Larvicidal Effect of Vitex ovata Thunb. (Lamiales: Lamiaceae) Leaf Extract towards Aedes (Stegomyia) aegypti (Linnaeus, 1762) (Diptera: Culicidae)

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Abstract: According to the WHO there are more than 700,000 deaths every year involving vector-borne diseases such as malaria, dengue, Chagas disease, yellow fever and Japanese encephalitis. Aedes aegypti, the principal vector of the dengue virus, is of great concern in various parts of the world, especially in tropical and subtropical countries. Vector control through insecticide application is one of the best ways to control the disease’s transmission. Thus, insecticide resistance in Ae. aegypti poses a significant threat to public health worldwide. The use of plant natural product-based insecticides that are less harmful to the environment, and without known resistance development, constitutes an alternative to chemical insecticides. Given this, a methanolic extract from Vitex ovata plants was prepared and tested for its larvicidal effect against Ae. aegypti. A susceptibility test on Ae. aegypti larvae was conducted using the standard WHO method. Results showed that the methanolic extract of V. ovata had larvicidal activity against Ae. aegypti with LC50 values of 2114 mg/L, and achieved 84% mortality with the highest concentration at 10,000 mg/L. This study showed that the crude extract of V. ovata bioactive molecules could be potentially developed as biolarvicides for Aedes mosquito vector control. This study recommends future research on using different solvents in the isolation of active ingredients from V. ovata, identification of phytochemicals with larvicidal properties, a toxicity study and lastly, an evaluation of the effectiveness of controlling Aedes in small-scale field trials for environmentally safe botanical insecticide intervention.

Keywords: methanolic extract; natural product-based insecticides; LC50; mosquitoes

1. Introduction

Aedes (Stegomyia) aegypti (Linnaeus, 1762), the principal vector of the dengue virus, is of great concern in various parts of the world, especially in tropical and subtropical countries [1]. The worldwide distribution of dengue epidemics includes over 100 countries, and up to 50–100 million infections are now estimated to occur annually [2]. In Malaysia, the number of dengue cases in 2019 was reported to be 127,407, with 176 deaths [3]. The increasing of this vector-borne disease is of great concern, as the incidence of dengue has increased 30-fold over the last 50 years [2]. Other than the dengue virus, Aedes (Stegomyia) aegypti s also known to transmit yellow fever virus, chikungunya virus, and Zika virus, putting more than half of the world’s population at risk.
Vector control through insecticide application is one of the best ways to control disease transmission. Most of the control measures for dengue vectors target adults by using permethrin, deltamethrin, and malathion, or using larvicide such as temephos and Bacillus thuringiensis israelensis [4]. The use of these insecticides is practiced by government operators, private companies and even household communities. Intense exposures of insecticide have contributed to insecticide resistance in Aedes populations. Evidence of resistance towards permethrin and temephos has been recorded from both Ae. aegypti and Ae. albopictus (Skuse, 1894) in a few localities of Malaysia [5–7].

Given insecticide resistance and the limited success of biocontrol programs on Aedes [8], a search for new insecticides has become necessary. The use of botanical insecticides has a long history and their use is as a safer solution to control various types of insect pests. Naturally occurring chemicals extracted from plants act quickly (compared to biological control), degrade rapidly and usually have low mammalian toxicity [9,10]. Botanical insecticides affect various insects differently and can be classified into six groups: repellents, feeding deterrents/antifeedants, toxicants, growth retardants, chemosterilants, and attractants [11,12].

Alternative uses of bio-insecticides derived from plants might provide a more suitable and sustainable solution against Ae. aegypti. Following this safer and greener alternative concept, the Vitex ovata Thunb. (synonym: Vitex rotundifolia L.f.) plant, which is ubiquitous at the shoreline of Malaysia, was evaluated. Vitex ovata was chosen considering several species from this plant genus were known to have larvicidal properties [13]. Leaf extract from V. trifolia L. consists of 89 phytochemical components, with the major component identified as eucalyptol, which is known as an insect repellant. Vitex ovata, also known as V. rotundifolia [14], possesses a biological compound named rotundial with potent repelling activity against Ae. aegypti [15]. Nevertheless, a study on the efficacy of V. ovata leaf extract as a larvicide against Ae. aegypti is not yet available. Hence, the purpose of this study was to evaluate the effectiveness of V. ovata crude extracts against the Ae. aegypti as a larvicide.

2. Results

The larvicidal effects of V. ovata methanolic extracts were concentration dependent. The average mortality of Ae. aegypti from three replicates for each of the concentrations are shown in Table 1. The entire larvicidal bioassay showed a significant increase in mortality with extract concentration (F = 38.68, p < 0.001). The two highest concentrations tested, 5000 mg/L and 10,000 mg/L, had the highest larvicidal activities, with 76% and 84% mortality achieved within 24 h, respectively (statistics showed no difference in mortality between the two concentrations). The lowest mortality of the larvae caused by the extract was 34% at a concentration of 1250 mg/L. No mortality was observed in the controls. In this study, the LC_{50} and LC_{90} values of V. ovata methanolic extracts were 2114 and 13,902 mg/L, respectively (Figure 1).

Table 1. Mortality of Ae. aegypti larvae exposed to V. ovata methanolic extract.

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>Actual Larvae Mortality n ± SE</th>
<th>Actual Larvae Mortality (%)</th>
<th>Arcsine Transformed Larvae Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>21.00 ± 0.89</td>
<td>84.00 ± 3.55</td>
<td>67.59 ± 2.64 a</td>
</tr>
<tr>
<td>5000</td>
<td>19.00 ± 0.74</td>
<td>76.00 ± 2.95</td>
<td>61.31 ± 2.21 a</td>
</tr>
<tr>
<td>2500</td>
<td>13.90 ± 0.50</td>
<td>55.67 ± 2.00</td>
<td>48.30 ± 1.17 b</td>
</tr>
<tr>
<td>1250</td>
<td>8.40 ± 1.16</td>
<td>33.67 ± 4.66</td>
<td>35.29 ± 2.85 c</td>
</tr>
</tbody>
</table>

Within column, means without common letters differ at p < 0.05. Different letters used here just an indication to show whether the means are same or different from each other.
Figure 1. Concentration-response curve for the larvicidal effect of V. ovata extract on Ae. aegypti.

Under microscopic observation, the larvae treated with the plant extract generally appeared darker, with the thorax and upper abdominal segment prominently dark in colour, compared to the larvae from the control groups. The lower abdominal segments of the treated larvae were as clear as the control larvae. Besides, the anal gills of the treated larvae had a dark discolouration compared to the larvae from the control group (Figure 2).

Figure 2. Aedes aegypti larva treated with V. ovata extract (Left) and control larva (Right) showing difference in the colouration of their midgut, in which the plant extract (greenish material) was visible in the treated larvae after 24 h.
3. Discussion

This study showed that the *V. ovata* methanolic extract might have larvicidal properties that can kill *Ae. aegypti* larvae. The crude extract of *V. ovata* used could be an innovative application as an alternative to synthetic chemical insecticides. The highest mortality of 68% achieved in this study was considered less effective. A larvicide is said to be effective if the mortality of larvae reaches at least 90% within 24 h [16]. Several authors have developed criteria to characterize mosquito larvicide potency for essential oils derived from plants, generally considered products with LC$_{50}$ < 100 mg/L as exhibiting a significant larvicidal effect [17–21]. However, there is no standard criterion established by the WHO for determining the larvicidal activity of natural products.

Several species of plants under *Vitex* are known to have larvicidal activity against mosquitoes. Several studies had been conducted on *Vitex agnus castus* L., *V. altissima* L.f., *V. grandifolia* Gürke, *V. negundo* L., *V. peduncularis* Wall. ex Schauer, *V. rotundifolia* Michx., *V. schiliebeni* Moldenke and *V. trifolia* L. against mosquito species such as *Culex quinquefasciatus* (Say, 1823), *C. pipiens* (Linnaeus, 1758), *Anopheles stephensi* (Liston, 1901), *A. gambiae* (Giles 1902), *Ae. aegypti*, and *Ae. albopictus* [22]. Overall, crude extracts are less effective than the essential oils of most plant extracts and less effective compared with only active compounds used [23,24]. Crude plant extracts might have lower killing power; however, the botanical blends of chemical compounds leave very little chance for the pests to develop resistance to such constituents.

The dark materials observed in the midgut of the larvae were similar to those observed in previous studies [25,26]. This indicated that the *Aedes* larvae ingested the *V. ovata* plant extract, suggesting the killing mechanism of this plant extract against the mosquito larvae. The absence of microorganisms in the leaf extract reveals that no bacteria or fungus were introduced in the larval environment together with the extract. Thus, the darkening of the *Aedes* larvae was not related to midgut melanization after incubation with the extract [27]. Therefore, the mortality of the larvae could be attributed to the extract components solely with deleterious effects in the midgut such as vacuolization, microvilli damage, and cell lysis, which eventually impair the gut homeostasis and extensive tissue disorganization [28,29].

The major constituents of *V. rotundifolia* essential oil are α-pinene and 1.8-cineole. These two monoterpenes have been demonstrated as eco-friendly larvicides against mosquitoes and houseflies [30,31]. However, contradictory results obtained from Waliwitiya et al. [32] and Huang et al. [33], concerning α-pinene and 1.8-cineole being the principal larvicidal components of *V. rotundifolia* against *Ae. aegypti* remain to be confirmed. The fruit is, by far, the most studied part of *V. rotundifolia*, followed by the leaves [34,35].

Chemical constituents of *V. ovata* methanolic leaf extract are not available. The bioactivity of plant phytochemicals can vary significantly depending on plant species, plant part, and even the type of solvent used in extraction [36]. Thus, further studies on the phytochemistry of the *V. ovata* methanolic leaf extract are required to elucidate this plant’s larvicidal potential further.

As mentioned above, the result from this study showed that *V. ovata* possess larvicidal components. However, methanol may not fully extract these components, as research has proved that solvent polarity mediates extraction yield and phytochemical contents [37–40]. A series of polar and nonpolar solvents tested to increase the extraction efficiency of phytochemicals with larvicidal properties from *V. ovata* might be an excellent approach to avoid missing out on any valuable compounds derived from the plant.

Many other factors could determine whether a plant extract is suitable to proceed to a field trial and eventual commercialization. For instance, the toxicity of *V. ovata* should be tested in-vitro and in-vivo to make sure it is environmentally safe does not cause toxicity to nontargeted organisms. Even though phytochemicals are relatively safe [41], a high dose could still be harmful [42]. Adequacy of the plant source is a primary concern when moving to commercialization. In this study, roughly 500 g of live *V. ovata* plant (leaves)
could produce 50 mL of the crude extract. Countries in which *V. ovata* is abundant could consider this plant for developing the bio-insecticide as suggested.

4. Materials and Methods

4.1. Plant Methanolic Extract Preparation

Whole plants of *V. ovata* (Figure 1) were collected from Pantai Sabak, Kota Bharu, Kelantan, Malaysia (6°9’59.6952” N, 102°20’31.4988” E), and the species were identified based on a morphological view of phyllotaxis. Next, the leaves of *V. ovata* were removed and washed under running water to remove soil. The cleaned leaves were then dried in an oven (50 ± 1 °C) for 24 h. The dried samples were pulverized into fine powders using an electrical grinding machine (Faber FBG-460 K, Faber, Fabriano, Italy). A total of 100 g of fine powder was put into conical flasks, and one litre of methanol was poured into the flask. The mixture was stirred every 24 h using a magnetic stirrer, in a process that lasted for 3 days. The mixture was then filtered using Whatman No. 1 filter paper to remove the sample waste and was evaporated in a rotary vacuum evaporator at 40 °C to obtain the crude extract. The concentrated extract was then stored in the refrigerator (4 °C) for further use. Extract purity was checked by streaking the extract on nutrient agar (NA) with a sterile swab and incubated at 37 °C for 24 h to ensure no contamination.

4.2. Mosquito Larvae Rearing

*Ae. aegypti* eggs were obtained from the Vector Control Research Unit (VCRU) at the University Sains Malaysia (USM), Penang, Malaysia. The eggs were hatched in dechlorinated water for 24 h and maintained at 28 ± 2 °C (room temperature), a pH of 6.9 to 7.0, and dissolved oxygen from 5.5 to 6.1 mg/L in the laboratory. Ground cat food was used as the food source for the larvae. After five to six days, the early 4th instar larvae were collected for the larvicidal bioassay.

4.3. Larvicidal Bioassay

Larvicidal bioassays were performed following the standard World Health Organization [43] guidelines on larval susceptibility test methods (distilled water and plant solution). The bioassays were carried out using 25 early 4th instar *Ae. aegypti* larvae (homogeneous population consisting of 5 mm to 6 mm in body length). The bioassays were replicated three times for each concentration, with methanol CH\textsubscript{3}OH as a solvent for the control. During the testing period, ground cat food was provided. In this study, four concentrations of 1250 mg/L, 2500 mg/L, 5000 mg/L and 10,000 mg/L were tested against the larvae. The control solutions were prepared with 1 mL of distilled water and 10% of the respective methanol solvent for each experimental replicate. The solvent control was to ensure that the mortality results were not due to the solvent. The experiments were conducted at a room temperature of 28 ± 2 °C, and larvae mortalities were recorded after 24 h. Death was considered as immobilization and total absence of movement from the larvae, even after touch. The data were analyzed using probit analysis for computing LC\textsubscript{50}, and LC\textsubscript{90} and one-way ANOVA was performed on data after arcsine transformation to discriminate the difference between the concentrations used (IBM SPSS Statistics 24, IBM, New York, NY, USA).

4.4. Microscopic Observation

The tested and control *Ae. aegypti* larvae were observed under a dissecting microscope for comparison on the physical changes.

5. Conclusions

Nowadays, environmental safety is considered to be of utmost importance. Insecticide that is eco-friendly with an acceptable mortality on the target organisms to ensure the pest population under the threshold level should be promoted. Leaf extract of *V. ovata* showed larvicidal activity on *Ae. aegypti* and may be developed as bio-larvicides for *Aedes* mosquito vector control. Since *V. ovata* is widely distributed in Malaysia, commercial exploitation
could provide an important step in developing a novel plant-based insecticide. Screening locally available medicinal plants for bio-insecticides could generate local employment, minimize dependence on imported products, and encourage local efforts to enhance the public health system.

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