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Evaluation of the Mosquito Repellent Activity of Nano-sized Microemulsion of *Eucalyptus globulus* Essential Oil Against *Culicinae*

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Abstract

Background: The mosquitoes of *Culicidae* family are serious vectors of several tropical diseases, such as malaria, filariasis, encephalitis, and nuisance. Control of mosquitoes and protection of people from their bites are of the most important ways to prevent transmitted diseases. Although the efficacy of N, N-diethyl-m-toluamide (DEET) is high and generally used as mosquito repellent, yet a number of biting diptera are tolerant to DEET. Furthermore, there are concerns about the safety of DEET and its allergic and toxic effects. Therefore, it is necessary to use other repellents like plant essential oils.
Objectives: The aim of this work was to develop a safe repellent with a long-lasting protection based on micro-emulsion of eucalyptus essential oil.
Methods: *Eucalyptus globulus* essential oil was obtained by water distillation in a Clevenger apparatus. The larvae of *Culicidae* were collected and adult mosquitoes reared for the repellency test. Preparation of micro-emulsions of *Eucalyptus globulus* essential oil was made by mixing the specified surfactant (Tween 80 and Span 20) and the appropriate amount of co-surfactant (propylene glycol) under the water titration method. The laboratory method, arm in cage, was used to estimate the time of protection of essential oil micro-emulsion against mosquitoes and DEET used as a standard repellent.
Results: Physicochemical properties of formulated micro-emulsions were appropriate and suitable for topical application. Particle size of eucalyptus oil 15% w/w micro-emulsion was lowest. When applying eucalyptus oil micro-emulsion at concentrations of 5, 10, and 15% w/w, time of protection against mosquitoes were 82 ± 15.8, 135.7 ± 26.4, and 170.7 ± 26 minutes, respectively. These times of protection were similar to DEET at same concentrations and significantly more than eucalyptus essential oil.
Conclusions: The formulated micro-emulsion of eucalyptus oil at a concentration of 15% w/w has potential repellency to the extent of DEET. It seems that nano-sized microemulsion is stable in terms of thermodynamics and kinetics. In conclusion, preparation of nano-sized microemulsion could delay the volatility of eucalyptus essential oil and volatile oil release from formulations and consequently increase protection time against mosquitoes.

Keywords: Mosquito Repellent, Essential Oil, Microemulsion, *Eucalyptus globulus*

1. Background

Mosquitoes have an important role in the transmission of diseases like malaria, dengue, chikungunya, Japanese encephalitis and filariasis, and cause millions of deaths, annually (1). Advantageous control of vector is an efficient tool to prevent transmission of disease. Repellents are an important part of these preventive actions (2). Among developed synthetic chemicals, DEET has a wide range of repellency to keep humans far from mosquito bites with most persistence and effectiveness on the body (3). At first, the US Department of agriculture developed DEET to protect army staff in 1946 and was approved for use for the public in 1957. Estimates indicate that nearly 75 million people in the US apply DEET, annually (4).

Human experiences and animal studies have demonstrated that DEET is generally safe, yet other evidence shows that use of DEET is associated with both systemic and local adverse effects. Dermal absorption of DEET has occurred in infants and children after applying pharmaceutical formulations containing DEET. There are cases of death and also toxicity, including encephalopathy, cardiovascular, dermal, and psychosis seizure (3). Although, DEET is used widely, however it is associated with environmental problems and human health risks. Accordingly, there are increasing efforts for the development of natural repellents and eco-friendly pharmaceutical formulations. In the past 50 years, thousands of plants have been screened for their repellency. Some natural products and plant-based formulations are more efficient than synthetic repellents. Essential oil repellents have short duration of action because of their volatility. As possible sources of repellents and insecticides, there are many preparations from natural origins that are repellent to certain insects (5). Essential oils are potentially good repellents and insecticides because of their selectivity, safety, and negligible adverse effects on the environment and non-target organisms (6).

The major component of *Eucalyptus globulus* is 1, 8-Cineol (Eucalyptol) and has been recognized as a high ovipositional repellent and mild mosquito feeding repellent (7). Chemical instability, volatility, propensity for oxidation and poor water solubility of essential oils make them inconvenient for extensive use (8). Consequently, the incorporation of essential oils in nano-formulations, such as micro-emulsions, could solve these problems through increase in dissolution rate, water solubility, dispersion uniformity and stability after topical application (9). Micro-emulsions are transparent isotropic formulations, which are thermodynamically stable and are prepared by dispersion of two immiscible liquids (water and oil) containing appropriate amounts of surfactant. The dispersed phase is composed of small nano-sized droplets with a dimeter of 10 to 100 nm (10). Because of their small droplet size, micro-emulsions may appear transparent, and Brownian motion prevents creaming or sedimentation, hence offering increased stability (11). Accordingly, this study was designed to evaluate the repellent activity of nano-sized micro-emulsion of *Eucalyptus globulus* essential oils.

2. Methods

2.1. Materials

The compound DEET was obtained from Sigma-Aldrich. Tween 80, Span 20, and propylene glycol (PG) were purchased from Merck (Germany).

2.2. Plant Material and Essential Oil Preparation

The fresh leaves of *Ecalyptus glubolus* were collected during autumn from the center for medicinal plants at college of pharmacy at Jundishapur University of Medical Sciences. The leaves were washed, dried in the shade, and chopped. The essential oil was isolated using the Clevenger apparatus through water distillation for about 4 to 5 hours. The prepared essential oil was dried under anhydrous sodium sulfate and stored in the dark at 4°C until use.

2.3. Nano-Sized Micro-Emulsion Preparation

The micro-emulsions were formed using five components: eucalyptus essential oil as the oil phase, a mixture of surfactants (Tween 80 and Span 20 1:1), and co-surfactant (PG) and distilled water as the aqueous phase. The water titration method was used to investigate concentration range of ingredients in micro-emulsion regions.

2.4. Construction of Phase Diagram

Different formulated micro-emulsions were optimized and selected based on the pseudo ternary phase diagrams in two different phase diagrams with weight ratios of 1:1 and 2:1 of surfactant and co-surfactant, respectively. If phase separation occurred or turbidity appeared, the formulation was assigned to be biphasic. The transparent and low viscosity sample was considered monophasic and this implies the existence of a micro-emulsion region (12, 13).

2.5. Determination of Particle Size

The mean particle size of droplets was measured by SCATTER SCOPE 1 QUIDIX (South Korea) at 25°C.

2.6. Determination of Viscosity

The viscosity of different formulations was measured at 25°C using a Brookfield viscometer (DV-II + Pro Brookfield., USA). Viscosity determination was made with spindle no. 34 at the shear rate of 50 rpm in triplicates.

2.7. Measurement of pH

The pH of each formulation was determined at 25°C (pH meter, Mettler Toledo seven easy, Switzerland).

2.8. Physical Stability of Nano-Emulsions

Ten milliliters of eucalyptus oil micro-emulsion samples was placed at room temperature. During 2 months, samples were evaluated for phase separation and turbidity.

2.9. Mosquito

Mosquito larvae were collected from nests right near the Jundishapur University in the fall. *Culicinae* larvae are visually distinguished from Anopheles larvae by a magnifying glass and a dropper drops. *Culicinae* larvae were transferred to plastic trays contained river water. The larvae were held in plastic trays to mature and were transferred to net cage and emerged as adults. Cages were placed at a temperature range of 25°C to 30°C with relative humidity at the range of 55% to 70%. Adults continuously provided with 10% sugar solution in water soaked on cotton pads. In this study 7-day-old female mosquitoes were used in the repellency test.

2.10. Repellent Activity

Repellency was evaluated using the arm-in-cage method. The arm-in-cage method refers to an environment with low density of mosquitoes per cage and unlike a high density, more accurately reflects the typical biting pressures (14). The repellent-treated arm of a volunteer is exposed to constant unfed mosquitoes in a cage for three-minute time intervals. If no mosquitoes bit or landed during the three-minute time interval, the arm was withdrawn from the cage and the repellency test period was continued 30 minutes. If at least 2 mosquitoes bit or landed during the three-minute study period, the time of repellency test was stopped. The time between application of formulated repellent to the arm and the two consecutive lands or bites was considered as the time of protection of each repellent against mosquitoes. The right forearm, which acted as a control, was not treated and was exposed for up to 30 seconds to the mosquito cage. The test was conducted on three human volunteers, who washed their arms with distilled water before testing. Forty female mosquitoes (disease free) that were starved for 12 to 24 hours were used in each experiment. A total of 1 gram of test repellents were applied on the left forearm from the elbow to the wrist (15).

2.11. Statistical Analysis

Data were analyzed using the one-way analysis of variance (ANOVA) and significant differences between groups were determined using the SPSS software (version 16.0). Post hoc Tukey's test and $P < 0.05$ were considered significant with 95% confidence intervals. All the experiments were repeated three times and data were expressed as mean value ± SD. Sigma plot 11 software was applied for providing ternary phase diagrams.

3. Results

3.1. Specification of Micro-Emulsions

3.1.1. Phase Studies

The phase diagram system consisted of the oil phase (eculyptus essential oil), surfactant (Tween80-Span20), and co-surfactant (PG). Surfactant and co-surfactant were chosen based on essential oil solubility, hydrophilic-lipophilic balance values, and ability to form micro-emulsion. The phase diagram was assigned to determine the micro-emulsion zones. Two phase diagrams made at ratio s/c of 1/1 and 2/1 have been mentioned in Figure 1. However, the weight ratio of surfactant/co surfactant as a critical and important parameter affects behaviors of micro-emulsion phase and higher concentration of surfactant increases the micro-emulsion region.

3.1.2. Physicochemical Properties of the Formulated Micro-Emulsions

The mean particle size of formulated micro-emulsions was at a range of 16 to 66 nm (Table 1). The pH value (5.65 ± 0.11) of micro-emulsion formulations was appropriate and suitable for topical application. The mean viscosity range of formulated micro-emulsions was from 237.6 ± 2.1 cps to 331.2 ± 1.5 cps (Table 1).

The physicochemical data showed that phase of formulated micro-emulsions were not separated and phases remained homogenous with acceptable physical stability.

3.2. The Abundance of Mosquito Species

After the repellency test, a total of 50 mosquito corpses were collected from cages randomly and the entomology laboratory determined their species by optical microscopy. Abundance of mosquitoes, according to species was *Culexpipiens* (62%), *Ochlerotatus caspius* (22%), *Culex pusillus* (10%) and *Culex tritaeniorhynchus* (6%).

3.3. Protection Time Against the Mosquito

As shown in Table 2, the protection times have been reported in three formulations at the same concentrations. These times are the time durations between application of formulated repellent to arm and occurrence of two consecutive lands or bites of mosquitoes (Figure 2).

4. Discussion

Based on this study, *Culex pipiens* was the first and *Ochlerotatus caspius* was the second abundant mosquitoes. To achieve the desired pressure biting, repellency test was done in darkness after at least 12 to 24 hours of starvation of mosquitoes to increase pressure biting on the human

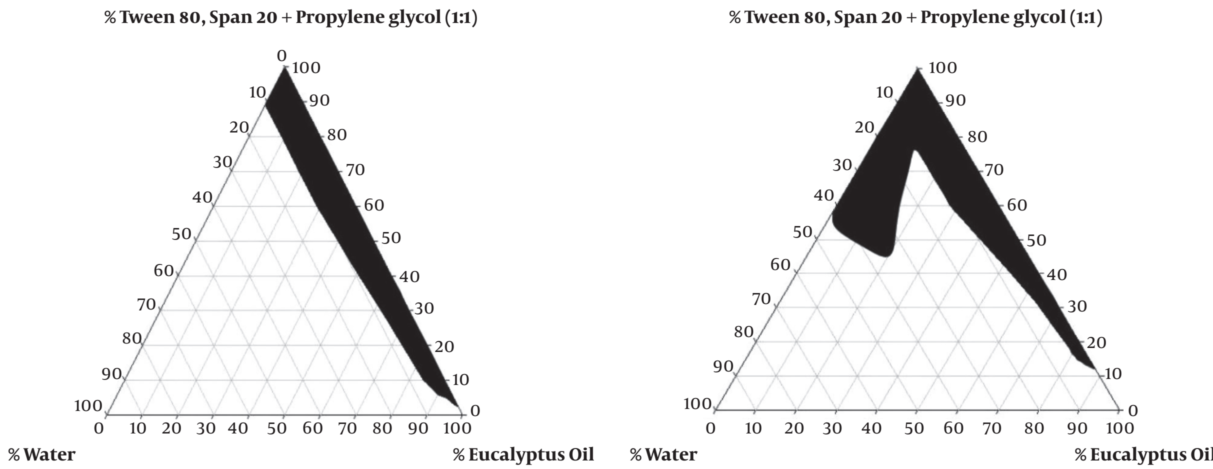

Figure 1. The pseudo-ternary phase diagrams of the essential oil/surfactant, co-surfactant/water system at the 1:1, 2:1 weight ratios of Span 20 - Tween 80 / PG at ambient temperature, dark area showing the micro-emulsion zone.

Table 1. Characterizations and Components of Selected Micro-Emulsions (% w/w), Particle Size, Viscosity and pH (Mean ± SD, N = 3)

Formulation	S/C	Oil, %	Water, %	S + C, %	Particle Size, nm	Viscosity, cps	pH
1	2:1	5	35	60	43.6 ± 3.7	1.5 ± 331.3	5.34 ± 0.13
2	2:1	10	30	60	53.7 ± 2.2	2.1 ±237.6	5.52 ± 0.04
3	2:1	15	25	60	17.1 ± 0.7	0.9 ± 305.8	5.86 ± 0.18

Table 2. Protection Time of Mosquito Repellents (Mean ± SD, N = 3)

Number	Repellent Formulations	Protection Time Against the Mosquito, Min
1	Ethanol[a]	Control < 30
2	DEET 5% w/w	115.75 ± 14.3
3	DEET 10% w/w	176.25 ± 15.35
4	DEET 15% w/w	211 ± 14.85
5	Eucalyptus essential oil 5% w/w	34.75 ± 2.99
6	Eucalyptus essential oil 10% w/w	47.5 ± 15.86
7	Eucalyptus essential oil 15% w/w	59 ± 11.89
8	Water + tween 80 + span 20 + PG[b]	Control < 30
9	Microemulsion of eucalyptus oil 5% w/w	82 ± 15.87
10	Microemulsion of eucalyptus oil 10% w/w	135 ± 26.46
11	Microemulsion of eucalyptus oil 15% w/w	170 ± 27

[a]Control for DEET and eucalyptus Oil.
[b]Control for microemulsion of eucalyptus Oil.

model. *Culex pipiens* is ornithophilic and the major carrier of West Nile fever virus in America (16). *Ochlerotatus caspius* has a high anthropophilic index and is considered an arbovirus vector in Europe, especially in Italy (17). Microemulsion of eucalyptus essential oils showed significantly more time of protection in comparison with the essential oil. Average time of protection against mosquitoes significantly increased when eucalyptus essential oil concentration increased from 5% to 15% in both eucalyptus essential oils diluted in ethanol and micro-emulsions of eu-

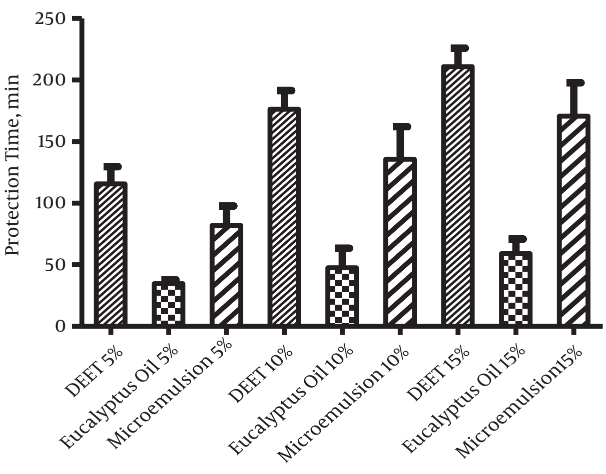

Figure 2. Protection Time of Mosquito Repellents of DEET, Eucalyptus Oil and Eucalyptus oil Micro-Emulsion at Three Same Concentrations

calyptus essential oils. However, more repellent material in the air caused greater exposure to chemical receptors in the antennae of female mosquitoes and their hunger for blood was reduced. For this reason, at higher doses of essential oils, the expected duration of protection against mosquitoes increase. A similar study on some plant essential oils was performed with concentrations of up to 100%, which increased the average duration of protection against mosquitoes (18). In the current study, three concentrations of eucalyptus oil 5%, 10% and 15% were used and human skin allergy was a limiting factor in the use of higher concentrations of eucalyptus oil. The maximum concentration of eucalyptus oil with petroleum ether solvent that should not cause skin allergy has been reported at 20% w/w (19). Furthermore, due to the two following reasons, lower doses of eucalyptus oil were used in this study, a, ethanol facilitates skin absorption when eucalyptus essential oils are diluted in ethanol; b, micro-emulsions of eucalyptus oil with particle diameter of 10 to 100 nm increases their penetration through the pores of the skin. In all three concentrations of 5%, 10%, and 15% w/w, average duration of protection against mosquitoes when using micro-emulsions of eucalyptus oil was significantly more than eucalyptus essential oils diluted in ethanol. A similar study by Sakulku with formulated citronella essential oil showed an increase in the time of protection against mosquitoes (20). Average duration of protection against mosquitoes when using micro-emulsions of eucalyptus oil was less than DEET diluted in ethanol at concentrations of 5%, 10%, and 15% w/w yet the difference was not significant. Although this solution form of DEET was not a good standard for the comparison, yet the peak of protection time of DEET (6 to 7 hours) against mosquitoes was at a concentrations of 20% to 30% w/w. Pleasant smell and odor of eucalyptus oil micro-emulsions at concentrations of 5, 10, and 15% w/w was acceptable for volunteers. Irritation and redness of volunteer skin did not occur by micro-emulsions of eucalyptus oil at concentrations of 5, 10, and 15% w/w. Micro-emulsions of eucalyptus oil at concentrations of 5%, 10%, and 15% w/w caused sticky and greasy skin. However, formulated eucalyptus oil micro-emulsions at the weight ratios of 2:1 of surfactant and co-surfactant was more tolerable than the 1:1 ratio of surfactant to co-surfactant for volunteers. In conclusion, thermodynamic and kinetic stability of eucalyptus oil micro-emulsions led to good time of protection against *Culicinae* and the micro-emulsion formulation resulted in a reduction in volatility of eucalyptus essential oil, delay in release of essential oil from formulation and reduction of evaporation. Furthermore, repellent materials remain for a longer duration in the air and subsequently the antennae of female mosquitoes are exposed to this material for a longer period and ultimately the duration of protection against mosquito bites is increased.

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Footnote

Conflict of Interests: The authors declare that they had no conflicts of interest.

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