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Research Article

Evaluation of Larvicidal Activity of Homoeopathic Formulations Against *Aedes aegypti* (Diptera-Culicidae)

Aberami G[1], Jegan T M[2], Yogeswari[3], Vettrivel Arul[4]

[1,2]Vinayaka Mission's Homoeopathic Medical College & Hospital, Vinayaka Mission's Research Foundation (DU), Salem, Tamil Nadu, India.
[3]Department of Repertory, Vinayaka Mission's Homoeopathic Medical College & Hospital, Vinayaka Mission's Research Foundation (DU), Salem, Tamil Nadu, India.
[4]Department of Community Medicine, Vinayaka Mission's Homoeopathic Medical College & Hospital, Vinayaka Mission's Research Foundation (DU), Salem, Tamil Nadu, India.
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I N F O

Corresponding Author:
Vettrivel Arul, Department of Community Medicine, Vinayaka Mission's Homoeopathic Medical College & Hospital, Vinayaka Mission's Research Foundation (DU), Salem, Tamil Nadu, India.
E-mail Id:
veldoc4565@gmail.com
Orcid Id:
https://orcid.org/0000-0002-2319-726X
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A B S T R A C T

Introduction: Mosquito-borne diseases, including dengue fever, Zika, and chikungunya, present significant public health challenges, particularly in tropical and subtropical regions. *Aedes aegypti*, the primary vector for these diseases, necessitates effective control methods. Traditional mosquito control approaches face issues like resistance development and environmental harm, prompting the search for safer alternatives.

Method: This study evaluates the larvicidal activity of homoeopathic formulations with six plant-based components: *Azadirachta indica*, camphor, *Eucalyptus globulus, Ocimum sanctum*, *Areca catechu*, and *Nigella sativa*. Three formulations were tested: Formulation 1 (equal proportions of all extracts), Formulation 2 (higher proportions of *Azadirachta indica* and *Areca catechu*), and Formulation 3 (only *Azadirachta indica* and *Areca catechu*). Larvicidal efficacy was assessed using late third to early fourth instar larvae of *Aedes aegypti*, with ethanol as a vehicle control.

Result: Results showed significant larvicidal activity for all formulations, with Formulation 2 exhibiting the highest efficacy (LC_{50}: 85.57 µg/mL, LC_{90}: 261.52 µg/mL). Statistical analysis confirmed the potency of Formulation 2, highlighting its potential as a natural insecticide.

Conclusion: The study underscores the promise of plant-based homoeopathic formulations as eco-friendly alternatives to synthetic insecticides, aiming to reduce environmental impact and mitigate resistance development.

Keywords: *Aedes Aegypti*, Homoeopathic Formulations, Larvicidal Activity, Natural Insecticides, Plant Extracts

Introduction

Mosquito-borne diseases, such as dengue fever, Zika, and chikungunya, pose significant threats to public health, particularly in tropical and subtropical regions.[1] *Aedes aegypti*, belonging to the family Culicidae and order Diptera, is the primary vector for these diseases, affecting millions of people annually.[2] Traditional methods of mosquito control, including mechanical and biological controls, as well as synthetic insecticides, have proven insufficient due to issues such as resistance development and environmental pollution.[3] This necessitates the exploration of natural substances that can act as effective larvicides and insecticides, offering safer and environmentally friendly alternatives.[4]

In this study, we focus on the larvicidal activity of homoeopathic formulations containing six plant-based components: *Azadirachta indica* (neem), camphor, *Eucalyptus globulus, Ocimum sanctum* (holy basil), *Areca catechu* (betel nut), and *Nigella sativa* (black seed). Neem is well-known for its insecticidal properties, primarily due to the active compound azadirachtin, which is effective against numerous mosquito species.[5] Camphor, derived from the camphor tree, has demonstrated insecticidal and repellent activities, particularly against mosquitoes.[6] *Eucalyptus globulus*, commonly used for its essential oils, contains 1,8-cineole, which has excellent larvicidal properties.[7] *Ocimum sanctum*, used in traditional Indian medicine, has shown significant larvicidal and repellent activities.[8] *Areca catechu* contains phytochemicals with high larval mortality rates, and *Nigella sativa*, known for its medicinal properties, has demonstrated insecticidal activity.[9,10]

We evaluated three specific homoeopathic formulations for their efficacy against *Aedes aegypti* larvae. Formulation 1 contains equal proportions (1.67 mL each) of all six plant extracts. Formulation 2 includes higher proportions of *Azadirachta indica* and *Areca catechu* (2.5 mL each) with lower amounts (1.25 mL each) of camphor, *Eucalyptus globulus, Ocimum sanctum*, and *Nigella sativa*. Formulation 3 consists solely of *Azadirachta indica* and *Areca catechu* (5 mL each). To avoid bias due to the presence of ethanol in the homoeopathic mother tinctures, we used 90% ethanol as the vehicle control in our experiments. This ensured that any observed larvicidal activity could be attributed to the plant extracts rather than the ethanol itself. By identifying the most effective formulations and the compounds responsible for larvicidal activity, this research aims to contribute to the development of safer, natural insecticides. These findings could support sustainable and eco-friendly approaches to mosquito management, reducing reliance on synthetic chemicals and their associated risks.

Materials and Method

Study Duration and Location

The study was conducted from January 3, 2024, to March 1, 2024, at Vinayaka Mission's Homoeopathic Medical College and Hospital, Salem, Tamil Nadu, and Trichy Research Institute of Biotechnology (P) Ltd., Trichy, Tamil Nadu. Necessary permissions were obtained from both institutions to carry out the research.

Mosquito Culture

To cultivate *Aedes aegypti* (Diptera-Culicidae), commonly known as the Yellow Fever mosquito, eggs were collected from stagnant water in rice fields using an "O"-type brush. These eggs were then taken to the laboratory and placed in enamel trays measuring 18 × 13 × 4 cm, each containing 500 mL of water to facilitate hatching. The rearing environment was strictly controlled, maintaining a temperature of 28 °C, relative humidity between 75% and 85%, and a light/dark cycle of 14 hours of light followed by 10 hours of darkness. The larvae were provided with a diet composed of Brewer's yeast, dog biscuits, and pond algae in a 3:1:1 ratio. This feeding regimen was sustained until the larvae progressed to the pupal stage.[11]

Preparation of Homoeopathic Formulations

Three different homoeopathic formulations were prepared using specific concentrations of six medicinal plant extracts in a 90% ethanol solution. Each formulation was prepared for a total volume of 10 mL as shown in Table 1.

These formulations were prepared in a solution of 90% ethanol, which also served as the vehicle control to avoid any bias due to the solvent's effects.

Table 1.Proportions of Homoeopathic Mother Tinctures in Three Formulations Evaluated for Larvicidal Activity Against *Aedes aegypti*

Formulation	*Azadirachta indica* (mL)	*Areca catechu* (mL)	*Camphor* (mL)	*Eucalyptus globulus* (mL)	*Ocimum sanctum* (mL)	*Nigella sativa* (mL)
Formulation 1	1.67	1.67	1.67	1.67	1.67	1.67
Formulation 2	2.50	2.50	1.25	1.25	1.25	1.25
Formulation 3	5.00	5.00	-	-	-	-

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Larvicidal Bioassay

The larvicidal activity of the homoeopathic formulations was evaluated using late third to early fourth instar larvae of *Aedes aegypti*. The bioassay followed the WHO guidelines with modifications. For the test, twenty-five larvae were introduced into 100 mL of each test solution placed in a 250 mL glass beaker. Each test solution, including the control, was replicated four times for statistical reliability.

Each formulation was diluted with distilled water to obtain concentrations of 1%, 0.5%, and 0.1%. Ethanol 90% was used as the vehicle control to account for any solvent effects. Larval mortality was recorded at 24 hours post-exposure. Larvae were considered dead if they did not respond to gentle prodding with a fine brush.[11]

$$\text{Percentage of Mortality} = \frac{(\text{Number of Dead LarvaeTotal})}{(\text{Total Number of LarvaeNumber})} \; x \; 100$$

Morphological Examination

Dead larvae were collected after 24 hours of treatment for the examination of morphological changes under light microscopy. The larvae were scrutinised after mounting with Hoyer's medium. Morphological changes in body segments including the head, setae, cuticle, abdomen, and anal gills were observed, photographed, and compared with those of the controls.[12]

Data Analysis

The statistical analysis employed in this study aimed to evaluate and compare the larvicidal efficacy of the homoeopathic formulations. Probit regression analysis was used to determine the LC_{50} and LC_{90} values for each formulation and the control, representing the concentrations required to kill 50% and 90% of the larvae respectively.[13] The 95% confidence intervals for these values were also calculated. Additionally, the Kruskal-Wallis test was conducted to assess variations in larvicidal activity across different concentrations of the formulations. Dunn's post-hoc test with Bonferroni correction was used for pairwise comparisons between different concentration levels. Comparative analysis between the different formulations (Formulation 1, Formulation 2, and Formulation 3) and the control was performed using one-way ANOVA. Tukey's post hoc test identified specific groups with significant differences. GraphPad Prism software was used for all statistical analyses, providing a robust evaluation of the efficacy and potency of the larvicidal formulations.

Result

The larvicidal activity of four samples (Formulation 1, Formulation 2, Formulation 3, and E) was evaluated using Probit regression analysis across five different concentrations (500, 250, 100, 50, and 10 µg/mL) with four replicates per concentration. The analysis utilised a log-logistic model with the lower limit fixed at 0 and the upper limit at 1, suitable for binomial response data (Figure 1).

For Formulation 1, the LC_{50} value was 139.40 µg/mL (95% CI: 39.35 to 239.46) and the LC_{90} value was 518.22 µg/mL (95% CI: -176.50 to 1212.93), both statistically significant (LC_{50} $p = 0.0063$, LC_{90} $p < 0.05$). Formulation 2 exhibited an LC_{50} value of 85.57 µg/mL (95% CI: 28.84 to 142.31) and an LC_{90} value of 261.52 µg/mL (95% CI: -27.52 to 550.56), also statistically significant (LC_{50} $p = 0.0031$, LC_{90} $p < 0.05$). For Formulation 3, the LC_{50} value was 95.99 µg/mL (95% CI: 33.34 to 158.63) and the LC_{90} value was 290.42 µg/mL (95% CI: -31.22 to 612.06), with significance levels of $p = 0.0027$ for LC_{50} and $p < 0.05$ for LC_{90}. Sample E, however, displayed an LC_{50} value of 563.59 µg/mL (95% CI: -387.04 to 1514.23) and an LC_{90} value of 4653.89 µg/mL (95% CI: -14448.46 to 23756.23), which were not statistically significant (LC_{50} $p = 0.2452$, LC_{90} $p > 0.05$), indicating a high level of uncertainty in the efficacy of sample E (Figure 2).

Comparative statistical analysis revealed that among the samples, Formulation 2 had the lowest LC_{50} value (85.57 µg/mL), indicating the highest efficacy at lower concentrations. The 95% confidence interval for Formulation 2's LC_{50} (28.84 to 142.31) was narrower, providing more reliability in the estimate. Formulation 3 followed with an LC_{50} value of 95.99 µg/mL (95% CI: 33.34 to 158.63), demonstrating significant but slightly less potent activity than Formulation 2. Formulation 1 had the highest LC_{50} value (139.40 µg/mL) with a confidence interval of 39.35 to 239.46, suggesting that it is less effective compared to Formulation 2 and Formulation 3 at lower concentrations (Figure 3).

While examining morphologically, Formulation 2 at 500 µg/mL was found to exhibit extensive damage to the digestive tract, respiratory tube, and anal gills, leading to rapid mortality; at 250 µg/mL, severe digestive and respiratory tract damage causing nutritional failure and oxygen deprivation, resulting in larval death; at 100 µg/mL, dark spots indicating areas of necrosis, highlighting severe cellular damage and the failing ability to maintain homeostasis, ultimately leading to larval death; the control showed no significant morphological damage, indicating a healthy condition (Figure 4).

The Kruskal-Wallis test revealed significant variations in larvicidal activity across different concentrations of Formulation 2 ($p < 0.01$). Dunn's post-hoc analysis, incorporating Bonferroni correction, pinpointed significant differences between the lowest concentration (10 µg/mL) and the higher concentrations (250 µg/mL, $p < 0.05$; 500 µg/mL, $p < 0.001$) (Figure 5). No notable disparities were observed between other concentration pairs, underscoring that elevated concentrations of Formulation 2 (250 µg/mL and 500 µg/mL) exhibit significantly heightened efficacy in larval eradication compared to the lowest concentration (10 µg/mL).

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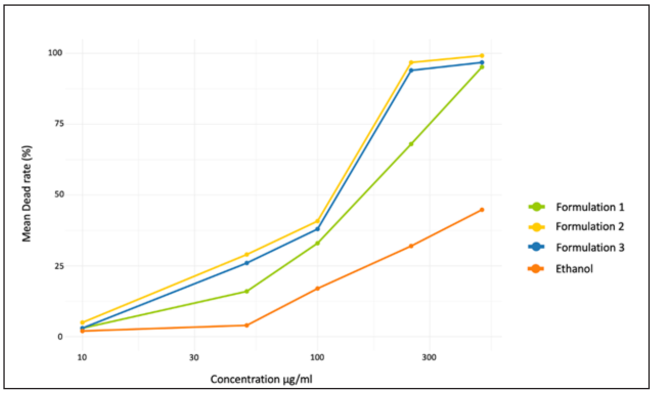

Figure 1.Mean Mortality Rate (%) of *Aedes aegypti* Larvae after 24-Hour Exposure to Various Concentrations of Three Homoeopathic Formulations and 90% Ethanol Control, with Formulation 2 showing the Highest Efficacy

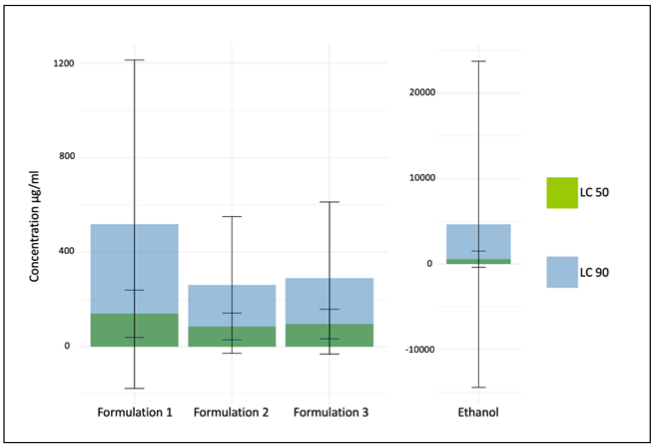

Figure 2.LC50 (Green) and LC90 (Blue) Values of Three Homoeopathic Formulations and Ethanol Against *Aedes aegypti* Larvae, showing Formulation 2 as the Most Effective with the Lowest Values

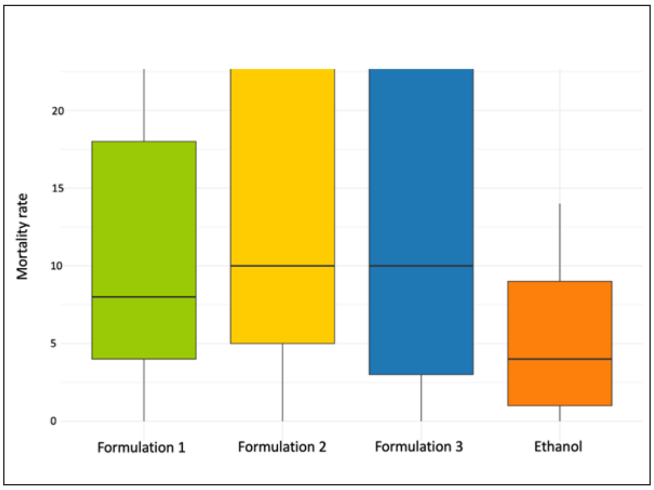

Figure 3.Mortality Rates of *Aedes aegypti* Larvae Exposed to Three Homoeopathic Formulations and Ethanol, with Formulation 2 showing the Highest Mortality Rate

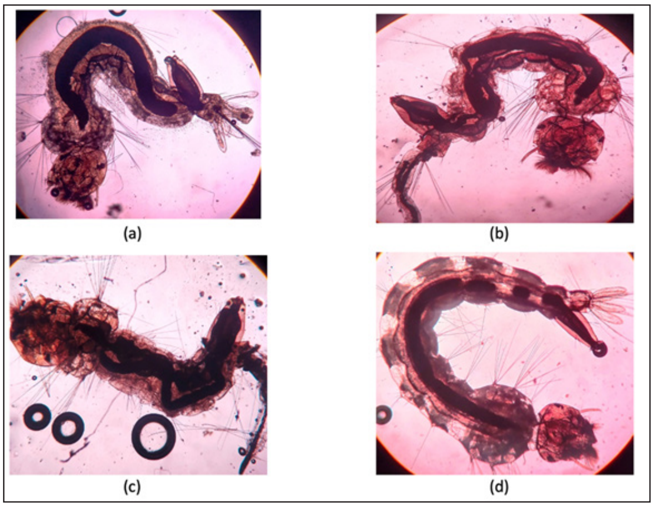

Figure 4.(a).500 µg/mL: Severe Digestive and Respiratory Damage, Leading to Rapid Mortality; (b).250 µg/mL: Necrosis and Structural Damage Causing Larval Death; (c).100 µg/mL: Dark Spots Indicating Necrosis, Leading to Cellular Damage and Larval Death; (d).Control: Intact, Healthy Larvae with Normal Function

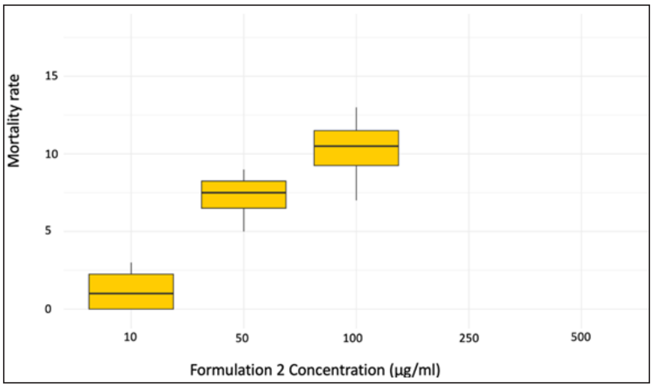

Figure 5.Box Plot showing the Dose-Dependent Increase in Mortality Rate with Increasing Concentrations (10, 50, 100, 250, and 500 µg/mL) of Formulation 2

Discussion

The present study demonstrated significant larvicidal activity of three homoeopathic formulations containing *Azadirachta indica, Areca catechu*, camphor, *Eucalyptus globulus, Ocimum sanctum*, and *Nigella sativa* against *Aedes aegypti* larvae, with Formulation 2 showing the highest efficacy (LC_{50}: 85.57 µg/mL, LC_{90}: 261.52 µg/mL). Comparatively, the emulsified *Azadirachta* oil indicated remarkable larvicidal effects against *Anopheles gambiae*, achieving 100% mortality at 500 ppm within three days and significant inhibition of pupae formation across all tested concentrations.[14] Additionally, *Metarhizium anisopliae* combined with *Azadirachta indica* extract showed enhanced larval mortality in *Anopheles albimanus*, with lethal concentrations (LC_{50}: 3.99 × 10^5 conidia/mL, 16 ppm) and

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a synergistic effect, reinforcing the potential of neem-based biocides in mosquito control.[15] Furthermore, ethanolic neem leaf extracts exhibited strong larvicidal properties against *Aedes aegypti*, with significant dose-dependent mortality (LC_{50}: 1.21 mL, LC_{90}: 9.03 mL), supporting neem's effectiveness across mosquito species.[16]

Essential oils from various *Cinnamomum* species, including *Cinnamomum camphora*, showed larvicidal activity against *Aedes aegypti* with LC50 values ranging from 17.4 µg/mL to higher concentrations, depending on the species and oil composition[17] *Eucalyptus globulus* essential oil, tested on major mosquito species, revealed potent larvicidal properties, with LC_{50} values ranging from 7.469 ppm to 30.198 ppm and sustained efficacy up to 48 hours post-treatment.[18] *Ocimum sanctum* demonstrated substantial larvicidal activity against *Aedes aegypti*, with granulated formulations showing up to 90.67% mortality (LC_{50}: 4405.803 ppm, LC_{90}: 6080.714 ppm).[19] *Areca catechu* extract also showed significant larvicidal effects against *Aedes* mosquitoes (LC_{50}: 621 mg/L for *Aedes aegypti* and 636 mg/L for *Aedes* albopictus), suggesting its potential for bio-larvicidal development.[9] Moreover, *Nigella sativa* essential oil and its selenium nanoparticles were effective against *Culex pipiens* and *Musca domestica larvae*, with significant larvicidal and antimicrobial properties.[20] These findings underscore the potential of homoeopathic and plant-based formulations as eco-friendly alternatives to synthetic larvicides, with Formulation 2 emerging as a particularly promising candidate for sustainable mosquito management.

Formulation 2's superior efficacy as a larvicidal agent can be attributed to the synergistic effects of its diverse components: *Azadirachta indica* (2.5 mL), *Areca catechu* (2.5 ml), camphor (1.25 mL), *Eucalyptus globulus* (1.25 mL), *Ocimum sanctum* (1.25 mL), and *Nigella sativa* (1.25 mL), each of which has demonstrated significant larvicidal properties in recent studies. *Azadirachta indica*, known for its potent bio-insecticidal properties, showed remarkable results in inhibiting larval development and pupae formation, while *Areca catechu's* extracts were highly effective against *Aedes larvae*.[21] The rapid and strong larvicidal activity of camphor essential oil, particularly against *Aedes aegypti*, and the sustained efficacy of *Eucalyptus globulus* oil against various mosquito species highlight their effectiveness.[22] *Ocimum sanctum's* notable larvicidal activity against *Aedes aegypti* further contributes to the formulation's strength. Moreover, the incorporation of *Nigella sativa*, which has shown significant larvicidal and antimicrobial effects, enhances the overall potency of the formulation. The combined action of these ingredients, each bringing unique and effective larvicidal mechanisms, results in the superior performance of Formulation 2.

Conclusion

This study reveals that homoeopathic formulations containing *Azadirachta indica, Areca catechu*, and other plant extracts exhibit significant larvicidal activity against *Aedes aegypti* larvae, with Formulation 2 being the most effective. These findings suggest that natural, plant-based compounds could serve as eco-friendly alternatives to synthetic insecticides, potentially reducing environmental impact and mitigating insecticide resistance. While the results are promising, further research is needed to confirm their practical application and scalability. Integrating these natural formulations into mosquito control strategies could contribute to a more sustainable approach to managing mosquito populations and reducing the spread of mosquito-borne diseases.

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Conflict of Interest: None

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