred color to *Ae. albopictus* [22]. Furthermore, presence of larva in the solution in the ovitraps may encourage gravid mosquitoes to lay their eggs inside the ovitraps [23].

In conclusion, our study indicated that *P. aduncum* extracts 1.0 ppm had good repellent effect on oviposition of *Ae. aegypti*, and *P. aduncum* extract and Temephos were less effective than grass infusion for oviposition of gravid *Ae. aegypti*.

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