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Eucalyptus oil nanoemulsion as potent ovicidal and pupicidal agent against *Aedes aegypti Linnaeus*

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ABSTRACT

Aedes aegypti is responsible for the spread of various public health diseases globally. Synthetic insecticides are commonly used to control Ae. aegypti, however regular and over use of these chemicals has resulted in mosquito resistance, human health issues and environment contamination. Essential oils (EOs) derived from plants are natural alternatives to replace the commercial synthetic insecticides. This study was conducted with the objective to extract oil from eucalyptus leaves, perform its chemical characterization, prepare its nanoemulsion and then study its ovicidal and pupicidal activity. GCMS analysis indicated Cineole (41.77%) and α-Terpilene (13.68%) as the major components of extracted eucalyptus oil. Out of the tested concentrations, 70 ppm of eucalyptus oil nanoemulsion exhibited 100% inhibition of egg hatching and exposure of this concentration also resulted in maximum per cent pupicidal mortality (77.33±6.11 in male and 70.67±8.33 in female). Efficient ovicidal and pupicidal properties of eucalyptus oil nanoemulsion indicated its potential to be used as bio-insecticide for the control of Ae. aegypti before their emergence.

Keywords- Aedes aegypti, eucalyptus oil, mosquito control, ovicidal potential, pupicidal efficacy

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INTRODUCTION

Aedes aegypti is an important mosquito species known to act as vector for the spreading of various deadly diseases such as dengue, chikungunya and zika fever across the globe. It is a diurnal mosquito species which is well adapted for urban and domestic environments [1]. Acceleration in rates of urbanization in tropical areas, unavailability of basic infrastructure and limited or non-existent sanitation, along with suitable climatic conditions for the mosquito growth, have collectively resulted in rise in cases of patients suffering from mosquito-borne diseases transmitted by Ae. aegypti [2]. Although, Food and Drug Administration (FDA) has approved a dengue vaccine (Dengvaxia) in 2019 but, its efficiency is limited to people who have been earlier infected by dengue and not as disease prevention for the entire population [3 and 4]. Therefore, the management of Ae. aegypti population remains the sole method for dengue prevention. Control of mosquito population usually relies on the application of insecticides such as the larvicide pyriproxyfen (Juvenil Hormone Analog–JHA) and the adulticides malathion (organophosphate) and Cielo® [5]. Although this strategy has contributed in saving thousands of lives of people suffering from mosquito-borne diseases, the rapid development of mosquito resistance and various ecological and public health risks associated with these chemicals has created an immediate requirement for alternative vector control products which are safe and eco-friendly [6]. Considering this fact, natural products of plant origin with insecticidal properties have been tried in the recent past for the control of various insect pests and vectors. The larvicidal efficacy of different indigenous plants has also been studied in many parts of India along with the repellent and antijuvenile hormonal activities [7]. Eucalyptus is one among these most significant plants and the material extracted from its leaves have shown significant larvicidal [8]. and repellent actions against mosquito vectors [9], while at the same time being very eco-friendly. Presently, interest has also been devoted to the development of nano-sized products for insecticidal activity as downsizing of natural oils to form nanoemulsions could be effective as insecticidal agents [10].

The main advantage of nanoemulsions is their ability to make highly homogenous dispersion from the immiscible substance in aqueous media including herbal oils [11 and 12]. Nanoemulsions may serve better for this purpose by reducing the quantity, increasing efficiency, ensuring biosafety and decreasing the vector population in turn declining the magnitude of epidemiology [13]. Larvicidal potential of eucalyptus oil nanoemulsion against Ae. aegypti has been already determined in our previous study [14], in continuation to that, the present study was planned to explore the its ovicidal and pupicidal effect against Ae. aegypti for its future usage as bio-insecticide.

MATERIALS AND METHODS

Collection of eucalyptus leaves and preparation of oil

Fresh leaves were collected from eucalyptus trees present in and around Punjab Agricultural University, Ludhiana, Punjab (India) during February to April, 2021. Infected leaves were discarded and fresh green leaves were cleaned using tap water to remove the dust and then shade dried by spreading on filter papers to wither for about 2-3 days at room temperature. After slight withering, their dry weight was noted and 250 g withered leaves were taken for oil extraction using Clevenger apparatus by following hydro-distillation technique. The flask with 500 ml capacity was filled up-to half with water, having 250g withered leaves and allowed to heat over a heating mantle for about 4-5 hours. The temperature was initially set at 100°C for 30 minutes till boiling was obtained and then a constant temperature 60°C was maintained. The extracted volume (ml) of oil was carefully collected in a clean glass vial and stored in the refrigerator at 4°C for its use and per cent oil yield was calculated using the following formula:

$$\text{Oil Yield (\%)} = \frac{\text{Volume of oil collected (ml)}}{\text{Weight of withered eucalyptus leaves taken initially (g)}} \times 100$$

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of eucalyptus oil

The composition of extracted eucalyptus oil was determined by GC-MS analysis at the Central University of Punjab, Bathinda (India) by GC program GCMS-QP2010 Ultra, using helium as carrier, gas flowing at a rate of 1ml/min with split ratio 10:0, injection temperature 250°C and oven temperature programming from 40 to 250°C. GC was equipped with a capillary column Rtx-5MS (30 mtr) and for this,1 µl of the extracted eucalyptus oil diluted in 1 ml methanol was injected in the GC-MS system.

Preparation of eucalyptus oil nanoemulsion

The nanoemulsion was prepared using freshly extracted eucalyptus oil, non-ionic surfactant (Tween 20) and distilled water by following the method already standardized in our laboratory (14). Firstly, coarse emulsion was prepared by adding water to organic phase having oil and surfactant in 1:2 (v/v) using a magnetic stirrer, which was later subjected to ultrasonication using 40 KHz Sonicator.

Testing the ovicidal efficacy of eucalyptus oil nanoemulsion against Ae. aegypti

Ovicidal potential of eucalyptus oil nanoemulsion was studied following the standard method(15). Egg strips of Ae. aegypti (containing 20 eggs each) procured from Department of Zoology, Acharya Narendra Dev College, New Delhi (India) were kept in plastic troughs having water containing an effective larvicidal concentration of eucalyptus oil nanoemulsion i.e. 70 ppm as determined by our previous study [14] and two sub effective concentrations (60 and 50 ppm). A control set (having de-chlorinated water) and vehicle-control set (having Tween 20 and de-chlorinated water) with egg strip (20 eggs) were also run simultaneously for comparison. De-chlorinated water was prepared by keeping the tap water in open large containers overnight, as chlorine in tap water (if present) gets evaporated. Three replicates of all the experimental sets viz. treated, control and vehicle-control were run for proper statistical analysis.

Hatching success in each replicate of the tested concentration was monitored up to 48 hours and hatched and unhatched eggs were counted under a microscope. The average hatching data of all the replicates was calculated to find the ovicidal potential. The ovicidal activity of each concentration was expressed in per cent by using the following formula:

$$\text{Ovicidal activity (\%)} = \frac{\text{Number of unhatched eggs after treatment}}{\text{Total number of eggs}} \times 100$$

Testing the pupicidal efficacy of eucalyptus oil nanoemulsion against Ae. aegypti

a) Maintaince of Ae. aegypti larvae and pupae in laboratory:

Pupae required to study pupicidal efficacy were arranged by collecting Ae. aegypti larvae from temporary water collections and then rearing them under laboratory conditions. For a collection of Ae. aegypti larvae, water samples were taken from various small fresh water collections such as desert coolers, roadside ditches, plate under pots, plastic containers and earthen pots lying in peri-domestic areas of urban regions of Ludhiana district of Punjab state (India). The water samples were collected in plastic bottles (300 ml capacity) and brought in the laboratory. From the collected water samples, Ae. aegypti larvae were identified and separated from other types of mosquito larvae (if present), on the basis of their morphological characters and maturity (larval instar) by following the standard keys [16 and 17]. Identified larvae were separated and reared in mosquito-rearing trays under laboratory conditions in 1L of de-chlorinated water. Water in rearing trays was refreshed every day by removing a little quantity of water and replacing it with fresh water so as to prevent scum formation on the water's surface. Growing mosquito larvae were fed on a mixture of dog biscuits and yeast ground in ratio 3:1 (2mg/100ml) [18]. After last molting of IVth instar larvae, pupae were picked with the aid of a pipette and placed in glass beakers and kept inside rearing cages.

b) Pupicidal assay of eucalyptus oil nanoemulsion against Ae. aegypti:

Newly transformed pupae were taken and male and female pupae were segregated based on sexual dimorphism by studying the morphological features under a microscope [19]. To study the pupicidal activity, 20 male and 20 female pupae were exposed separately to effective larvicidal concentration (70 ppm) as determined by previously conducted research study in our laboratory (14). and two sub effective concentrations (60 and 50 ppm) of eucalyptus oil nanoemulsion. A control set (having de-chlorinated water) and vehicle-control set (having Tween 20 and de-chlorinated water) containing 20 pupae of respective sex were also run simultaneously for comparison. All sets (treatment, control and vehicle-control) were run in triplicate. Pupae were considered dead when failed to respond to mechanical stimulation or to metamorphose into the adult stage. Number of dead pupae were counted after 48 hours of exposure in different sets and per cent pupal mortality was calculated by using the following formula:

$$\text{Pupal mortality (\%)} = \frac{\text{Number of dead pupae recorded}}{\text{Number of pupae taken intially}} \times 100$$

Statistical analysis

Data were statistically analyzed by comparing per cent unhatched eggs and pupal mortality in eucalyptus oil nanoemulsion treatment sets with control and vehicle-control sets by using ANOVA (Duncan multiple range test) at a 5% level of significance.

RESULTS

General characteristics and GC-MS analysis of eucalyptus oil nanoemulsion

Prepared eucalyptus oil was transparent, light yellow in color, with camphor-like odour and on an average 0.89±0.10% (v/w) oil yield was obtained. The chromatogram obtained by GC-MS analysis of eucalyptus oil showed 111 peaks indicating the presence of 111 compounds in it (Figure 1) and out of these 10 compounds were considered as the major one because these were having an area of more than 1%. Eucalyptol/ cineole was the most abundant compound with 41.77% area followed by α-Terpilene (13.68%) > β-Pinene (6.05%) > D-Limonene (4.41%)> Benzenamine (3.42%) > α-Terpineol (2.75%) > Octadiene (2.37%) > Carvone (1.44%)> Octanoic acid (1.14%) > Terpinen-4-ol (1.13%) as given in Table 1.

Table 1: Major compounds reported in extracted eucalyptus oil analysed by GC-MS

S. No.	Peak No.	Retention time (min)	Area (%)	Molecular formula	Name of compound
1.	10	14.717	41.77	C10H18O	Cineole
2.	9	15.493	13.68	C10H16	α-Terpinene
3.	3	10.759	6.05	C10H16	β-Pinene
4.	8	14.664	4.41	C10H16	D-Limonene
5.	7	14.476	3.42	C15H5NH2	Benzenamine
6.	32	21.460	2.75	C10H18O	α-Terpineol
7.	58	27.093	2.37	C8H14	Octadiene
8.	40	23.212	1.44	C10H14O	Carvone
9.	75	32.006	1.14	C8H16O	Octanoic acid
10.	29	20.811	1.13	C10H18O	Terpinen-4-ol

Ovicidal potential of eucalyptus oil nanoemulsion against Ae. aegypti

Exposure of eggs to the tested concentrations of eucalyptus oil nanoemulsion @50, 60 and 70 ppm resulted in reducing the egg hatching to variable extent (Table 2). At the tested lowest concentration (50 ppm), only 28.33±10.41 per cent egg hatching (larval emergence) was recorded, in median tested concentration (60 ppm) increase in ovicidal rate was recorded, as it resulted in 58.33±7.64 per cent killing of ova. While, 70 ppm of eucalyptus oil nanoemulsion was found to inhibit 100% egg hatching, as no larval emergence was observed from the eggs after their exposure to 70 ppm for 48 hours. However, in control and vehicle-control sets 100% egg hatching was recorded (Table 2 and Figure 2).

Table 2: Effect of different concentrations of eucalyptus oil nanoemulsion on hatching of Aedes aegypti eggs

Concentration of eucalyptus oil nanoemulsion (ppm)	Average number of hatched eggs (n=20)	Average number of unhatched eggs (n=20)	Per cent egg hatching/larval emergence (Mean±S.D)	Per cent ovicidal activity (Mean±S.D)
50	14.67±2.52	4.67±1.53	71.67±10.41	28.33 ± 10.41^{b}
60	8.33±1.53	11.67±1.53	41.67±7.64	58.33 ± 7.64^{c}
70	0.00±0.00	20.00±0.00	0.00±0.00	100.00 ± 0.00^{d}
Control	20.00±0.00	0.00±0.00	100.00±0.00	0.00 ± 0.00^{a}
Vehicle - control	20.00±0.00	0.00±0.00	100.00±0.00	0.00 ± 0.00^{a}

- n= Total number of eggs taken for each experiment in triplicate
- Figures followed with different superscripts indicate significant difference ($p<0.05$) with respect to Control and Vehicle-control sets by using Duncan multiple range test

Identification of sex of Ae. aegypti pupae

Sex of the laboratory-reared Ae. aegypti pupae was determined based on sexual dimorphism by observing them under a microscope [19]. Two morphological features used for identification were; pupal body size and shape of the genital lobes (present at the end of the pupal abdominal segments just below the paddles). Body size of male pupae was comparatively smaller than female pupae. The observation regarding the shape of genital lobes indicated that in males genital lobes were slightly triangular where as lobes were completely oval in female pupae. Moreover, space between the left and right lobes also mark the identification of sex, as a gap was found between two lobes in males, while the lobes were observed to be overlapping in the case of female pupae (Table 3 and Figure 3).

Table 3: Morphological identification of sex of Aedes aegypti pupae

Morphological character	Male	Female
1. Pupal body size	Small	Large
2. Shape of the genital lobes	Slightly triangular	Completely oval
3. Space between left and right lobes	Visible gap between left and right lobes	Overlapping left and right lobes

Pupicidal potential of eucalyptus oil nanoemulsion against Ae. aegypti

When identified male and female pupae (n= 20 from each sex, in triplicate) were exposed to the selected concentrations of eucalyptus oil nanoemulsion i.e 50, 60 and 70 ppm, it resulted in 52.00±4.00, 66.67±6.11 and 77.33±6.11 per cent mortality in males, and 45.33±6.11, 54.67±2.31 and 70.67±8.33 per cent mortality in females, respectively. Toxicity was found to increase statistically with increase in the concentration of the oil. However, per cent pupal mortality showed non-significant variation concerning the gender of the pupae which indicated that eucalyptus oil treatment has no pupal sex-specific effect. No pupal mortality was observed in control and vehicle-control sets (Table 4).

Table 3: Effect of different concentrations of eucalyptus oil nanoemulsion on mortality of Aedes aegypti pupae

Concentration (ppm)	Per cent pupal mortality (Mean±S.D)	
	Male (n=20)	Female (n=20)
50	52.00 ± 4.00^{b}	45.67 ± 6.11^{b}
60	66.67 ± 6.11^{c}	54.67 ± 2.31^{c}
70	77.33 ± 6.11^{d}	70.67 ± 8.33^{d}
Control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Vehicle - control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

- n= Total number of pupae taken for each sex separately in triplicate
- Figures followed with different superscripts indicate significant difference ($p<0.05$) with respect to Control and Vehicle-control sets by using Duncan multiple range test

Fig. 1

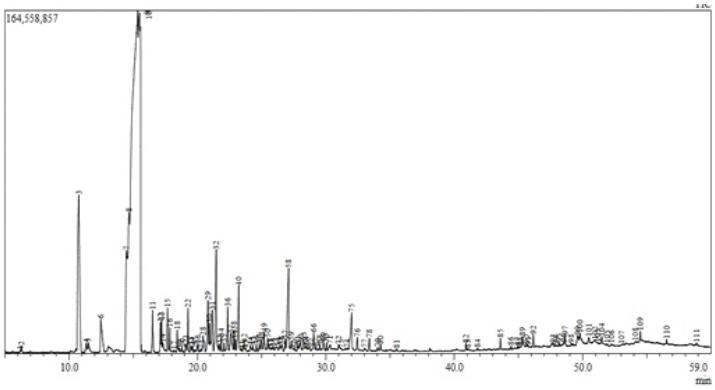
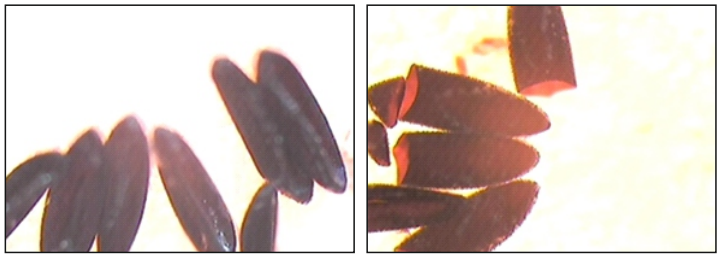
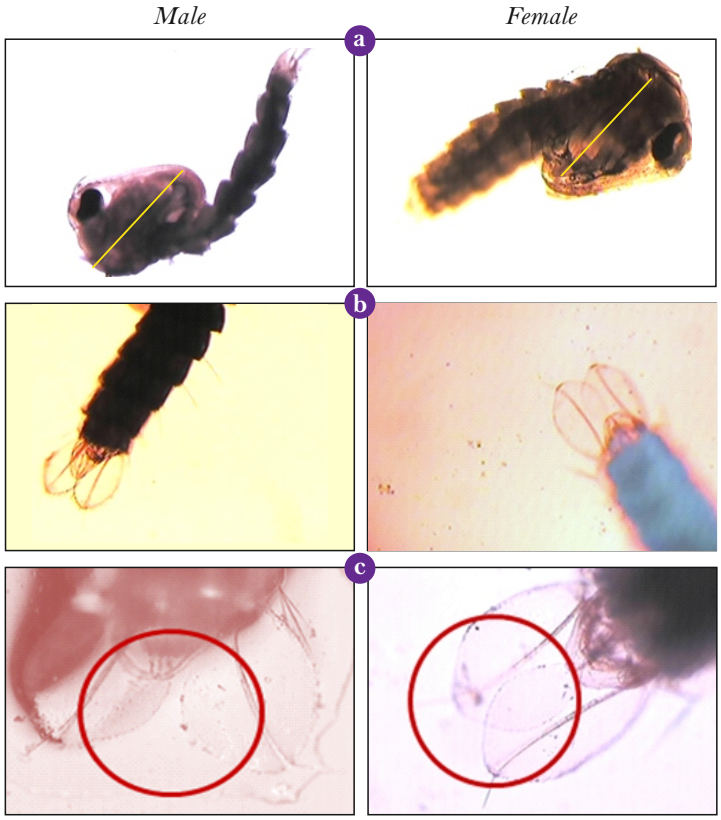


Fig. 2



No egg hatching *Egg Hatching*

Fig. 3



Legends to Figures

Fig. 1: GC-MS Chromatogram of extracted eucalyptus oil

Fig. 2: Unhatched and hatched eggs of Aedes aegypti observed under microscope (10xX4x)

a) No egg hatching due to treatment with 70 ppm of eucalyptus oil nanoemulsion

b) Egg hatching in control set

Fig. 3: Morpholgical identification features of male and female pupae of Aedes aegypti under microscope

a) Body size of the male and female pupae at 10xX4x

b) Difference in genital lobes of male and female pupae at 10xX4x

c) Difference in genital lobes of male and female pupae at 10xX10x

DISCUSSION

Due to the various harmful effects of synthetic pesticides along with the increasing problem of resistance among mosquitoes, developing plant-based products which are environmental friendly is an urgent requirement. Eucalyptus oil is known for various insecticidal properties [20 and 21]. Downsizing the particle size to the nanoscale improves the efficiacy and stability of essential oils. The present study highlight the importance of eucalyptus oil nanoemulsion as an effective ovicidal and pupicidal agent against Ae. aegypti. The larvicidal potential of eucalyptus oil nanoemulsion has already been evaluated in our laboratory [14] and further study has also revealed its effect in terms of significantly delaying the development of Ae. aegypti [22]. In the present study, major compounds observed in extracted eucalyptus leaf oil by GC-MS were Cineole and α-Terpilene and Piene. Similar composition of eucalyptus leaf extracts with predominant compounds as 1,8-cineole, γ-Terpinene, α- Pinene and Globulol, which are responsible for larvicidal and repellent activities against mosquitoes has also been reported in literature [23]. Exposure of fresh viable eggs to 70 ppm of eucalyptus oil nanoemulsion resulted in 100% inhibition of egg hatching as given in table 2, this may be attributed due to the various toxic components present in this oil. The mechanism of general ovicidal action of essential oils is due to the lipophilic nature of these oils which interacts with cellular lipid membranes of eggs and resulting in destabilization of their integrity [24]. Moreover, it is also proposed that spontaneous formation of molecular complexes promotes an increase in cellular permeability that enhances the circulation of ions and other macromolecules causing functional failure, thus leading to mortality at different immature stages of the growing embryo [25]. Similarly, exposing pupae to toxic doses of the eucalyptus oil resulted in their significant mortality due to its chemical constituents as observed during the present study.

Secondary metabolites of many plant species show effect on growth and development in various life stages of mosquitoes like inhibiting molting, morphological abnormalities and mortality by interfering the molting to next the stage eventually leading to the death of pupae [26]. Plant extracts also show a juvenile hormone analogue action, resulting in certain morpho-genetic abnormalities in treated larvae like larval-pupal intermediates, decolorization and extension of pupal stage and incomplete emerged adult [27].

Pupal abnormalities have been reported when Ae. albopictus pupae were exposed to LC50 dose of citrus extract [28]. Larval treatment with E. citriodora also resulted in a significant decrease in body weight of the emerged pupae along morphological deformities [29]. Comparative study regarding pupicidal effect of neem and eucalyptus oil against Ae. aegypti revealed eucalyptus oil as a more efficient pupicidal agent [30]. Treatment with eucalyptus oil nanoemulsion resulted in a significant delay in the development of pupal and adult stages of Ae. aegypti [22]. Ovicidal and pupicidal efficiency of eucalyptus oil nanoemulsion reported during the present study revealed the significance of eucalyptus oil for its usage as a bio-insecticide.

CONCLUSION

The present study concludes that eucalyptus oil nanoemulsion can be used as an effective plant-based insecticide that target different immature stages of Ae. aegypti inhabiting aquatic habitats, as it showed markedly significant ovicidal and pupicidal efficacy and is already known to have effective larvicidal potential. So, eucalyptus oil-based formulations could be developed and used as an alternative to synthetic insecticides for the management of Ae. aegypti population to manage the spreading of dengue in the future.

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