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# Bioefficacy of plant essential oils for the ovicidal, larvicidal and pupicidal activities against the Dengue vector *Ae. aegypti*

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**Abstract**
The present study was conducted to investigate the bioefficacy of three commercially obtained plant essential oils viz. Neem, Eucalyptus and Clove for the control of the medically important dengue vector *Ae. aegypti*. A standard solution was used to make 50 ml solutions ranging from 50 to 1000 ppm. Twenty five eggs as a batch were added to each essential oil solution and a mortality count was conducted 48hrs post treatment. Essential oils $LC_{50}$ and their confidence limits at 95% probability were calculated according to the methods of Reed-Muench and Pizzi, respectively to assess the toxicity levels. Maximum bioefficacy was observed in Neem and Clove oil, probably due to the action of minor compounds present in the plant oils. A 1000ppm concentration of all the oils was found to be 80-95% larvicidal. Overall, the three oils achieved over 80% larval and pupal mortality within 48hrs of treatment and there was no significant difference in lethal doses. Also, none of the oils were effective at 5% or 10% concentration at all developmental stages. The study highlighted that the ideal method of controlling mosquitoes is by targeting them at the stage at which they are vulnerable i.e., the oval/larval or pupal stage.

**Keywords:** Essential oils, *Ae. aegypti,* larvicidal activity, ovicidal activity, pupicidal activity

## Introduction

Mosquito-borne diseases cause significant morbidity, mortality and economic burden to humankind and animals. Comprising approximately 3500 species, mosquitoes are found beyond the tropical and subtropical regions of the world with which they are classically associated [1]. Mosquitoes are distributed globally and most female mosquitoes take blood meals from vertebrates to obtain the necessary nutrition to produce their eggs, injecting saliva (which may contain pathogens) into the host animal [2]. To find a host, these mosquitoes are attracted to chemical compounds emitted by mammals. These compounds include ammonia, carbon dioxide, lactic acid, and octenol. Scientists at the Agricultural Research Service have studied the specific chemical structure of octenol to better understand why this chemical attracts the mosquito to its host. They found the mosquito has a preference for "right-handed" (dextrorotatory) octenol molecules [3]. While many mosquitoes are distinctly selective feeders, restricted to one or a few closely related species, some feed in a less restrictive manner, varying between mammals, birds, and reptiles [4]

Mosquitoes breed in water, occasionally depositing eggs directly on water, but generally using a variety of moist surfaces, tree holes, and containers [3-5]. *Aedes aegypti* (L.) is generally known as a vector for an arbovirus responsible for dengue fever, which is endemic to Southeast Asia, the Pacific island area, Africa, and America [6]. Dengue fever has become an important public health problem as the number of reported cases continues to increase. The fatality rate is 1–5%, and less than 1% with adequate treatment; however those who develop significantly low blood pressure may have a fatality rate of up to 26%. [7]. For the decade of the 2000s, 12 countries in Southeast Asia were estimated to have about 3 million infections and 6,000 deaths annually. It is reported in at least 22 countries in Africa; but is likely present in all of them with 20% of the population at risk. This makes it one of the most common vector-borne diseases worldwide [2]

*Ae. aegypti* eggs can withstand very dry conditions (desiccation) and remain viable for many months in the absence of water [8]. The European strain can undergo a period of reduced development (diapause) during the winter months. Like most arboviruses, dengue virus is maintained in nature in cycles that involve preferred blood-sucking vectors and vertebrate hosts [7]. The viruses are maintained in the forests of Southeast Asia and Africa by transmission from female *Aedes* mosquitoes—of species other than *Ae. Aegypti* –to their offspring and to lower primates [5]. In towns and cities, the virus is primarily transmitted by the highly domesticated *Ae. Aegypti* whereas in rural settings the transmission happens through other species of *Aedes* such as *Ae. albopictus* as well. Both these species had expanding ranges in the second half of the 20th century. In all settings the infected lower primates or humans greatly increase the number of circulating dengue viruses, in a process called amplification [8]

Plants offer an alternative source of insect control agents because they contain a range of bioactive chemicals [6], many of which are selective and have little and no harmful effect on non-target organisms and the environment [7]. Interest in the development of botanical insecticides started in the early 1930s and was sustained through the late 1950s [7-9]. This effort was halted after that time because of the appearance, development, and use of synthetic insecticides. However, interest in botanical pesticides revived during recent years because of some of the drawbacks of synthetic insecticides, including lack of selectivity, impact on the environment, and the emergence and spread of pest resistance [10]. At the present time, isolation, identification, and development of natural products are the focus of numerous research programs around the globe. To date, about 2,000 plant species have been reported to possess pest control properties [9] and of these about 344 species of plants have been studied and found to contain bioactive materials showing some activity against mosquitoes [11]. In this context, essential oils have received much attention as potentially useful bioactive compound against insects showing a broad spectrum of activity against insects; low mammalian toxicity and degrading rapidly in the environment [13]

The use of chemical insecticides in controlling mosquitoes has been encountered by many problems due to detrimental hazards of organic synthetic pesticides to human, domestic animals, wildlife and the environment [12]. In addition to adverse environmental effects from conventional insecticides, most mosquitoes and other pest species has become physiologically resistant to many of these compounds [13]. These problems have highlighted the need for the development of new strategies for selective mosquito control. Phytochemicals are advantageous due to their eco-safety, target-specificity, non development of resistance, reduced number of applications, higher acceptability, and suitability for rural areas [14].

In view of the recently increased interest in developing plant origin insecticides as an alternative to chemical insecticide, this study was undertaken to assess the bioefficacy of three commercially obtained plant essential oils for larvicidal, pupicidal, ovicidal and growth inhibitory activities against the different developmental stages the of dengue vector *Aedes aegypti*. The present study also aimed to address main gaps in our knowledge on the properties of the commercially available oils and their possible threats to non target species.

## Materials and Methods

Egg strips/ovipads of *Aedes aegypti* were obtained from Entomological Research Institute (ERI), Loyola College, Chennai. The eggs were carefully separated to create several batches. Each batch contained 25 eggs and such batches were used for the experiment.

**Collection of essential plant oils:** Three commercially available essential oils namely Neem (Azadirachta *indica*) Eucalyptus (*Eucalypts*) and Clove (*Syzygium aromaticum*) were obtained from Thangam Departmental Stores, Triplicane, Chennai.

**Larvicidal bioassay:** The larvicidal bioassay was performed according to the method proposed by the WHO, with slight modifications [6]. The stock solution of the essential oils was prepared by emulsifying 1mL of the oil with three drops of acetone. The mixture was then made up with distilled water to make 1 litre. The working concentrations - 1000, 500, 250, 125 and 75 mg/L (ppm) were then prepared from the stock solution. Three batches of eggs were allowed to hatch and twenty larvae of *Ae. aegypti* from each batch were reared to reach 2$^{nd}$, 3 $^{rd}$ and 4$^{th}$ instar larval stages. 1-2 drops of milk were diluted in 2ml of tap water and left out in open. This allowed the growth of protozoa in it. This solution was used in the form of feed for the growing larvae. Further, twenty larvae of each instar stage were exposed into 3 different 500 mL Styrofoam cups containing 50 mL of each concentration. The control experiment was set up with twenty larvae of each stage, containing tap water and acetone. The bioassay was replicated five times for each larval stage. Larval mortality was recorded after 48 hrs.

**Pupicidal bioassay:** Procedure similar to larvicidal bioassay was followed with different concentrations – 250, 200 and 100 ppm. The 4$^{th}$ instars from one batch of eggs were allowed to reach pupal stage and 20 pupae were exposed into 500 mL Styrofoam cups containing 50 mL of each oil concentration. The control experiment was set up with twenty larvae containing tap water and acetone. The bioassay was replicated five times and pupal mortality was recorded after 48 hrs.

**Ovicidal bioassay:** For ovicidal bioassay, slightly modified method of Su and Mulla [15] was performed. The eggs/egg rafts of *Aedes aegypti* were obtained from ERI, Loyola College. The different oils were diluted to achieve various concentrations - 50, 100 and 150 ppm. Eggs (20 in number) were exposed to each concentration of all the three oils. After treatment, the eggs from each concentration were individually transferred to cups containing dechlorinated water for hatching assessment. Each experiment was replicated five times along with appropriate control. The egg hatching was assessed 48 hr post treatment.

All the experiments were conducted in open on a terrace to acclimatize field conditions instead of under laboratory conditions. The containers were interspersed over a table and left under a natural light-dark regime but protected from direct sun. The mosquito larval and pupicidal mortality was checked at 6 and 12 hrs intervals and complete dead and moribund larvae/pupae were recorded after a total exposure period of 48 hours. Mosquito larva/pupa was considered dead when no movement was observed after repeated prodding with a capillary tube or a needle. The larvae were considered moribund if they only moved a little without showing any kind of swimming or vigorous movement. Similarly a pupa was said to be moribund when it failed to show its characteristic dicing movements in water. The moribund larvae/pupae could never revive and thus were also counted as dead.

**Statistical analysis:** The insect mortality percentage was calculated using the Abbotts [16] and $LC_{50}$ value was calculated after 48 hr by Probit analysis [17] using the PROBIT software Statistical Package and according to the methods of Reed-Muench and Pizzi, $LC_{50}$ values represent the concentration at which 50% of the larvae/pupae were immobilized. Means were compared with Duncan's Multiple Range test [18]. Assumed 95% of upper confidence limit (UCL), lower confidence limit (LCL) and Chi-square were also calculated. Results with $p<0.05$ were considered to be statistically significant.

## Results

The present study reflected spectrum of activity with different essential plant oils tested against the *Ae. aegypti* mosquito larvae, pupae and eggs. The results of relative toxicity after 48 hours of treatment and the 95% confidence limits $LC_{50}$ (LCL-UCL) values are presented and in Table 1-4. When the oils were compared for their toxicity, a significant difference ($p<0.05$) in the toxicity was found for all essential oils (p=0.0001). The chi-square values in the bioassays indicated probably the heterogeneity of the test population.

**Table 1:** Relative Toxicity of the Essential Oils against *Ae. Aegypti* – 2nd Instar Larva, 48hrs post treatment

| Essential Oils | $LC_{50}$ (Ppm) | 95% Confidence Limits (Ppm) | Fit of Probit Line | | |
|---|---|---|---|---|---|
| | | LCL-UCL | $\chi^2$ | df | P |
| Neem | 1.86 | 1.532-2.206 | 15.489 | 3 | $P<0.0001$ |
| Eucalyptus | 1.50 | 1.193-1.811 | 1.969 | 3 | $P<0.0001$ |
| Clove | 1.85 | 1.534-2.179 | 4.054 | 3 | $P<0.0001$ |

**Table 2:** Relative Toxicity of the Essential Oils against *Ae. Aegypti* – 3rd Instar Larva after 48hrs of Treatment

| Essential Oils | $LC_{50}$ (ppm) | 95% Confidence Limits (ppm) | Fit of Probit Line | | |
|---|---|---|---|---|---|
| | | LCL-UCL | $\chi^2$ | df | P |
| Neem | 2.04 | 1.71-2.38 | 1.90 | 3 | $P<0.0001$ |
| Eucalyptus | 1.78 | 1.46-2.09 | 0.82 | 3 | $P<0.0001$ |
| Clove | 1.87 | 1.54-2.19 | 7.41 | 3 | $P<0.0001$ |

**Table 3:** Relative Toxicity of Essential Oils against *Ae. Aegypti* – 4th Instar Larva After 48hrs of Treatment

| Essential Oils | $LC_{50}$ (ppm) | 95% Confidence Limits (ppm) | Fit of Probit Line | | |
|---|---|---|---|---|---|
| | | LCL-UCL | $\chi^2$ | df | P |
| Neem | 1.89 | 1.57-2.22 | 4.08 | 3 | $p<0.0001$ |
| Eucalyptus | 1.73 | 1.42-2.05 | 10.71 | 3 | $p<0.0001$ |
| Clove | 2.06 | 1.71-2.40 | 6.27 | 3 | $p<0.0001$ |

**Table 4:** Relative Toxicity of Essential Oils against *Ae. Aegypti* – Pupa after 48hrs of Treatment

| Essential Oils | $LC_{50}$ (ppm) | 95% Confidence Limits (ppm) | Fit of Probit Line | | |
|---|---|---|---|---|---|
| | | LCL-UCL | $\chi^2$ | df | P |
| Neem | 3.17 | 2.25-4.10 | 6.18 | 1 | $P<0.0001$ |
| Eucalyptus | 2.90 | 1.99-3.81 | 9.35 | 1 | $P<0.0001$ |
| Clove | 2.38 | 1.51-3.25 | 10.74 | 1 | $P<0.0001$ |

Chi-squared test, comparing experimental and control group, with a significance level established at $P<0.05$ and chi-square value were significant at $p<0.0001$ level.

Among the three plant essential oils, the maximum bioefficacy was observed in Eucalyptus and Clove oil against *Ae. aegypti* at second and third instar stage. Neem oil showed optimum mortality at fourth instar stage, probably because of the Azadirachtin, a predominant insecticidal active ingredient present in it. As expected, the percent larval and pupal mortality increased with increase in concentration of the oil. At high concentrations (1000 ppm), the mortality was not significantly different. A concentration of 1000 ppm of all essential oils was found to be 80-95% larvicidal. However, at a concentration of 250 ppm, Eucalyptus and Clove oil was able to kill more than 50% of third late instars, as compared to Neem oil.

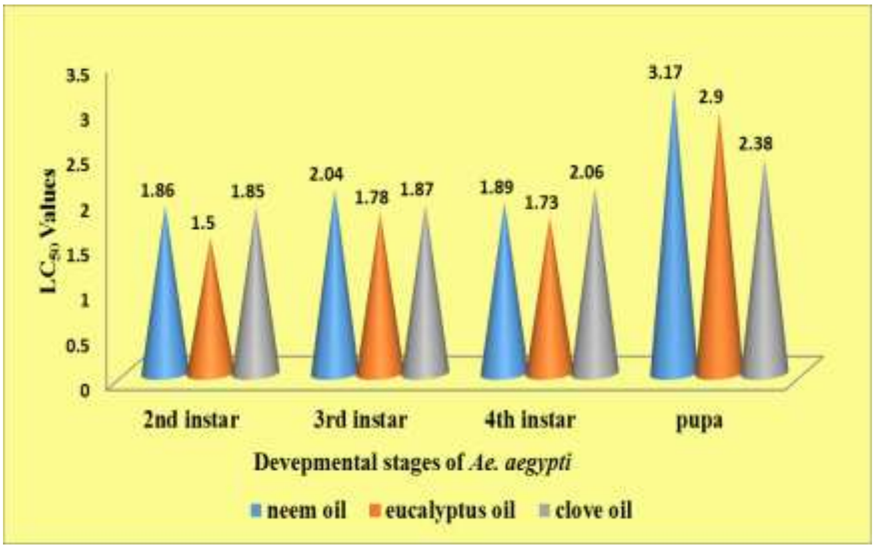


**Fig 1:** Relative Toxicity ($Lc_{50}$ Values) of Essential Oils against *Ae. Aegypti* developmental Stages - 48 Hrs Post Treatment

## Discussion

Many approaches have been developed to control the mosquito menace. One such approach to prevent mosquito-borne disease is to kill at its oval/larval or at the pupal stage. Many studies made use of plant extracts for mosquito control. Insecticides of botanical origin may serve as suitable alternative biocontrol techniques in the future. Larval control can be the effective and an appropriate way to control the mosquitoes in man-made breeding habitats [20]. Essential oils are composition of volatile components having minor constituents contain pleasant fragrance which are responsible for mosquito repellency and inhibit the orientation of blood sucking insects [13]

All essential oils used in this study reduced the egg hatching in mosquito eggs. However clove oil was most toxic at this life stage and also showed different kinds of activity. It is evident from the present data that the exposure of mosquito eggs to the essential plant oils elicits egg mortality. At the highest concentration (150ppm), egg hatching was reduced to 90%. It can be concluded that dose of 150 ppm could be used for achieving the desired level of control of this mosquito by ovicidal action. Differences in susceptibility to ovicides are due to differential rates of uptake, penetration through the chorine, and conversion to active inhibitor, detoxification and failure of the toxicant to reach the target. Efficacy to act on the embryo inside the egg shell depends on efficient penetration of the insecticides, which in turn is influenced by the exposure period [21].

In the present study clove and neem oil showed prominent ovicidal activity, this might be due to the volatile compounds present in the oils. It is reported that the neem products containing azadirachtin showed oviposition deterrent activity against *C. tarsalis* and *C. quinquefasciatus* [14]. The present investigation elicited optimum mortality rate of neem oil against the fourth instar larva. This was probably due to the anti-feedent, growth disruption, sterility and larvicidal activities which becomes active towards the 4$^{th}$ larval stage. Eucalyptus oil showed maximum larvicdal properties towards the initial instar stages

Similar reports on ovicidal activity of crude extract of *Ervatamia coronaria* exerted 100% mortality at 250, 200 and 150 ppm against *C. quinquefasciatus*, *Ae. aegypti* and *An. stephensi*, respectively [22]. Essential oils contain more than 20 to 80 minor and major highly volatile chemical constituents of which the major components showed effective repellent against *Ae. aegypti* mosquito[14]. Plant oil which contains limonene, linalool, citronellol showed effective repellent activity against different mosquito species [12, 16, 17]. Due to high volatile property, essential oils exhibited effective but short duration of protection against mosquitoes.

The composition and yield of the essential oils of each plant species may vary according to the genetic, environmental and physiopathological traits influencing the plant oil metabolism. The total quantity, as well as the relative proportions of secondary metabolites, in the oils can be regulated by biotic and abiotic factors such as the season, area and time of collection of samples [23] This demonstrates the relevance of chemical and biochemical studies. A major drawback in the synthetic insecticide is that they are non-selective and could be harmful to other beneficial organisms, animals and humans. Further they are not easily biodegradable. This study showed that no detrimental effect was observed on the neighbouring flora and fauna in field study of these oils. The non target species did not receive any harm at low to moderate concentrations of the essential oils. These plant products are ecofriendly and do not leave a residues in the environment.

The use of essential oils as insecticides is a highly promising initiative to develop and preserve the regions of high mosquito infestations. They are known to be complex mixtures of secondary metabolites that can be obtained at low costs using renewable technology, often displaying higher activities than the individual isolated compounds [24]. Focal treatment, which involves the eradication of immature forms of *Ae. Aegypti* by using larvicides (such as the organophosphate temefos) in the specific places where larvae can be found is a very efficient approach [25].

Some previous studies have been performed with the aim to evaluate only the larvicidal activity of essential oils, in general. This study focuses on the sustainable use of the essential oils and aims to produce a plant based mosquitocide to combat the spread of the dengue and chikungunya virus.

## Conclusion

The findings of this study demonstrated the mosquitocidal potentials of the 3 chosen plant essential oils against the dengue vector *Ae. aegypti*. Also the current study clearly indicated that the ovicidal activity of the plant oils against the egg rafts of *Ae. aegypti* may depend upon three key factors viz., dose of the plant oils, age of egg rafts and period of exposure. These locally sourced essential oils can therefore be incorporated in the mosquito control measures, mostly in local areas where access to health facilities is extremely difficult

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