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NANOEMULSIFIED *EUCALYPTUS GLOBULUS* ESSENTIAL OIL AGAINST MOSQUITO *AEDES AEGYPTI* (L.)

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ABSTRACT

In the present study, nanoemulsified essential oil of *Eucalyptus globulus* was analysed for its chemical composition and larvicidal activity against mosquito *Aedes aegypti.* GC-MS analysis revealed presence of total 22 compounds, of which the major ones were 1, 8-cineole (42.16%) and P-cymene (14.81%). Non polar fractions of oil were sonicated by mixing it in water with Tween 20 in different ratios, and 1:2 ratio was found to be the most stable. This was characterized using TEM and found to have spherical droplets of 20-40 nm. Nanoemulsified oil was found to be effective at 70 ppm with LC_{50} and LC_{90} of 51.95 and 64.21 ppm, respectively. SEM studies showed significant shrinkage and disruption of various larval body parts post treatment.

Key words: *Aedes aegypti*, *Eucalyptus globulus*, 1, 8-cineole, larvicidal assay, nanoemulsion, sonication, GC-MS, LC_{50}, LC_{90}, SEM, TEM, spherical droplets, size

Aedes aegypti mosquito vector is well known for transmitting severe viral diseases in humans (Leta et al., 2018; Shanmugaraj et al., 2019) like dengue fever, chikungunya fever, dengue hemorrhagic fever (Yang et al., 2009). Prior to adult stage, its larvae have been found to develop well in aquatic bodies such as grassy ditches, the edges of streams, rivers and small temporary rain pools (CDC, 2004). Larval mosquitoes are controlled by biocontrol agents like aquatic predators, including young odoantes' instars, tadpoles, fishes, copepods and water bugs (Bowatte et al., 2013; Kalimuthu et al., 2014). Regulation of the mosquito population in the early aquatic stages with botanicals is advantageous (Djenontin et al., 2014) over insecticides (Tikar et al., 2008; Sarwar et al., 2009). In recent times, the ability of botanical products against mosquito vectors had been investigated (Azizullah et al., 2014; Benelli et al., 2015; Pavela, 2015). Essential oils are hydrophobic liquids containing volatile and aromatic compounds (Weinzieri, 2000), and these are easily extractable, ecofriendly and biodegradable. Essential oils with lethal activity against mosquitoes are known with plant families viz., Lamiaceae, Cupressaceae, Rutaceae, Apiaceae and Myrtaceae. In particular, the oil extract of eucalyptus leaves possesses an extensive variety of antimicrobial, fungicidal, insecticidal/insect repellent, acaricidal, larvicidal, herbicidal and nematocidal activities (Zhou et al., 2016). The leaf essential oil from *Eucalyptus* spp. had shown excellent inhibitory effects against both *Ae. aegypti* and *Ae. albopictus* larvae (Cheng et al., 2009).

The main drawback of using essential oils as larvicidal agent in the natural habitat of larvae, which are water bodies is immiscibility of water and oil. This problem can be overcome by downsizing the oils using nanotechnology. The use of nanotechnology in insect pest control is being emphasized (Prabhakar et al., 2017), and against mosquitoes by formation of nanoparticles from plant extracts (Priya and Santhi, 2014). This study proposes an effective strategy for *Ae. aegypti* larval control using formulated aqueous eucalyptus oil nanoemulsion by demonstrating the chemical composition of *E. globulus* and documenting the morphological changes in larvae by SEM imaging.

MATERIALS AND METHODS

The pure form of eucalyptus oil (*Eucalyptus globulus* Labillardiere) was obtained from Loba Chemie Private Limited, Mumbai, India. Chemical composition of *E. globulus* oil was determined by Gas Chromatography-Mass Spectroscopy (GC-MS) as per standard procedure (Joshi et al., 2016). Thin layer chromatography of eucalyptus oil was performed (Rossini et al., 1995). Eucalyptus oil was transferred into 250 ml separatory funnel for fractionation of polar and non polar components. The oil was partitioned thrice using 50 ml of hexane (non polar) then added 5 ml of eucalyptus oil followed by addition of 50 ml of acetonitrile (polar). Hexane and acetonitrile layer was drained out in separate beakers. Afterwards hexane layer was added in separatory funnel and added more

acetonitrile for pure separation of non polar fraction. By using rotary evaporator, solvent traces were removed leaving behind the non polar fractions. Similar process was done in case of acetonitrile to get the polar fraction of oil. The polar fraction of oil being water soluble was not used for nanoemulsion synthesis.

The aqua nanoemulsion was formulated using non polar eucalyptus oil, non- ionic surfactant (Tween 20) and distilled water. The concentrations of these components to form a stable nanoemulsion were standardized using varying concentration of Tween 20 and water with fixed concentration of (6%) eucalyptus oil for all the formulations. Firstly, coarse emulsion was prepared by adding water to organic phase which contains oil and surfactant in different ratios 1:1, 1:2, 1:3 (v/v) using a magnetic stirrer, which was later subjected to ultrasonication using 40 KHz Sonicator. The morphology and size of hybrid nanoemulsions were recorded in Hitachi Transmission electron microscope Hi 7650 (TEM) at an accelerated voltage of 80 kV by casting a drop of hybrid nanoemulsion which was negatively stained with phosphotungstic acid and placed on a 200-mesh carbon coated copper grid in the Electron Microscopy and Nanotechnology (EMN) Laboratory, PAU, Ludhiana. TEM micrographs were acquired using TEM with a tungsten source.

Ae. aegypti larvae were collected from various peridomestic water collection sites such as desert coolers, earthen pots, fire buckets and side ditches on the road from areas of Ludhiana, Punjab from June to November 2017. The larvae along with water samples were collected using plastic droppers in 300 ml plastic bottles and brought to the laboratory. *Ae. aegypti* larvae were identified on the basis of different morphological characteristics as per standard keys (Becker et al., 2010; Bar and Andrew, 2013). Twenty *Ae. aegypti* larvae at 4th instar stage were extracted from water samples and placed in 250 ml of de-chlorinated water in plastic beakers. The concentrations of eucalyptus oil in water @ 100, 90, 80, 70, 60 and 50 ppm were prepared by diluting the most stable nanoemulsion using de-chlorinated water in triplicate for getting the effective concentration of prepared oil nanoemulsion. A vehicle-control set (having Tween 20 in de-chlorinated water in same ratio as treatment set) and a control set (having 250 ml de-chlorinated water only) containing twenty 4th instar larvae each in beakers were also run simultaneously (in triplicate). All mosquito larvae were adequately fed with mixture of dog biscuits and yeast ground in ratio 3:1 (2 mg/100 ml). Mortality of larvae after 3, 6, 12, 24 and 48 hr of treatment were recorded in oil nanoemulsion treated, vehicle-control, control sets and the concentration of oil in water nanoemulsion with highest mortality within lesser time duration was considered as the effective concentration.

To determine the LC_{50} and LC_{90} values of stable aqua nanoemulsion of non polar eucalyptus oil against *Ae. aegypti* larvae, the results of preliminary bioassay of all the tested concentrations were used. The range of toxic levels of the oil in water nanoemulsion was determined with the help of computer programme POLO software. To compare the morphological changes in treated and control larvae, samples were prepared for SEM by the method of Schaper and Hernandez-Chavarria (2006) and examined under Scanning Electron Microscope (SEM-S3400N) operated at an accelerating voltage of 15kV. Data obtained was statistically analyzed by comparing the mortality observations using ANOVA (DMRT) on SPSS software version 16. For calculating the LC_{50} and LC_{90}, the log concentration-mortality regression was conducted by log probit method (Finney, 1971) using the POLO (Robertson et al., 1980) computer program.

RESULTS AND DISCUSSION

Pure commercial form of eucalyptus essential oil (*E. globulus*) was colourless to pale yellow liquid having refractive index, density, optical rotation and boiling point of 1.457-1.469, 0.909 g/cm^3, - 5º to + 10º and 175º C respectively. The oil was insoluble in water, sparingly soluble in hexane and completely soluble in ethanol with aromatic odour. In the chromatographic chamber having non polar and polar solvent system i.e. benzene and ethyl acetate revealed the presence of five spots having R_f values 0.18, 0.36, 0.54, 0.72, 0.90 and one spot with R_f value 0.92 respectively. In the benzene-ethyl acetate (97:3) solvent system, two coloured spots having R_f values 0.49 (yellowish) and 0.91 (brown) revealed the presence of two major compounds. The non polar fractions showed five spots and polar fraction showed one spot due to presence of five non polar and one polar compound.

GC-MS data of eucalyptus oil showed the presence of 1, 8-cineole (42.16%) and P-cymene (14.81%) as principal compounds. There were a total of 22 compounds representing 99.99% of the total oil. Other essential compounds present were α-Pinene (9.53%), o-cymene (7.79%), α-Terpineol (6.27%) and carbonic acid (3.44%). The retention time and % area of compounds present in eucalyptus essential oil are

given in Table 1. The GC-MS analysis of essential oil of *Eucalyptus globulus* showed that 1, 8-cineole (59.45%) and terpinene (10.91%) are the main components (Mohammadi et al., 2019). Chemical composition by GC-MS of the essential oil of *Eucalyptus globules Labill*., grown in Montenegro, resulted in the identification of a total of 11 constituents, 1, 8 cineole (85.8%), α-pinene (7.2%), and α-myrcene (1.5%) being the main components. Other compounds identified in the oil were α-pinene, limonene, α-phellandrene, α-terpinene, linalool, pinocarveol, terpinen-4-ol, and α-terpineol (Damjanovic-Vratnica et al., 2011). Recently, Kassahun and Feleke (2019) also studied composition of essential oils of *Eucalyptus globulus* by using GC-MS and revealed total twenty compounds. Eucalyptol (55.43%), α-Pinene (25.55%) and D-Limonene (5.687%) were the main components identified by them.

The aqua nanoemulsions of non polar eucalyptus oil were obtained after sonicating coarse emulsion having oil, water and Tween 20 for 30 minutes. The prepared nanoemulsions were screened on the basis of certain parameters like phase separation and appearance. Among the prepared nanoemulsions having 6% non-polar eucalyptus oil and Tween 20 in different ratios i.e. 1:1, 1:2 and 1:3, the nanoemulsion with 1:2 ratio was found to be stable after thermodynamic stabilization process. This aqua nanoemulsion (1:2) was found to have maximum optical clarity and after the heating and cooling cycles, no phase separation was seen while the other two ratios were turbid and slightly milky in appearance and showed phase separation. Transmission electron micrographs showed the spherical shape of droplets with size ranging from 20-40 nm in non polar eucalyptus oil nanoemulsions (Fig. 1).

Overall % larval mortality was found to increase significantly with increase in concentration of non polar eucalyptus oil based aqua nanoemulsion. The target of the present study was to find out the lower concentration of aqua nanoemulsion which can kill 100% larvae within 24 hrs and the one determined during this study was 70 ppm. Thus, 70 ppm concentration of aqua nanoemulsion based on non polar eucalyptus oil was found to be statistically efficient in comparison to other concentrations, as all larvae were killed within 24 hrs, before their conversion to the next stage of development i.e. the pupae. Exposure of 50 and 60 ppm concentrations of non polar eucalyptus oil based aqua nanoemulsion

Table 1. Composition eucalyptus oil

S. No.	Name	Molecular Formula	Retention Time. (min)	Area. (%)
1	1, 8-cineole	C10H18O	6.67	42.16
2	α-Pinene	C10H16	11.01	9.53
3	P-cymene	C10H14	11.81	14.81
4	1,3,8 p-Menthatriene Ethanone	C10H14	12.81	0.76
5	P-cymene	C10H14	12.97	0.62
6	o-cymene	C10H14	13.13	7.79
7	Myrtenyl acetate	C12H18O2	13.88	2.75
8	Linalyl acetate	C12H20O2	14.28	0.64
9	Tetralin	C10H12	14.58	0.40
10	Linalyl isobutyrate	C14H24O2	14.75	0.55
11	Bicyclo(3.1.1)heptan3ol	C10H16O	15.22	0.68
12	Bicyclo(3.1.0)hexan3ol	C12H18O2	15.63	0.65
13	1Cyclopentene1methanol	C10H18O	16.10	1.41
14	α-Terpineol	C10H18O	16.45	6.27
15	carbonic acid	C15H26O3	16.70	3.44
16	Trans Carveol	C10H16O	16.96	0.41
17	Trans-2Caren-4-ol	C10H16O	17.12	0.46
18	D-Carvone	C10H14O	17.39	1.34
19	Camphenone	C10H14O	17.55	0.85
20	Ethyltetramethylcyclopentadiene Cyclohexene	C11H18	18.43	0.40
21	2-Methylisoborneol	C11H20O	18.57	0.31
22	Limonene oxide	C10H16O	19.39	3.76

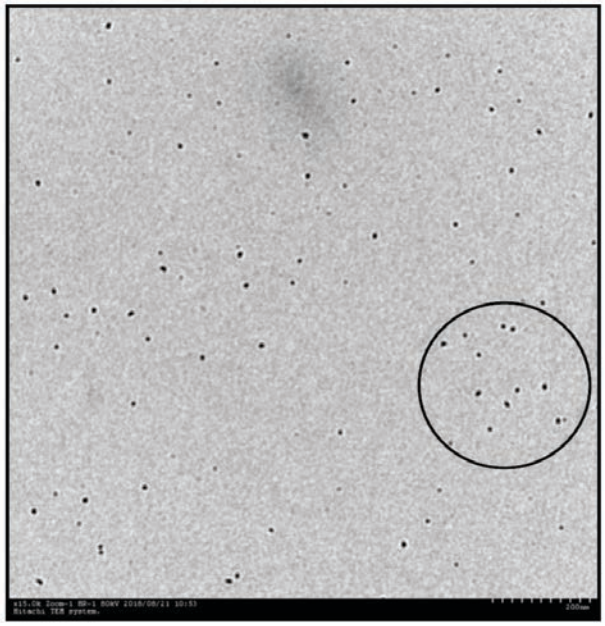

Fig. 1. TEM image of stable aqua nanoemulsion of non polar eucalyptus oil (1:2)

resulted in killing of larvae within 12 to 48 hours whereas larval mortality was observed within 24 hrs of exposure in case of 70, 80 and 90 ppm of concentrations. However, in control and vehicle-control sets no larval mortality was recorded (Table 2). The values for LC_{50} and LC_{90} of non polar *E. globulus* oil based aqua nanoemulsions worked out to be 51.95 and 64.21 ppm against *Ae. aegypti* larvae as calculated by use of POLO computer programme (Robertson et al., 1980) based on log-probit technique (Finney 1971).

Eucalyptus oil contains numerous components such as γ–Terpinene, 1,8-cineole, Globulol and α–Pinene. The most prominent of these is 1,8-cineole which is responsible for the larvicidal properties of this oil against insects (Franich 1985). Through their surface and tracheal systems, essential oils enter the larval body. The body wall of insects can absorb toxic substances in large volumes according to Lu and Kacew (2002) and Matsumura (1985). These substances can then easily penetrate the cuticle and further into the insect body because insects are generally so small that the body surface area is exposed to relatively higher concentrations than that of mammals (Martinez et al., 2013). The middle gastrointestinal tract (GIT) or midgut is protected by epithelial tissue and this tissue's cells can be lysed by toxic substances and subjected to necrosis in effect. Kaur et al., (2019) also studied that nanoemulsion of eucalyptus oil have larvicidal potential at lower concentration as compared to crude eucalyptus oil. They found 70 ppm concentration as most effective concentration which is in strong agreement to the present study. In another study, Kocher and Riat (2017) have reported that 90 ppm of eucalyptus crude oil emulsion resulted into 100% killing of *Culex* larvae within 48 hours. The synergistic interaction of the essential oils constituents can lead to larvicidal effect through contact, fumigation or ingestion methods (Mendes et al., 2017).

The effect of oil nanoemulsion treatment on the larval surface was clearly visualized using Scanning electron microscopy. Fig. 2 compares the structural as well as morphological changes in the oil nanoemulsion treated larvae with the untreated one. The larva without any treatment showed smooth body surface with well differentiated head, thorax and abdominal region (Fig. 2a). Fig. 2b depicts abdominal segment of the control larvae having smooth surface with abdominal spines. However, in Fig. 2c treated larvae have shown shrinkage and cracking in the abdominal segment. Also, contracted

Table 2. Effect of aqua nanoemulsion of non-polar eucalyptus oil on 4th instar *Ae. aegypti* larvae

Oil concentration (ppm)	% mortality (Mean± S.D) (n=20)					Range of mortality (within hours)
	3 hr	6 hr	12 hr	24 hr	48 hr	
50	0.00± 0.00^{f}	8.33± 2.89^{d}	31.67± 2.89^{d}	46.67± 5.77^{c}	81.67± 7.64^{b}	3-48
60	5.00± 0.00^{e}	15.00± 5.00^{d}	48.33± 2.89^{c}	70.00± 5.00^{b}	98.33± 2.89^{a}	3-48
70	16.67± 2.89^{d}	41.67± 7.64^{c}	83.33± 7.64^{b}	100.00± 0.00^{a}	-	3-24
80	28.33± 2.89^{c}	45.00± 5.00^{c}	81.67± 7.64^{b}	100.00± 0.00^{a}	-	3-24
90	45.00± 5.00^{b}	53.33± 5.77^{b}	85.00± 5.00^{b}	100.00± 0.00^{a}	-	3-24
100	65.00± 5.00^{a}	83.33± 7.64^{a}	100.00± 0.00^{a}	-	-	3-12
0 (Control)	0.00± 0.00^{f}	0.00± 0.00^{e}	0.00± 0.00^{e}	0.00± 0.00^{d}	0.00± 0.00^{c}	-
0 (Vehicle-control)	0.00± 0.00^{f}	0.00± 0.00^{e}	0.00± 0.00^{e}	0.00± 0.00^{d}	0.00± 0.00^{c}	-

n = number of larvae taken, Figures followed with various superscripts indicate significant difference ($p<0.05$ DMRT)

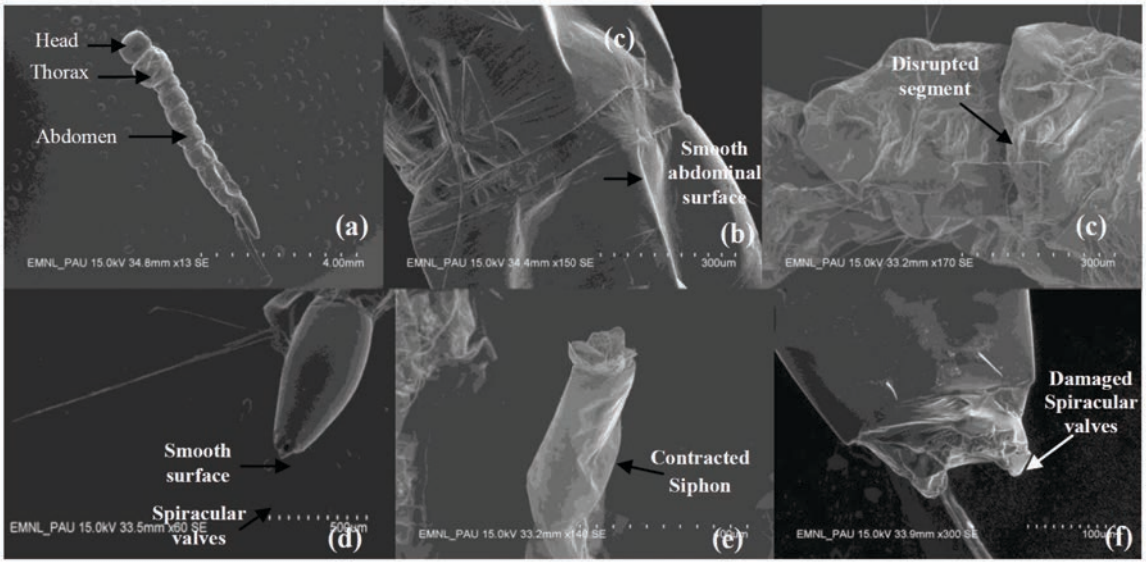

Fig. 2. *Ae. aegypti* larvae-SEM images: (a) Entire larvae without oil nanoemulsion treatment (Control), (b) Abdominal segment of control larvae, (c) Abdominal segment of treated larvae (d) Terminal region highlighting siphon of control larvae, (e and f) Terminal region highlighting siphon of treated larvae

siphon and damaged spiracular valves were observed in *E. globulus* oil based nanoemulsion treated larvae (Fig. 2e, f) while untreated larvae were devoid of any type of cracking and shrinkage. Observations of SEM studies indicate that contact with eucalyptus oil nanoemulsion causes significant damages and shrinkage on the surface of the *Aedes* larvae. Insun et al. (1999) demonstrated similar damage to the surface morphology of *Culex quinquefasciatus* larvae after treatment of *Kaempferia galanga* extract. In another study by Kumar et al. (2012) housefly larvae treated with *E. globulus* oil showed surface shrinkage by SEM technique. Thus, present study has shown good larvicidal activity of *E. globulus* nanoemulsion against the larvae of *Ae. aegypti*. The good killing potential was supported by the enrichment of 1,8-cineole in the non polar part of the oil nanoemulsion. Therefore, *E. globulus* nanoemulsion provides a strong alternative to harmful chemical insecticides as an economically-viable and non-toxic product for controlling vector, *Ae. aegypti*.

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