Original Article

Evaluation of Nanoemulsion of *Eucalyptus globulus* Oil as Potent Botanical Larvicide against Malaria Vector, *Anopheles stephensi* and West Nile Vector, *Culex pipiens* Under Laboratory and Semi-Field Conditions

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Abstract

Background: Due to undesired environmental impact of insecticides as well as resistant of vectors to them, the development of organic and natural insecticides has been more considered. In the current study, we developed nanoemulsion of eucalyptus and investigated lavicidal activity of it against malaria vector, *Anopheles stephensi* and *Culex pipiens* under laboratory as well as semi-field conditions.

Methods: An optimized nanoemulsion was prepared by mixing Eucalyptus oil, Tween 80 and ethanol at ratio of 1:2:1.5 in distilled water, then, stirred for 20 minutes at room temperature. The product was then used for bioassay tests against 3-4th instar larvae of *Anopheles stephensi* as well as *Culex pipiens*. Furthermore, a semi-field trial was carried out to evaluate larvicidal activity of nanoemulsion of eucalyptus.

Results: Nanoemulsion of eucalyptus showed significantly high lavicidal activity comparing with bulk eucalyptus essential oil. The LC_{50} and LC_{90} value of nanoemulsion against *An. stephensi* were 111.0 and 180.8 ppm respectively and 29.5 and 73.7 ppm for *Cx. pipiens*, respectively. In the semi field condition, the Nanoemulsion of eucalyptus decreased 1-2nd instar larval density of Culicines and Anophelines to 90.1% and 85.2%, respectively.

Conclusion: The nano formulation of eucalyptus oil showed high larvicidal activity. Therefore, nanoemulsion of eucalyptus oil can be used as an eco-friendly larvicide against mosquitoes.

Keywords*: Eucalyptus*; Essential oil; Nanoemulsion; Larvicide; *Anopheles stephensi*; *Culex pipien*s

Introduction

Mosquitoes are a serious threat to public health as they act as vectors that help in transmission of diseases that can be lethal (1,2). Owing to the lack of proper medication and vaccines for treating mosquito-borne diseases, an alternative and effective approach used to control the vector population at the larvicidal stage is necessary because, during this stage,

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the mosquitoes are in a stationary phase (3-5). The resistance to synthetic pesticides and harmful effects of their accumulation in the environment has created the need for natural and non-persistent insecticides. The essential oils extracted from plants are suitable as they are economically reasonable and have high activity in certain cases and are also bio-degradable (4,6,7). Eucalyptus is a diverse genus of flowering trees and shrubs in the Myrtle family, Myrtaceae. The oil extracted from eucalyptus leaves possesses allelopathic property and prevents insects from attacking it, thereby, acting as a natural pesticide (8,9). The fumigation activity (10) and repellency (11) and insecticidal activity of eucalyptus oil has been demonstrated (12). Nanoemulsion of natural oils could enhance pesticide activity of the component (13). Nanoemulsions are emulsions whose droplet size is uniform and extremely small with the size ranging from 20 to 100 nm. Nanoemulsions are metastable and their stability is determined by the method of preparation (14). Nanoemulsions can be formulated by two kinds of methods such as high-energy and low-energy emulsification methods. The high-energy emulsification method comprises high-pressure homogenization and ultrasonication (15). Ultrasonication is the most widely used method owing to its ease of use and it is an economical method. The low-energy emulsification technique comprises methods that exploit the chemical properties of a system to convert a microemulsion into a nanoemulsion (16,17). The use of nanopesticides would be a contemporary measure for the control of pests and reducing the toxic effect of synthetic bulk pesticides on the environment (18). Recently, controlling for vector borne diseases using neem oil nanoemulsion will be of good alternative against *Culex quinquefasciatus* compared to the synthetic pesticides (13). The present study was carried out to develop a nano formulation of eucalyptus oil as an ecofriendly larvicide and evaluate its larvicidal activity against the larvae of malaria vector, *Anopheles stephensi* and vectors of West Nile virus (WNV), *Culex pipiens* in laboratory and semi-field conditions.

Material and Methods

Preparation of nanoemulsion

Eucalyptus oil (*Eucalyptus globulus*) was purchased from Barich Co., Iran and stored at room temperature under laboratory conditions; Tween 80 (Polyoxyethylene 20 monooleate) was supplied from Sigma. All other chemicals used were of analytical reagent grade. The oil-in-water nanoemulsion was formulated using eucalyptus oil, non- ionic surfactant (tween 80) and water. The concentration of eucalyptus oil (6%, v/v) was fixed for all the formulations. Initially, coarse emulsion was prepared by adding water to organic phase containing oil, surfactant and co-surfactant in ratios 1:1: 1.5 (v/v) using a magnetic stirrer, which was then subjected to ultrasonic emulsification using a 20 kHz Sonicator (Ultrasonics, USA) with a power output of 750 W. Energy input was given through sonotrode containing a piezoelectric crystal with a probe diameter of 13 mm. Sonicator probe generates disruptive forces that reduce the droplet diameter converting coarse emulsion to nanoemulsion. Then the characterization of nanoemulsions was carried out and the emulsion stability was investigated.

Droplet size distribution and polydispersity index

The droplet size distribution (analysis by volume) and poly dispersity index (PDI) of eucalyptus oil nanoemulsion formulation (1:2) was determined using a 90-plus particle size analyzer. The PDI is a measure of the homogeneity and stability of the droplet size in the nanoemulsion system. PDI values below 0.2 indicate a narrow size distribution and thus provide long-term stability to the formulated nanoemulsion. Prior to experiment, formulated emulsion was diluted with milli-Q (Millipore corporation) double-distilled water to trim down multiple scattering effects.

Morphology of emulsion droplets

To visualize the shape and morphology of

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the formulated nanoemulsions, Atomic force microscopy (AFM) was carried out. One drop of emulsion was negatively stained with phosphotungstic acid and positioned on a copper grid.

Larvicidal bioassay

Mosquito rearing: Mosquitoes consisting of *An. stephensi* and *Cx. pipiens* were reared under a uniform condition including larvae and adult nutrition, temperature (28±2 °C), humidity (70±10 %) and on a 12-h light-dark cycle. Larvae were grown in bowls at a density of 200 larvae / 500 ml of distilled water with 0.01% table salt, and fed on fish food. The pupae were transferred to cages made of muslin cloth before eclosion to the adult stage. The female mosquitoes were fed on 10% fructose and the females were fed on guinea pig blood.

Larvicidal activity: The larvicidal activity of eucalyptus nanoemulsion against third instar larvae of *An. stephensi* and *Culex pipiens* were treated with different concentrations of nano and bulk eucalyptus oil emulsions. The bioassays were carried according to the guide line of World Health Organization for laboratory and field testing of mosquito larvicides (19). Initially, serial dilutions of nanoemulsion (5, 50, 100, 160, and 240 ppm) were prepared in sterile glass beakers (250 mL) containing 200 ml of water. Then 20 larvae (stage 3-4) of the mosquito species were placed into each beaker. The same procedure was conducted for the bulk of eucalyptus oil. The mortality rate of larvae was recorded after 24 and 48 hours. Each test was performed in four replicates. The percentages of larval mortality and standard deviation were calculated for each concentration of nanoemulsion and bulk emulsion.

Semi-field larval bioassays

Twelve artificial breeding places each 1×1 meters were provided in a semi-field condition in the Kazeroun area, southern Iran and allow the wild mosquitoes to lay their eggs on the surface of breeding places. The density of larvae 10 per dipper were measured in each breeding place before any intervention. The spray of three times of obtained lethal concentration (LC_{90}) of bulk-eucalyptus oil (≈114 ppm) and nano-

eucalyptus oil (≈90 ppm) were sprayed randomly on the surface of each breeding place. Each treatment was replicated in four breeding places and four breeding places of free oils as the control.

Statistical analysis

The mortality quantities of 50% and 90 % of imagicides (LC_{50} and LC_{90}) and the level of confidence of 95%, the equation of the regression line were estimated using a regression probit analysis as described by Finney (20).

Mosquito identifications

All the adults females were identified using morphological identification key identification (21).

Results

Characterization of the selected nano-emulsion

Based on the thermodynamic stability study, 1:2 ratio nanoemulsions was selected, and their average diameter is 18 nm andtheir size distribution as presented in Fig.

1. The polydispersity index (PI) of the nanoemulsions is 0.060 which shows that it is uniform. The particles are presented in Atomic force microscopy (AFM) from 22 to 40 nm, and the droplets are spherical in nature (Fig. 2).

Larvicidal activity of eucalyptus oil nano-emulsion

The larvicidal activity of both nanoemulsion eucalyptus oil and bulk eucalyptus oil against larvae of *An. stephensi* and *Cx. pipiens* was varied. High mortality rate was observed among larvae of *An. stephensi* exposed to 160 ppm of nanoemulsion of eucalyptus oil within 24 hours, while the mortality rate for eucalyptus oil was 74% at the concentration of 160 ppm after 24 hours (Table 1). Moreover,

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100% mortality occurred for the larvae exposed to both nanoemulsion eucalyptus oil and eucalyptus oil at a concentration of 240 ppm. Overall, the larvicidal activity of nanoemulsion oil against larvae of *An. stephensi* within 24 hours was significantly higher than eucalyptus at 160 ppm, but absolute mortality was observed among the larvae of *An. stephensi* after 48 hours in both nanoemulsion formulation and bulk oil of eucalyptus (Table 1). Similarity, the larvicidal activity of nanoemulsion oil against larvae of *Cx. pipiens* was higher than eucalyptus oil at 160 ppm within 24 hours while 100% mortality occurred by 240 ppm for both formulations of eucalyptus oils (Table 2). Lethal concentration ($LC_{50}$and LC_{90}) for nanoemulsion of eucalyptus oil and eucalyptus oil were significantly different within 24 and 48 hours after treatment (Table 3). Moreover, LC_{50} and LC_{90} of nanoemulsion of eucalyptus oil against larvae of *An. stephensi* significantly lower than eucalyptus oil within both 24and 48 hours treatment (Table 3). Similarly,

lower LC_{50} and LC_{90} was observed for larvae of *Cx. pipense* exposed to nanoemulsion of eucalyptus oil which was significantly different with LC_{50} and LC_{90} for the larvae exposed to eucalyptus oil (Table4).

Semi field larval bioassays

Both nano-eucalyptus and bulk eucalyptus oils decreased the density of mosquito larvae in the treated breeding places one day after

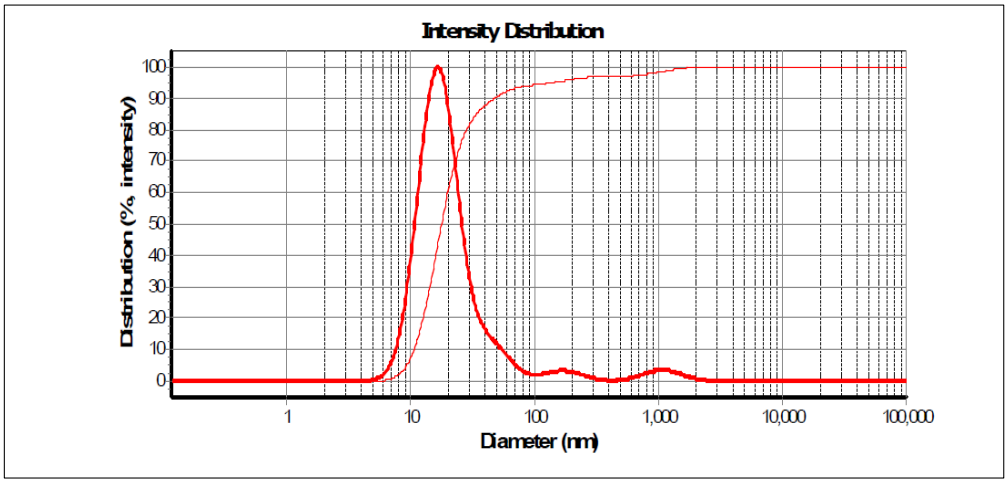

Fig. 1. Size distribution of eucalyptus nanoemulsion oil measured by Dynamic Light Scattering

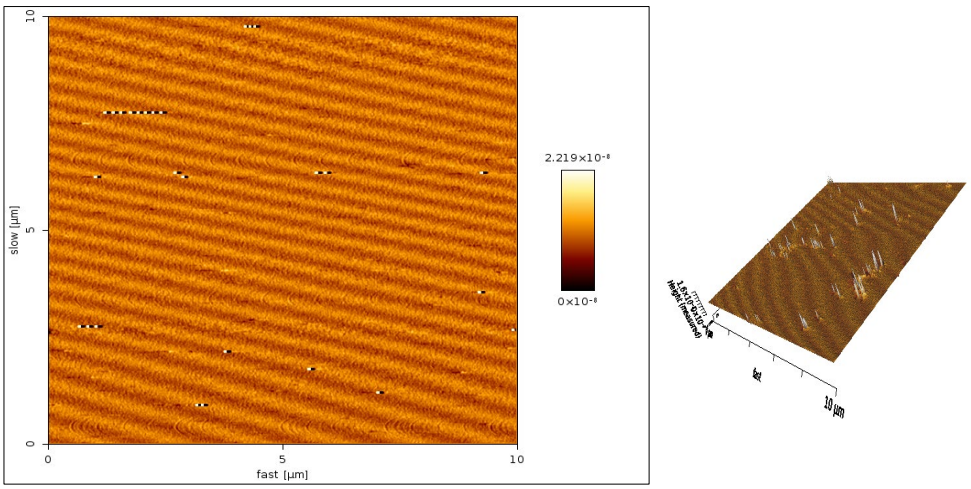

Fig. 2. Atomic force microscopy image and size of eucalyptus nanoemulsion oil

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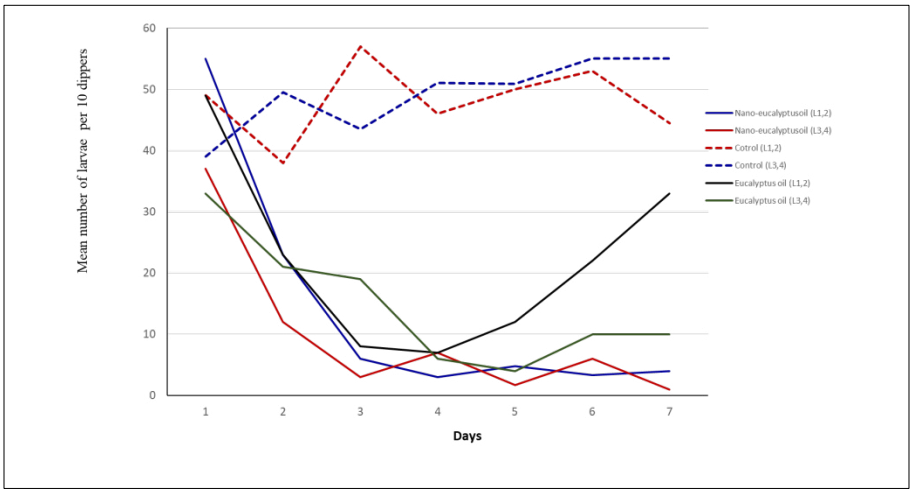

Fig. 3. Residual larvicidal activity of Nanoemulsion of eucalyptus oil and bulk eucalyptus oil against different stages of mosquito larvae at semi-field condition. Twelve artificial breeding places (1×1 meters) were prepared and then allowed the wild mosquitoes laid eggs. L1, 2 = Larvae stage 1 & 2, L 3,4= Larvae stage 3 & 4

Table 1. Probit analysis of larvicidal activity of essential and nanoemlusion of *Eucalyptus globulus* oil on 3rd and 4th instar larvae of *Anopheles stephensi* after 24 hours

Concentration (ppm)	Essential oil			Nanoemlusion oil		
	Mortality (%)	Observed probit mortality	Expected probit mortality	Mortality (%)	Observed probit mortality	Expected probit mortality
60	9	3.659	3.246	8	3.595	3.384
160	74	5.643	5.658	91	5.025	5.204
240	100	7.526	6.654	100	7.576	7.024
360	100	7.526	7.651	100	7.576	8.089
Control	0	-	-	0	-	-

Table 2. Probit analysis of larvicidal activity of essential and nanoemlusion of *Eucalyptus globulus* oil on 3rd and 4th instar larvae of *Culex pipiens* after 24 hours

Concentration (ppm)	Essential oil			Nanoemlusion oil		
	Mortality (%)	Observed probit mortality	Expected probit mortality	Mortality (%)	Observed probit mortality	Expected probit mortality
60	8	3.595	3.384	11	3.773	3.520
160	51	5.025	5.204	86	4.900	5.161
240	100	7.576	7.024	100	7.576	6.802
360	100	7.576	8.089	100	7.576	8.443
Control	0	-	-	0	-	-

spraying. The high larvae population at stage 1 and 2 in the control breeding sites indicated that the wild mosquitoes laid eggs frequently during the bioassay period (Fig. 3). Therefore, the density of mosquito larvae was relatively high in the control breeding site while rapidly declining in the treated sites. However, nano-eucalyptus emulsion kept the larvae density low for 6 days post spraying while the number of larvae at stage 1 and 2 increased two days after eucalyptus oil spraying (Fig. 3). The results indicating that nano-eucalyptus oil

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Table 3. Lethal concentration (LC_{50} and LC_{90}) and in the 24 and 48 hours bioassay tests of *Eucalyptus* oil and Nano-*Eucalyptus* against 3rd and 4th instar larvae of *Anopheles stephensi*

	Exposure time (hours)	p-Value	χ^2 table (df)	χ^2 (Heterogeneity)	LC_{50} (ppm) .± 95%C.L	LC_{90} (ppm) ± 95%C.L.	b ± SE
***Eucalyptus* oil**	**24**	0.01	15.086 (5)	15.611*	103.7845 **122.8343** 145.0833	169.1937 **206.2336** 295.0632	5.6608 ± **0**.764
	48	0.00	15.035 (5)	14.623*	93.761 **101.663** 145.0833	142.402 **155.973** 175.732	2.399 ± 1.023
Nano-*Eucalyptus* oil	**24**	0.01	9.210 (2)	5.1802*	63.7924 **80.8730** 105.7719	102.8420 **111.0171** 119.7206	6.0457 ± 0.539
	48	0.00	9.145 (2)	4.258*	68.235 **76.869** 95.582	46.235 **58.595** 65.548	7.0457 ± 0.652

Table 4. Lethal concentration (LC_{50} and LC_{90}) and in the 24 and 48 hours bioassay tests of *Eucalyptus* oil and Nano-*Eucalyptus* against 3rd and 4th instar larvae of *Culex pipiens*

	Exposure time (hours)	p-Value	χ^2 table (df)	χ^2 (Heterogeneity)	LC_{50} (ppm) ± 95%C.L	LC_{90} (ppm) ± 95%C.L.	b ± SE
***Eucalyptus* oil**	**24**	0.01	8.235 (2)	10.546*	47.3526 **68.6321** 76.1356	20.3120 **39.4112** 78.6321	6.5324 ± 1.034
	48	0.01	9.210 (2)	10.678*	42.4037 **64.2180** 74.0175	14.7673 **37.3711** 86.4146	.4508 ± 1.127
Nano-*Eucalyptus* oil	**24**	0.01	9.210 (2)	15.611*	43.3775 **53.6733** 70.0869	25.7675 **29.4644** 33.0781	3.2200 ± 0.313
	48	0.01	9.451 (2)	15.646*	31.6885 **34.5681** 39.0263	19.9645 **23.2523** 26.2879	5.1234 ± 0.4123

has slightly longer residual larvicidal activity rather than bulk eucalyptus.

Discussion

Due to numerous limitations to control of adult mosquitoes, the ideal method to control them is targeting the larval stage. Control of mosquito larvae is based largely on the use of synthetic chemicals. Generally, synthetic pesticides have some disadvantages such as health problems, harmful to the environment, pests may develop tolerance to certain chemicals over time and contamination of soil and water resources (22-24). However, the indiscriminate and injudicious use of pesticides has led to the widespread development of resistance among pests as well as insect vectors (25). Thus, alternative components to be needed for controlling mosquito vectors. Botanical pesticides are considered as safe, easily

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biodegradable, environmentally friendly and with low toxicity (26,27). However, usage of them is often limited due to instability and rapid degradation, application frequency as well as requiring higher application rates. Hence, the use of botanical insecticides associated with nanotechnology offers considerable potential for increasing efficacy of plant based insecticide (27-29). Eucalyptus oil is the oil distilled from the leaves of eucalyptus, a genus of the plant family Myrtaceae. The repellent activity of eucalyptus oil was demonstrated against *Cx. quinquefasciatus* (30,31). The seed and leaf extract of eucalyptus oil contain compounds that are toxic to mosquito larvae (32,33). Since they do not cause any adverse health effects; they are used as insect repellents and a safe and eco-friendly alternative to synthetic pesticides (34). Therefore, in this study, we used eucalyptus oil as a safe and non-toxic larvicide. Generally, botanic pesticides have low effective durability; therefore, we utilized nanotechnology to overcome this problem. We synthesized nano-eucalyptus oil to increase larvicidal durability and effectiveness. The method of preparation determines the stability of the formulated nanoemulsion. Based on atomic force microscopy analysis, the size of our product was from 22 to 40 nm (Fig. 2), and the larvae mortality we achieved in laboratory and semi field bioassays (Tables 1 , 2 and Fig. 3) was as the results of these nano-size. Nevertheless, the larvicidaleffects of nano-eucalyptus oil are likely to change as the nano-particle size changes.

The stabilization of the nanoemulsions is also dependent on the steric effect of the non-ionic surfactant (35). Therefore, the effectiveness of nano-eucalyptus oil may be influenced by chemical component of water in the breeding sites. In semi-field assay, we did not observe 100% mortality of mosquito larvae a day after treatment of nano-eucalyptus oil and eucalyptus. This result may be due to components of water in the breeding sites. In addition, only a single concentration of nano-eucalyptus oil (≈90 ppm) was tested against wild mosquito larvae. According to WHO guide line, the larvicide dosage for the field trial should be three times of LD_{90} concentration in a laboratory scale. However, we did not observe convenient results for larvicidal effect of nano-eucalyptus oil in the field condition, Thus, more serial concentrations of the nano emulsion should be assays to overcome the highest larvae mortality in the field condition. In this study, eucalyptus oil (6%, v/v) is mixed with a non-ionic surfactant, tween 80 along with water, which is used as an aqueous phase. This emulsion is then subjected to ultrasonication that breaks down the bulk emulsion into an emulsion comprising droplets having the size in the nanometer range. This increases the surface area of the droplets, thereby, increasing the reactivity; thus, making nanoemulsions more effective than its bulk counterpart. However, the insecticidal and antimicrobial activity of eucalyptus oil is linked to its chemical composition such as 8-cineole (eucalyptol), which its concentration ratio varies in eucalyptus trees from different locations (36).

Conclusion

The nano formulation of eucalyptus oil showed high larvicidal activity against mosquito larvae when compared to its bulk counterpart. Therefore, nanoemulsion of eucalyptus oil can be used as an eco-friendly larvicide against mosquitoes. From this study, it can be concluded that eucalyptus oil nanoemulsion is a safe and effective alternative larvicide to control mosquito-borne diseases.

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University of Medical Sciences.

Competing interests

The authors declare that they have no competing interests.

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