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# An *in vitro* study of the bioefficacy of essential oil blends against *Aedes aegypti* (Linn.) and *Anopheles dirus* (Peyton and Harrison) by using membrane feeding apparatus

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ABSTRACT

**Objective:** To determine the bioefficacy of plant essential oils on *Aedes aegypti* and *Anopheles dirus*. Repellency was determined by measuring reduction in feeding and mortality. A novel *in vitro* bioassay apparatus was developed that had a sausage-casing membrane feeding system.
**Methods:** Mixtures of three essential oils were evaluated: turmeric (*Curcuma longa*), eucalyptus (*Eucalyptus globulus*), and orange (*Citrus aurantium*). The oils were mixed in pairs or all together at equal volume for a total of 10% volume and then formulated with 90% virgin coconut oil. Completed formulations were evaluated with and without an additional 5% vanillin. The formulations were applied to the sausage casing membranes and female mosquitoes provided (expose) blood meals (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h) to assess the percentage repellency over time.
**Results:** The strongest repellency was at shorter exposure periods. For *Aedes aegypti*, the strongest feeding reduction was with the turmeric and eucalyptus combination and with the addition of vanillin (97.6%–99.6%). For *Anopheles dirus*, the strongest repellency was when all three oils were combined (98.4%–99.6%).
**Conclusions:** Vanillin increased the effects of repellency and mortality for all formulations and demonstrated an increased potential to enhance the bioefficacy of essential oil repellents. This study also demonstrated an *in vitro* membrane feeding system that can be used to screen essential oils.

## 1. Introduction

Mosquito-borne infectious diseases, such as dengue fever and malaria, are increasing each year, which may be due to the effects of global warming and climate change[1]. Dengue virus is primarily transmitted by *Aedes aegypti* (L.) (*Ae. aegypti*) mosquitoes and is the primary vector throughout the global distribution of dengue[2]. Malaria is transmitted by anopheline mosquitoes and the primary vectors are unique to different geographical locations. *Anopheles dirus* (Peyton and Harrison) (*An. dirus*) is considered one of the most important vectors in Thailand and Southeast Asia[3]. Both of these diseases are difficult to manage because there are no available vaccines, and in the case of dengue, there are no therapeutic drugs[4]. Efforts to control these diseases often focus on vector control and preventive strategies to minimize mosquito bites.

The use of topical insect repellents applied to the skin is a proven method to reduce mosquito bites. There is a long history of using plant derived extracts to reduce mosquito bites. However, since the development of modern synthetic repellents in the 1940's, natural repellents have been replaced largely by synthetic chemicals[5]. Currently there is a renewed interest in using plant-based insect repellents due to concerns about safety and the preference for products that are considered more natural[6]. Several essential oils and volatile compounds from a multitude of plants have been found to possess repellent properties against arthropods[7]. These plants derived chemicals often repel mosquitoes, but there is a wide variability between mosquito species[8]. Compounds that repel mosquitoes have been found in the following plant families: Graminae[9], Labiatae[10], Lamiaceae[11], Myrtaceae[12], Poaceae and Rutaceae[13], Umbelliferae[14], and Zingiberaceae[15].

This study evaluated essential oils from the plants turmeric (*Curcuma longa* L., family: Zingiberaceae), eucalyptus (*Eucalyptus*

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*globulus* Labill, family: Rutaceae) and orange (*Citrus aurantium* L. family: Myrtaceae). It is known that turmeric contains the chemical ar-turmerone that is repellent to arthropods[16]. The eucalyptus plant contains important active ingredient such as 1,8-cineole, α- and β-pinene that can repel various mosquito species[17].

Mosquito repellents are often tested by using the arm in cage technique[18]. This method allows mosquitoes to feed directly on human volunteers and has several disadvantages, such as the pain and discomfort associated with mosquito feeding, the requirement for Institutional Review Board approval, the limited number of candidate repellents that can be screened at one time[19]. Even though the direct evaluation of repellents on human skin remains essential for evaluating repellents, artificial membrane feeding systems can serve as a useful alternative when pre-selecting candidate repellents[20]. The use of artificial membrane feeding systems is largely dependent on the types of membranes, including animal tissues, Parafilm-M® films, and collagen membranes[21,22].

This paper evaluated the efficacy of essential oils from turmeric rhizomes (TU), eucalyptus leaves (EU) and orange peels (OR). These oils were evaluated individually in a previous study using the arm in cage method compared to the synthetic repellent diethyltoluamide (N,N-diethyl 1-3 methylbenzamide 25% w/w; Kor Yor 15)[23]. This study looked at the same three chemicals but combined them in mixtures to determine if there was a synergistic effect. Also each mixture was evaluated with or without 5% vanillin extract. Vanillin was added because other studies have found that it extends the amount of time that certain natural products are effective against mosquitoes[15].

## 2. Materials and methods

### 2.1. Mosquitoes rearing

*Ae. aegypti* and *An. dirus* were reared in the insectary of the Entomology Department, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. The photoperiod was maintained at 12 h light/12 h dark with a temperature of 25 ± 2 °C and a relative humidity of 60%–80%. Filter papers containing eggs of *Ae. aegypti* were placed in plastic trays (30 cm × 35 cm × 5 cm) with 2500 mL of distilled water and larvae were provided fish food tablets (OPTIMUM®, Bangkok, Thailand). After one day, newly hatched larvae were diluted to about 500 larvae per tray for density and population. For *An. dirus*, approximately 150 eggs were added to a plastic tray and larvae provided fresh powdered fish food until pupation. The pupae of both species were collected and placed in holding cages until adult emergence. Freshly emerged adults were allowed to feed on soaked cotton pads containing a 5% multivitamin solution *ad libitum*. All testing was performed using 5–7 day-old post-emergent females that were denied sugar and only provided water for 8 h before testing.

### 2.2. Preparation of plant essential oils

Extracts from many of the plants are available commercially. Eucalyptus leaf oil (New Directions Aromatics Inc., Mississauga, Canada), orange peel oil (New Directions Aromatics Inc., Mississauga, Canada) and vanillin (Borregaard Industries Ltd. Company, Sarpsborg, Norway) were purchased from Chanjao Longevity Co., Ltd., Bangkok, Thailand. Extracts from the turmeric plant were not available commercially. Therefore, TU were collected from Suwan Farm, Pak Chong, Nakhon Ratchasima Province, Thailand. Essential oils were extracted by water distillation[24]. The different essential oils were blended at equal ratios for a total volume of 10% and then mixed with virgin coconut oil (Agrilife Co., Ltd., Bangkok, Thailand) using a vortex mixer (Vortex-Genie 2, Scientific Industries Inc., New York, USA) (Table 1). The coconut oil was chosen because it created a formulation similar to what would be applied to human skin. All formulations were kept at room temperature before testing.

**Table 1**

List of selected essential oil blends without and with 5% vanillin against *Ae. aegypti* and *An. dirus.*

| Code No. | | Formulation in coconut oil |
|---|---|---|
| Without 5% vanillin | TU:EU | 5% Turmeric + 5% Eucalyptus |
| | TU:OR | 5% Turmeric + 5% Orange |
| | EU:OR | 5% Eucalyptus + 5% Orange |
| | TU:EU:OR | 3.33% Turmeric + 3.33% Eucalyptus + 3.33% Orange |
| With 5% vanillin | TU:EU + V | 5% Turmeric + 5% Eucalyptus + 5% Vanillin |
| | TU:OR + V | 5% Turmeric + 5% Orange + 5% Vanillin |
| | EU:OR + V | 5% Eucalyptus + 5% Orange + 5% Vanillin |
| | TU:EU:OR + V | 3.33% Turmeric + 3.33% Eucalyptus + 3.33% Orange + 5% Vanillin |

TU: Turmeric; EU: Eucalyptus; OR: Orange; V: Vanillin.

### 2.3. Repellency assay by feeding membrane apparatus

Repellency of essential oil blends was examined for *Ae. aegypti* and *An. dirus* under laboratory conditions using a membrane feeding system. Fifty of 5–7 day-old female mosquitoes were selected and placed in plastic cups (8 cm diameter × 8 cm high) covered with netting. A membrane feeding system was used with a sausage membrane stretched over a standard membrane feeder with a surface area of 3.14 $cm^2$ ($r$ = 1) and secured with a rubber band. Before feeding, either 10 µL of each mixture or 10 µL of coconut oil (negative control) was pipetted onto the sausage-casing membrane and spread evenly with the tip of the pipette. The treated membranes were allowed to dry and mosquitoes provided blood meals (exposed) at eight different time intervals (0.5, 1.0, 1.5, 2, 2.5, 3, 3.5 and 4.0 h) after application. A water feeding jacket was used to maintain the temperature of the feeding system at 37 °C. Approximately 1.5 mL of refrigerated (25 °C) human blood (Thai Red Cross Society, Bangkok, Thailand) was added to the glass feeder and allowed to warm to 37 °C. Then screened plastic cups of 50 female mosquitoes were allowed to feed for 5 min undisturbed. After the 5-min interval the membrane feeder was removed and unengorged mosquitoes were removed. Fully engorged mosquitoes and provided a sugar source and maintained in their containers in the insectary at 25 ± 2 °C for 24 h and then the number of dead mosquitoes counted.

### 2.4. Data analysis

Each mixture of essential oils was replicated five times ($n$ = 5) and results presented as the mean ± SD. To assess the significance of differences among groups, data were analyzed as a complete randomized design with a One-way ANOVA followed by Duncan's multiple range test. A $P$-value of < 0.05 was considered to indicate statistical significance using SPSS statistics for Window Version 16.0.

For comparison, percentage repellency was calculated for each test using the following formula:

$$\% \text{ Repellency} = \frac{A}{B} \times 100 \quad (1)$$

Where A is the number of mosquitoes that did not feed on the treated membrane and B is the total number of mosquitoes exposed.

Feeding was calculated as:

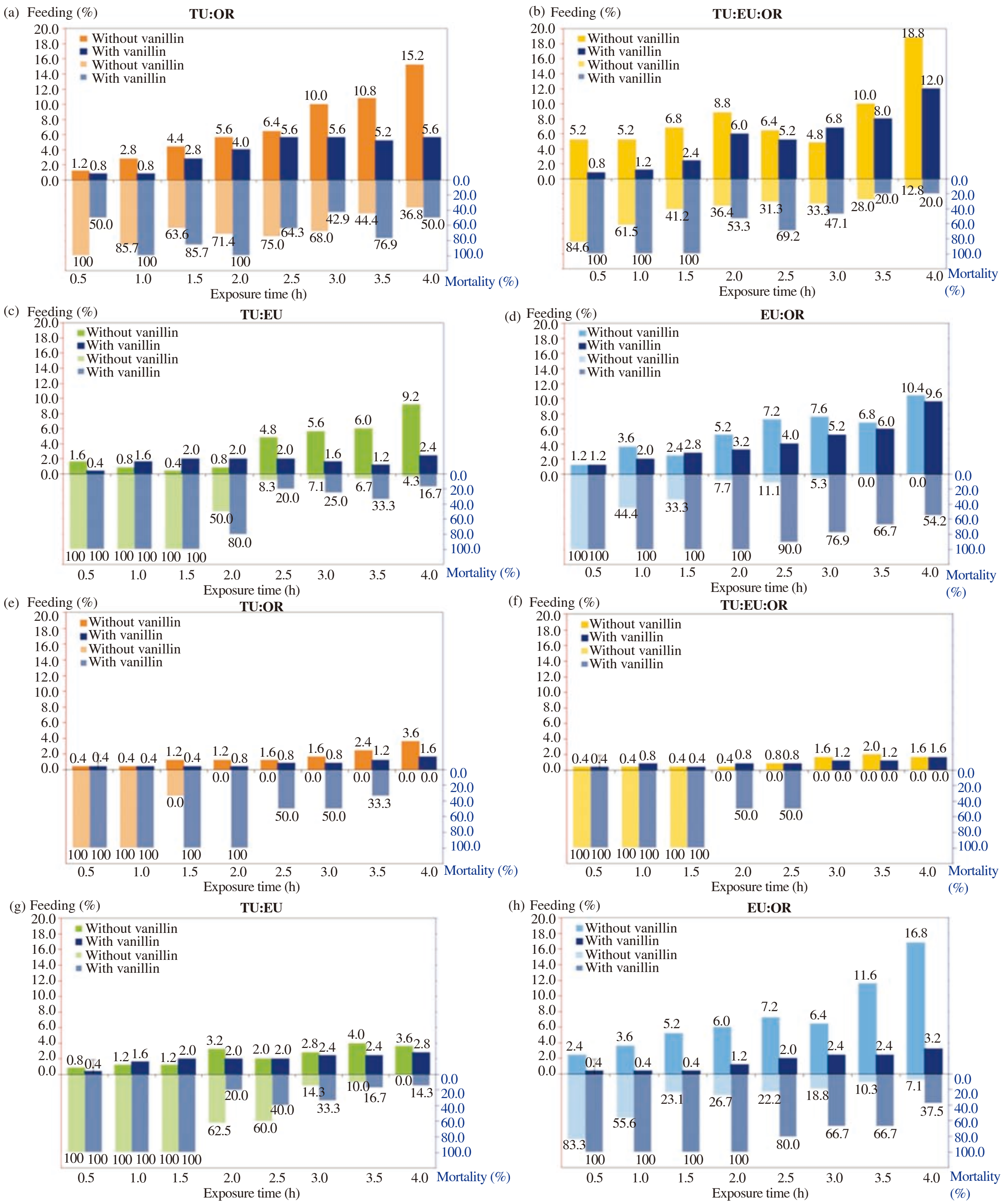


**Figure 1.** Comparative efficacy of four formulations of selected essential oils without and with 5% vanillin.

a, e: Turmeric:orange (TU:OR); b, f: Turmeric:eucalyptus:orange (TU:EU:OR); c, g: Turmeric:eucalyptus (TU:EU); d, h: Eucalyptus:orange (EU:OR) for *Ae. aegypti* and *An. dirus*.

$$\% \text{ Feeding} = \frac{F}{B} \times 100 \quad (2)$$

Where F is the total number of mosquitoes which fed on treated membranes, and B is the total number of mosquitoes exposed.

Mortality was calculated as:

$$\% \text{ Mortality} = \frac{D}{F} \times 100 \quad (3)$$

$$\text{Mortality per hour} = \frac{D/F}{h} \times 100 \quad (4)$$

Where D is the number of dead mosquitoes 24 h after blood feeding, F is the total number of mosquitoes which fed on treated membranes, and h is the period of time that the extracts were left on the membrane.

The number of mosquitoes which not feed on the membrane of each formulation was used to calculate the repellency per hour after application by the following formula:

$$\text{Repellency per hour} = \frac{A}{h} \times 100 \quad (5)$$

Where A is the number of mosquitoes that did not feed on the membrane and h is the period of time that the extracts were on membrane.

## 3. Results

The efficacy of various formulations of selected essential oils (10% total volume) with and without 5% vanillin, were presented in Figure 1. Shorter exposure times consistently resulted in lower feeding rates of both *Ae. aegypti* and *An. dirus* to all formulations. Feeding rates increased over time after application of formulations to the membranes. The addition of vanillin decreased feeding rates for all formulations. The greatest reduction in *Ae. aegypti* feeding was with the turmeric and eucalyptus mixture (TU:EU, Figure 1c). The greatest reduction in *An. dirus* feeding was with turmeric, eucalyptus, and orange mixed together (TU:EU:OR, Figure 1f).

Mortality rates were also shown in Figure 1. Mortality rates were decreased based on time after application of formulations to the membranes. The greatest and most consistent mortalities for *Ae. aegypti* were the mixtures of TU:OR with and without vanillin (Figure 1a) and the mixture of EU:OR with vanillin (Figure 1d). The greatest mortality for *An. dirus* females was with the mixture of EU:OR with vanillin (Figure 1h).

The percentage of repellency of all formulations against *Ae. aegypti* decreased with increasing exposure times (Table 2). The strongest percentage of repellency without vanillin was observed with the mixture of TU:EU (90.8%–98.4%), followed by EU:OR

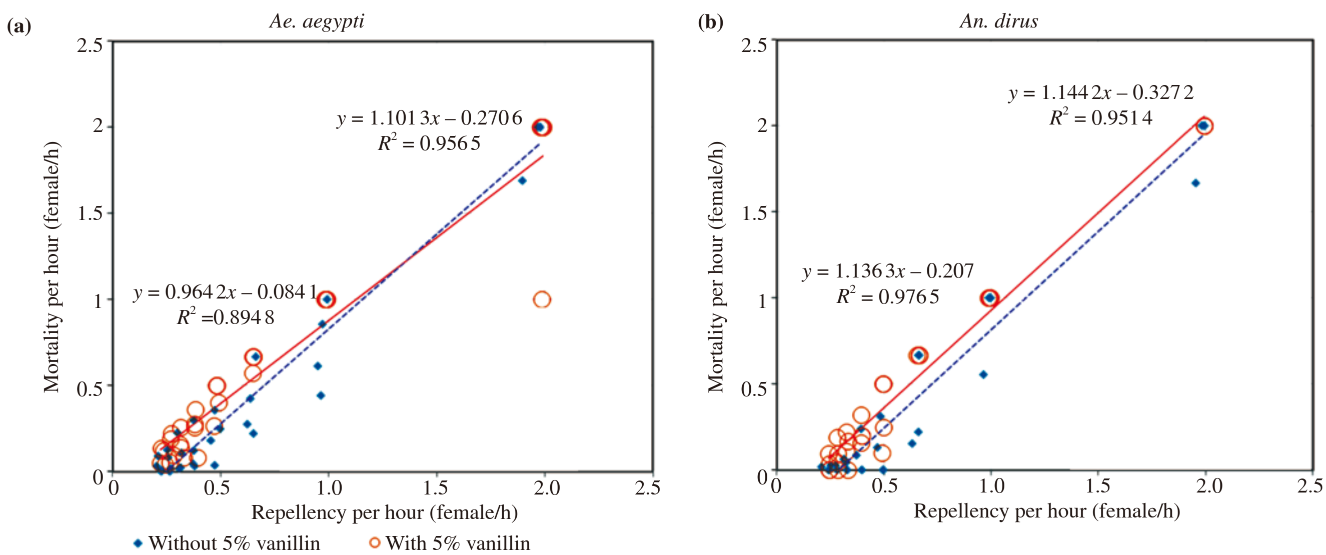


**Figure 2.** The relationship between feeding per hour and mortality per hour after application to sausage membranes for all formulations with and without vanillin against *Ae. aegypti* (a) and *An. dirus* (b).

**Table 2**

The percentage repellency of four formulations of selected essential oils without and with 5% vanillin at different exposure times after application for *Ae. aegypti*.

| Exposure time (h) | Control | | Formulation without vanillin | | | | $P$-value | Formulation with vanillin | | | | $P$-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No treat | Negative | TU:EU | TU:OR | EU:OR | TU:EU:OR | | TU:EU + V | TU:OR + V | EU:OR + V | TU:EU:OR + V | |
| 0.5 | 4.8 ± 3.0 | 62.0 ± 4.0 | 98.4 ± 2.6$^{a}$ | 98.8 ± 1.8$^{a}$ | 98.8 ± 1.1$^{a}$ | 94.8 ± 5.9$^{a}$ | 0.22 | 99.6 ± 0.9$^{a}$ | 99.2 ± 1.1$^{a}$ | 98.8 ± 1.8$^{a}$ | 99.2 ± 1.1$^{a}$ | 0.80 |
| 1.0 | 10.0 ± 8.9 | 64.0 ± 6.4 | 99.2 ± 1.8$^{a}$ | 97.2 ± 3.0$^{a}$ | 96.4 ± 4.1$^{a}$ | 94.8 ± 3.6$^{a}$ | 0.23 | 98.4 ± 2.6$^{a}$ | 99.2 ± 1.1$^{a}$ | 98.0 ± 2.4$^{a}$ | 98.8 ± 1.1$^{a}$ | 0.79 |
| 1.5 | 6.8 ± 3.3 | 67.2 ± 2.7 | 99.6 ± 0.9$^{a}$ | 95.6 ± 3.8$^{a}$ | 97.6 ± 3.6$^{a}$ | 93.2 ± 7.6$^{a}$ | 0.20 | 98.0 ± 2.4$^{a}$ | 97.2 ± 2.3$^{a}$ | 97.2 ± 2.7$^{a}$ | 97.6 ± 2.6$^{a}$ | 0.95 |
| 2.0 | 10.8 ± 7.9 | 63.2 ± 2.7 | 99.2 ± 1.1$^{a}$ | 94.4 ± 3.0$^{ab}$ | 94.8 ± 3.0$^{ab}$ | 91.2 ± 9.1$^{b}$ | 0.04 | 98.0 ± 3.5$^{a}$ | 96.0 ± 3.7$^{a}$ | 96.8 ± 2.3$^{a}$ | 94.0 ± 4.9$^{a}$ | 0.41 |
| 2.5 | 21.6 ± 5.2 | 62.0 ± 4.2 | 95.2 ± 1.1$^{a}$ | 93.6 ± 3.0$^{a}$ | 92.8 ± 3.6$^{a}$ | 93.6 ± 10.0$^{a}$ | 0.92 | 98.0 ± 2.4$^{a}$ | 94.4 ± 5.4$^{a}$ | 96.0 ± 2.0$^{a}$ | 94.8 ± 4.1$^{a}$ | 0.55 |
| 3.0 | 6.4 ± 3.3 | 58.8 ± 5.1 | 94.4 ± 1.7$^{a}$ | 90.0 ± 6.8$^{a}$ | 92.4 ± 3.8$^{a}$ | 95.2 ± 3.3$^{a}$ | 0.27 | 98.4 ± 2.6$^{a}$ | 94.4 ± 2.6$^{a}$ | 94.8 ± 3.3$^{a}$ | 93.2 ± 4.4$^{a}$ | 0.12 |
| 3.5 | 13.6 ± 8.9 | 59.2 ± 3.4 | 94.0 ± 1.4$^{a}$ | 89.2 ± 3.3$^{a}$ | 93.2 ± 3.6$^{a}$ | 90.0 ± 5.1$^{a}$ | 0.14 | 98.8 ± 1.8$^{a}$ | 94.8 ± 2.7$^{b}$ | 94.0 ± 3.2$^{b}$ | 92.0 ± 2.8$^{b}$ | 0.01 |
| 4.0 | 8.8 ± 5.4 | 56.4 ± 1.6 | 90.8 ± 2.3$^{a}$ | 84.8 ± 7.2$^{ab}$ | 89.6 ± 3.6$^{ab}$ | 81.2 ± 9.7$^{b}$ | 0.01 | 97.6 ± 1.7$^{a}$ | 94.4 ± 2.6$^{b}$ | 90.4 ± 2.2$^{c}$ | 88.0 ± 2.4$^{c}$ | 0.00 |

Mean of each row with different subscript letters are significantly different ($P$ < 0.05, by One-way ANOVA and Duncan's multiple range test), and data were expressed as mean ± SD. Negative control is virgin coconut oil. TU: Turmeric; EU: Eucalyptus; OR: Orange; V: Vanillin.

**Table 3**

The percentage repellency of four formulations of selected essential oils without and with 5% vanillin at different exposure times after application for *An. dirus*.

| Exposure time (h) | Control | | Formulation without vanillin | | | | *P*-value | Formulation with vanillin | | | | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No treat | Negative | TU:EU | TU:OR | EU:OR | TU:EU:OR | | TU:EU + V | TU:OR + V | EU:OR + V | TU:EU:OR + V | |
| 0.5 | 37.6 ± 16.3 | 70.0 ± 3.3 | 99.2 ± 1.8$^a$ | 99.6 ± 0.9$^a$ | 97.6 ± 0.9$^b$ | 99.6 ± 0.9$^a$ | 0.04 | 99.6 ± 0.9$^a$ | 99.6 ± 0.9$^a$ | 99.6 ± 0.9$^a$ | 99.6 ± 0.9$^a$ | 0.00 |
| 1.0 | 35.2 ± 24.1 | 68.0 ± 3.4 | 98.8 ± 1.8$^a$ | 99.6 ± 0.9$^a$ | 96.4 ± 1.7$^b$ | 99.6 ± 0.9$^a$ | 0.01 | 98.4 ± 1.7$^a$ | 99.6 ± 0.9$^a$ | 99.6 ± 0.9$^a$ | 99.2 ± 1.1$^a$ | 0.36 |
| 1.5 | 34.8 ± 20.8 | 71.2 ± 2.1 | 98.8 ± 1.8$^a$ | 98.8 ± 1.1$^a$ | 94.8 ± 3.0$^b$ | 99.6 ± 0.9$^a$ | 0.00 | 98.0 ± 2.8$^a$ | 99.6 ± 0.9$^a$ | 99.6 ± 0.9$^a$ | 99.6 ± 0.9$^a$ | 0.33 |
| 2.0 | 31.2 ± 22.6 | 71.2 ± 3.4 | 96.8 ± 2.3$^{ab}$ | 98.8 ± 1.1$^a$ | 94.0 ± 5.8$^b$ | 99.6 ± 0.9$^a$ | 0.06 | 98.0 ± 2.8$^a$ | 99.6 ± 0.9$^a$ | 98.8 ± 1.1$^a$ | 99.2 ± 1.1$^a$ | 0.50 |
| 2.5 | 37.2 ± 21.9 | 72.8 ± 1.1 | 98.0 ± 1.4$^a$ | 98.8 ± 1.8$^a$ | 92.8 ± 3.6$^b$ | 99.2 ± 1.1$^a$ | 0.00 | 98.0 ± 1.4$^a$ | 99.2 ± 1.8$^a$ | 98.0 ± 2.4$^a$ | 99.2 ± 1.1$^a$ | 0.53 |
| 3.0 | 31.6 ± 16.3 | 72.4 ± 2.9 | 97.2 ± 1.1$^a$ | 98.4 ± 1.7$^a$ | 93.6 ± 2.2$^b$ | 98.4 ± 1.7$^a$ | 0.00 | 97.6 ± 2.6$^a$ | 99.2 ± 1.1$^a$ | 97.6 ± 2.6$^a$ | 98.8 ± 1.8$^a$ | 0.54 |
| 3.5 | 35.6 ± 21.1 | 71.2 ± 3.5 | 96.0 ± 2.4$^a$ | 97.6 ± 2.6$^a$ | 88.4 ± 2.2$^b$ | 98.0 ± 2.8$^a$ | 0.00 | 97.6 ± 1.7$^a$ | 98.8 ± 1.1$^a$ | 97.6 ± 1.7$^a$ | 98.8 ± 1.8$^a$ | 0.56 |
| 4.0 | 32.0 ± 23.2 | 76.4 ± 1.6 | 96.4 ± 2.2$^a$ | 96.4 ± 3.0$^a$ | 83.2 ± 2.7$^b$ | 98.4 ± 1.7$^a$ | 0.00 | 97.2 ± 1.1$^a$ | 98.4 ± 1.7$^a$ | 96.8 ± 2.3$^a$ | 98.4 ± 2.2$^a$ | 0.57 |

Mean of each row with different subscript letters are significantly different ($P < 0.05$, by One-way ANOVA and Duncan's multiple range test), and data were expressed as mean ± SD. Negative control is virgin coconut oil. TU: Turmeric; EU: Eucalyptus; OR: Orange; V: Vanillin.

(89.6-98.8%), and TU:OR (84.8%–98.8%). The lowest percentage of repellency was observed for the mixture of all three plant extracts (TU:EU:OR, 81.2%–94.8%). The percentage of repellency for all essential oil combinations with vanillin was not statistically different in any of the formulations without vanillin. The mixture of TU:EU provided the most repellency at 3.5 and 4 h with and without vanillin.

There were no statistical differences between the repellency of any formulations against *An. dirus* with or without vanillin, except for EU:OR + vanillin after 3 h (Table 3). The highest percentage of overall repellency was observed for the combination of all three plant extracts (TU:EU:OR) and with the mixture of TU:OR.

Overall, there were a positive correlation for formulations that produced the most repellency and increased mortality (Figure 2). For both *Ae. aegypti* (Figure 2a) and *An. dirus* (Figure 2b), the ratio of mortality per hour of formulations with vanillin was greater than without vanillin. Similarly, the repellency per hour of the four formulations with vanillin was stronger than without vanillin.

## 4. Discussion

Essential oils can have a significant effect on mosquito feeding rates (repellency) and mortality. For all formulations, exposure at 0.5 h after application of plant extracts to the artificial membrane resulted in greater repellency and higher mortality. Whereas, at 4 h after application there was increased feeding (decreased repellency) and lower mortality. These are consistent with many other studies demonstrating that plant extracts are volatile and lose their efficacy over time after application[25,26].

The different mixtures led to different outcomes. The combination of TU:EU resulted in the highest repellency for *Ae. aegypti*, while the combination of all three essential oils (TU:EU:OR) gave the least repellency. Currently, the combination of all three extracts (TU:EU:OR) provided the greatest repellency against *An. dirus*. These results showed that the two mosquito species have different responses to the three plant-derived essential oils tested. It also demonstrated that the efficacy of each formulation is based on the compatibility of active ingredients and these compounds produce different effects when combined together. There are other reports showing that essential oils from plants are synergistic. The synergistic actions of three plant essential oils were used in this study that increased the repellency of formulations that would be expected from individual essential oils[27]. The repellent activity of mixing essential oils from Japanese mugwort (*Artemisia princeps*) and cinnamon (*Cinnamomum camphora*) was greater against *Sitophillus oryzae* and *Bruchus rugimanus* (Coleoptera: Curculionidae) than that elicited by individual oils[28].

The results also showed that the addition of 5% vanillin significantly decreased feeding rates and increased mortality. Studies have shown that vanillin reduces the evaporation rate of active ingredients and therefore extends the protection time (repellency)[15]. It is believed that vanillin changes the volatile composition of essential oils and also has an effect on the gustatory processes of mosquitoes[29,30]. In this study the effect of vanillin was often more evident at increased time after application. It is recommended that vanillin be considered included as an ingredient for future natural product repellents.

Finally, this study presented a unique method to evaluate repellent formulations using a sausage membrane casing as part of a membrane feeding system. The arm in cage method has been used to evaluate many essential oil formulations[31]. While the "arm in cage" method is the "gold standard" for evaluating repellents, a membrane feeding system offers several advantages in that it can be quickly performed, standardly replicated, and does not require human volunteers[32,33]. This method could be developed to rapidly screen, evaluate, and select the most promising formulations before they are tested on human volunteers.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

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