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Original Article

Development, Characterization and Evaluation of a Stable *Eucalyptus globulus* Essential Oil–Permethrin Nanoemulsion with Repellent Activity Against *Aedes aegypti*

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Abstract

Background: *Aedes aegypti* is a primary vector of several clinically significant arboviruses. This study aimed to develop a nanoemulsion-based co-delivery system incorporating permethrin and *Eucalyptus globulus* essential oil for mosquito repellent applications.

Methods: The essential oil composition of *E. globulus* was determined using gas chromatography-mass spectrometry (GC-MS). Nanoemulsions were formulated using a low-energy spontaneous emulsification method and characterized for stability, droplet size distribution and zeta potential. The optimal formulation was further examined by transmission electron microscopy (TEM) and evaluated for cytotoxicity. The repellent activity of eucalyptus essential oil, permethrin, their combinations, and the eucalyptus essential oil-permethrin nanoemulsion was assessed against female *Ae. aegypti* mosquitoes using the arm-in-cage assay.

Results: The nanoemulsions exhibited a mean droplet size of 93.5 nm and a zeta potential of 60–70 mV, indicating good stability. Transmission electron microscopy (TEM) confirmed the presence of spherical and uniformly dispersed droplets. The eucalyptus essential oil-permethrin nanoemulsion demonstrated strong repellency. A 3% permethrin concentration in the eucalyptus-permethrin nanoemulsion provided the maximum protection rate at 91.8%. In stability testing, the 1.5% version of the same nanoemulsion proved to be the most stable.

Conclusion: *Eucalyptus globulus* essential oil, especially in nanoemulsion form and combined with permethrin, shows promise as a natural repellent formulation against *Ae. aegypti*. However, field trials are essential to confirm these findings under natural conditions.

Keywords: Essential oil-based nanoemulsion; Insect repellents; Dengue vector; Pyrethrins; Yellow fever mosquito

Introduction

Aedes aegypti is one of the most important vectors of arthropod-borne viral diseases, including dengue fever, chikungunya, Zika and yellow fever, worldwide. The global spread of this species in recent decades, influenced by factors such as urbanization, climate change, increased international travel and the high adaptability of this mosquito to human environments,

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has become a major public health concern (1). Currently, there is no specific antiviral treatment for dengue fever; management is supportive and dengue vaccines are under development, but available vaccines still have important limitations (2, 3). Therefore, prevention of mosquito bites remains one of the main pillars of mosquito-borne disease control.

Among personal protection methods, the use of mosquito repellents is considered one of the most effective strategies to reduce human- mosquito contact. Synthetic repellents such as DEET, picaridin, IR3535 and pyrethroid-based compounds have shown significant efficacy in preventing mosquito bites and are recommended by health organizations (4). DEET is the most commonly used commercial topical repellent. It is not insecticidal, but it serves as the gold standard in testing repellent effectiveness (5). Using insect repellent on clothing is preferable to using the substance on the skin, as it poses no skin risks and allows for higher doses to be used if needed (6).

However, concerns related to possible side effects, environmental impacts, unpleasant odors of some products and the increasing demand for environmentally friendly products have led to increasing attention to plant-based compounds. Essential oils extracted from aromatic plants have been considered as potential alternatives due to their natural origin and suitable biological activity. Among them, eucalyptus essential oil contains active compounds that can disrupt host-seeking behavior in mosquitoes and reduce landing and blood-feeding rates. However, high volatility, low water solubility and sensitivity to oxidation are the most important limitations of these compounds, which reduce their protection time compared to synthetic repellents (7, 8).

Permethrin, a synthetic pyrethroid, is widely used in public health mosquito control programs and residential applications because of its strong insecticidal activity against mosquitoes (9). Studies have shown that pyrethroid compounds can prevent mosquitoes from approaching the host by providing contact and spatial repellents. However, in recent years, studies have reported resistance of *Ae. aegypti* to this insecticide (10, 11). Certain plant essential oils can synergistically enhance the insecticidal activity of permethrin against adult *Ae. aegypti* mosquitoes, thereby improving its efficacy. Combining botanicals with synthetic compounds may produce synergistic effects, increasing efficacy while reducing the amount of chemical required (12).

In recent years, nanotechnology has provided new solutions to overcome the limitations of essential oils. Nanoemulsions can improve the protective effects of these materials by increasing the stability, solubility, controlled release and bioavailability of active ingredients. Various studies have shown that nanoemulsions containing essential oils have greater repellent and insecticidal activity against mosquitoes compared to conventional formulations (13, 14).

Nano-based delivery systems, including nanoemulsions, liposomes, and polymeric nanoparticles, can enhance the physicochemical stability of active compounds, enable controlled and sustained release, improve skin penetration and reduce the therapeutic dosage required to achieve the desired effect. Consequently, compared to conventional formulations, these systems may reduce local skin irritation and minimize systemic exposure (15). Regarding eucalyptus essential oil, nano-formulations have been shown to reduce volatility and degradation while enhancing antimicrobial and insecticidal activities (16). Similarly, nano-formulations of permethrin have demonstrated improved bio-efficacy at lower concentrations and reduced toxicity toward non-target organisms (17). Although these findings highlight the potential of nanotechnology to enhance efficacy and safety, further well-designed clinical studies are still required to confirm the safety and long-term therapeutic benefits of these nano-formulations in humans (15). This approach involves the use of plant-

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based compounds and stabilizing systems designed to maintain their efficacy over extended periods.

The combined use of a synthetic insecticide and a repellent plant essential oil within a nanoscale system is both a key focus of recent studies and the central hypothesis of the present research. Given that insecticide resistance and limited public acceptance of certain chemical compounds pose serious challenges, such systems can provide a better balance between efficacy, safety and consumer acceptance.

Accordingly, this study aimed to evaluate and compare the repellent efficacy of eucalyptus essential oil, permethrin, a eucalyptus + permethrin combination and a eucalyptus-permethrin nanoemulsion against adult *Ae. aegypti* mosquitoes in a controlled laboratory setting.

Materials and Methods

Extraction of essential oil of *Eucalyptus globulus*

Fresh leaves of *E. globulus* were collected from Bandar Abbas County (56º16′E, 27º11′N), Hormozgan Province, Iran. The collected plant material was initially air-dried at room temperature and subsequently ground into a fine powder using a mechanical grinder.

Eucalyptus essential oil (EEO) extraction was performed through hydrodistillation for 3 hours using a Clevenger-type apparatus according to standard protocol (18) in the Vector Control Laboratory, School of Public Health, Tehran University of Medical Sciences. The extracted essential oil was stored in amber vials at 4 °C until further analysis.

Preparation of eucalyptus nanoemulsion

Oil-in-water (O/W) nanoemulsions were formulated using eucalyptus essential oil as the dispersed phase, with a non-ionic surfactant (Tween 80), Ethanol (70%) as a co-surfactant, and water as the continuous phase (19). Compositions were varied systematically to identify ratios capable of producing optically clear and physically stable dispersions.

Polysorbate 80 (Tween 80) is a nonionic surfactant and emulsifier that is directly used in pharmaceutical and topical nanoemulsion formulations due to its excellent safety profile, low skin irritation potential and high biocompatibility (20, 21). It provides solubility, stability, and bioavailability of poorly soluble compounds and is particularly used in pharmaceuticals, vaccines and nutritional therapies (22). Furthermore, Tween 80 is promising in targeted drug delivery systems (22, 23) and is particularly used as an emulsifier in food and cosmetic products (24).

Nanoemulsions were generated using a low-energy spontaneous emulsification approach (25). During formulation, the oil phase was premixed with the surfactant, followed by incorporation of the co-surfactant. The aqueous phase was subsequently introduced under gentle agitation to promote the development of nano-sized droplets.

To select the optimal eucalyptus nanoemulsion, different ratios of essential oil, emulsifiers and distilled water were prepared. After preparation, the nanoemulsions were kept at room temperature for 24 hours; then the sample that remained transparent was used in the permethrin loading stage with different concentrations; and finally, the eucalyptus-permethrin nanoemulsion that was transparent was examined and studied for stability tests for two months.

Preparation of eucalyptus–permethrin nanoemulsion

Nanoemulsions containing eucalyptus essential oil and permethrin were formulated using a spontaneous emulsification approach. In this method, the oil phase comprising eucalyptus oil and surfactant was blended to obtain a homogeneous mixture, after which a co-surfactant was incorporated. The aqueous phase, containing distilled water and permethrin, was subsequently introduced under controlled mix-

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ing conditions to promote the formation of nano-sized droplets. A magnetic stirrer set at 800 rpm was utilized throughout the entire process to ensure uniform mixing and droplet formation at the Department of Advanced Technologies, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran.

To prepare the nanoemulsion via the spontaneous emulsification method (25), a magnetic stirrer was used at room temperature with a stirring speed of 800 rpm for one hour (19). The materials employed included 5% oil (eucalyptus essential oil), 20% Tween-80 (surfactant), 3.8% ethanol (co-surfactant), 65.2% deionized water and 1.5% permethrin. First, an appropriate amount of eucalyptus essential oil was placed in a penicillin vial. Then, the selected surfactant was added to the oil extract over a period of 30 minutes. At this stage, permethrin was added, followed by ethanol (as a co-surfactant) in a specific volume; finally, distilled water (as the aqueous phase) was added dropwise to the mixture over one hour. A magnetic stirrer operating at 800 rpm was used throughout the process. The formulation was mixed until complete dispersion was achieved at room temperature. Tween-80 was used as the surfactant to enhance efficacy. Following the preparation of the nanoemulsions, the samples were stored at room temperature for 24 hours to allow for the observation of any sedimentation, creaming, or flocculation. Formulations were visually inspected and only those exhibiting clarity and absence of phase separation were selected for subsequent physicochemical analyses and stability evaluation (19).

Initial physical stability assessment

Following preparation, all formulations were mixed until visually homogeneous, and then the Samples were stored at room temperature for 24 hours to assess their initial physical stability. The occurrence of instability phenomena such as sedimentation, creaming and flocculation was evaluated visually. Only samples that remained transparent and showed no signs of phase separation after 24 hours were selected for thermodynamic stability tests and particle size analysis.

Droplet size and distribution analysis

The mean hydrodynamic diameter and particle size distribution of the nanoemulsions were determined using Dynamic Light Scattering (DLS) technology. The analysis was performed using a DLS-9900 instrument (manufactured by K-ONE, South Korea). Nanoemulsion samples were placed in a quartz cuvette for individual analysis. Measurements were conducted at a temperature of 25 °C and a fixed scattering angle of 90° on days 1, 10, 30 and 60 after preparation. The acquired data, based on the light intensity scattered by the nanoparticles, provided the mean particle diameter (d50) in nanometers and the corresponding particle size distribution plot.

Thermodynamic stability assessment

Thermodynamic stability was assessed through temperature-dependent cycling procedures. In the heat storage test, the transparent formulations were kept at 40 °C for two months following production. Similarly, in the cold storage test, the clear formulations were stored at 4 °C for two months after production. Furthermore, during the room temperature storage test, the transparent formulations were maintained at 25 °C for two months after production. On days 1, 10, 30 and 60, the nanoemulsions' transparency, phase separation and droplet diameter were evaluated.

Following both 60-day cycles, the formulations that retained their clarity and showed no instability after the full evaluation period were identified as thermodynamically stable nanoemulsion formulations and advanced to further characterization.

Physicochemical characterization

pH measurement: The pH of each nanoemulsion was determined using a pH meter calibrated for aqueous dispersions. Measurements were conducted on the undiluted formulations

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to assess their acidity or alkalinity within the expected physiological range.

Viscosity determination: Viscosity was evaluated using an appropriate viscometric method at controlled temperature conditions. Measurements were performed on representative aliquots of each formulation to characterize rheological behavior relevant to stability and application performance.

Turbidity analysis: Turbidity was determined using a Spectrophotometer (model: IZI, K-Lab, Korea) to measure the light absorbance at 600 nm.

Zeta potential measurement: Zeta potential analysis was conducted to determine the surface charge of the dispersed droplets in the Comprehensive Research Laboratory of East of Iran, Mashhad University of Medical Sciences. This parameter was used as an indicator of electrostatic stability, providing information about the likelihood of droplet aggregation within the colloidal system.

Morphological characterization: The morphology and structural characteristics of the nanoemulsion droplets were examined using transmission electron microscopy (TEM; model LEO-910, Germany) by the Comprehensive Research Laboratory of East of Iran, Mashhad University of Medical Sciences. Micrographs were used to visualize droplet shape, confirm nanoscale dimensions and support DLS-derived particle size data.

Gas chromatography-mass spectrometry analysis

The chemical composition of the essential oil was determined using gas chromatography-mass spectrometry (GC-MS) on a Shimadzu GC-MS-QP2010 system. The instrument was equipped with an RTX-5MS capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness). The carrier gas was helium at a flow rate of 0.9 mL/min. The initial oven temperature was set at 50 ºC for 2 min and ramped at 3 ºC/min to 250 ºC. The injector was set to 250 ◦C.

Mass spectrometry conditions were ionization energy: 70 eV, MS interface temperature 250 °C, Detector voltage 0.9 kV, Mass range 35–300 m/z, Scan time 0.5 s/scan, Scan speed 769 u/s and Acquisition interval 0.50 s (2 Hz).

Compound identification and quantification

Retention indices (RI) were calculated using the retention times of a homologous series of n-alkanes (C_5–C_{30}) injected under identical chromatographic conditions, following the Van Den Dool and Kratz method. Individual compounds were identified by comparing their calculated RIs and mass spectral data with those available in the NIST and Wiley mass spectral libraries, as well as published literature values for RTX-5MS and similar non-polar columns. Quantitative analysis was performed based on the relative peak areas obtained from the GC chromatogram (7), with results expressed as percentages of the total essential oil composition. The major components were identified based on their relative abundance and characteristic mass spectral dispersion patterns. GC-MS and Compound identification and quantification were performed at the Natural Products and Medicinal Plants Research Center, North Khorasan University of Medical Sciences.

Cytotoxicity assessment

Cell culture conditions: Mouse fibroblast cells (L-929) were obtained from Gene Iran and cultured in T25 plastic flasks containing Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was replaced four times a week to maintain optimal growth conditions. Cell counting was performed using a hemocytometer.

MTT assay: To determine cytotoxicity, the required number of cells was trypsinized and seeded into three 96-well plates, with each well containing 4.1×10^4 cells. The cells were incubated overnight under the aforementioned conditions. Following incubation, the medium

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was replaced with eucalyptus nanoemulsion (5%) or eucalyptus-permethrin nanoemulsion (1.5%) treatments.

The cytotoxic effects on fibroblast cells were evaluated using the MTT reduction assay at 24, 48 and 72 hours post-treatment. This assay relies on the conversion of the MTT salt into the insoluble formazan product. For the assay, 100 µL of the MTT stock solution (5 mg/ml) was added to each well, and the plates were incubated for 4 hours at 37 °C. Subsequently, 100 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was then measured at a wavelength of 570 nm using a microplate reader (Fig. 1). This test was performed at the Central Laboratory of Tehran University of Medical Sciences.

Repellent assays

Mosquito rearing

Aedes aegypti mosquitoes were reared at 27–28 °C, 65–75% relative humidity and a 12:12h light: dark photoperiod in the insectary of the Bandar Abbas Health Education and Research Station, Tehran University of Medical Sciences, Iran. The Bandar Abbas population of *Ae. aegypti* larvae was bred from a laboratory colony. Larvae were fed with fish food. The pupae were transferred to 50×50× 50 cm cages and were provided with a 10% sugar solution via soaked tissue paper. Female mosquitoes were blood-fed on a rabbit twice weekly (26).

Compositions and concentrations

The treatments and concentrations used in the arm in cage tests were as follows: eucalyptus essential oil at concentrations of 1%, 3%, 5%, 7% and 10%; permethrin at concentrations of 0.25%, 0.75%, 1.5% and 3%; and combinations of eucalyptus essential oil (5% and 10%) with permethrin (1.5% and 3%). Concentrations of 1%, 3%, 5%, 7% and 10% eucalyptus essential oil were used to prepare the optimal eucalyptus nanoemulsion. Finally, the 5% formulation was selected because of its transparent appearance, and eucalyptus-permethrin nanoemulsion was prepared.

Arm-in-cage tests

Mosquito repellency was evaluated using the arm-in-cage method, following the standard protocol established by the World Health Organization (27). Two- to five-day-old unfed female *Ae. aegypti* mosquitoes were used in the repellent assays. The repellent activity of eucalyptus essential oil, permethrin, their combination and the eucalyptus-permethrin nanoemulsion was evaluated in a 50×50×50 cm cage equipped with transparent mesh, allowing direct observation of mosquito landing and biting behavior. Fabrics impregnated with the treatments were prepared, then the fabrics were shaped into gloves. Volunteers (including five males and five females) washed their hands with unscented soap and water, disinfected with 70% alcohol and allowed to dry. Each volunteer then inserted their hand into a glove up to the wrist, and the glove’s end was secured with a rubber band. The gloved hand was placed inside the cage for 3 minutes, which contained thirty 2- to 5-day-old unfed female mosquitoes that had not been provided with blood meals or sugar solution. Cotton fabric without any active ingredient served as the negative control (one control replicate for each concentration tested). Tests were performed in triplicate biological replicates for each concentration. The mosquito repellency test was performed according to a modified WHO protocol. Each evaluation comprised three biological replicates, each consisting of a fresh group of 30 unfed female mosquitoes. The percentage protection provided by the samples was calculated using the following equation (27):

$$\text{Protection \%} = \frac{C-T}{C} \times 100$$

Where C is the number of landings in the control sample, and T is the number of landings in the treatment.

https://doi.org/10.18502/jad.v20i2.22409

Results

Chemical composition of *Eucalyptus globulus* essential oil

The chemical composition of *E. globulus* essential oil was analyzed by GC-MS. The constituents were identified by comparing their mass spectra and retention indices (RI) with those reported in spectral libraries and published literature. Quantitative analysis was performed based on the relative peak areas obtained from the GC-MS chromatogram, and the composition of each constituent was expressed as a percentage of the total essential oil.

A total of 30 compounds were identified, accounting for 83.74% of the total essential oil. The identified constituents and their relative percentages are presented in Table 1, while the corresponding GC–MS chromatogram is shown in Fig. 2.

The essential oil was predominantly composed of oxygenated monoterpenes, with eucalyptol (1,8-cineole) identified as the major constituent, followed by monoterpene hydrocarbons. Eucalyptol was the most abundant compound, representing 28.33% of the total oil, with a retention time (RT) of 17.54 min. Other major constituents included α-pinene (6.92 %), spathulenol (5.07%) and terpinen-4-ol (4.29 %). The earliest eluting compound was α-thujene (RT= 15.40 min), whereas β-eudesmol exhibited the longest retention time (RT= 26.86 min).

Overall, the chemical profile of *E. globulus* essential oil was characterized by a high proportion of oxygenated monoterpenes, particularly 1,8-cineole, which is recognized as the principal bioactive constituent responsible for many of the biological and pharmacological properties attributed to eucalyptus essential oils.

Characterization of the optimized nanoemulsion, transmission electron microscopy (TEM) and zeta potential analysis

Based on preliminary optimization studies aimed at maximizing stability and minimizing droplet size, a nanoemulsion formulation containing 20% Tween 80, 8.3% Ethanol, 5% eucalyptus essential oil, 1.5% permethrin and deionized water was prepared. The DLS analysis of eucalyptus nanoemulsion (Fig. 3A) and eucalyptus-permethrin nanoemulsion (Fig. 4A) showed a median particle diameter (d_{50}) of 155 nm and 93.5 nm, respectively.

The average particle size of eucalyptus-permethrin nanoparticles is less than 100 nm, which is generally considered optimal for enhancing stability and bioavailability in pharmaceutical formulations and insect repellent products. The role of Tween 80 here is as a nonionic surfactant, reducing the interfacial tension between the oil and water phases and stabilizing the particles at the nano-size. This prevents the coalescence of the droplets and the formation of a separated phase.

The eucalyptus and eucalyptus-permethrin nanoemulsion exhibited a single, narrow zeta potential peak centered at approximately 60–70 mV (Figs. 3B and 4B), indicating a highly stable formulation with strong electrostatic repulsion between droplets.

The heterogeneous globule distribution and droplet aggregation were investigated using TEM. The produced nanoemulsion was stable, nearly spherical and widely distributed (Figs. 3C and 4C).

The nanoemulsion particles appear as bright and dark spots scattered in the background, indicating the presence of a large number of spherical particles with a nanometer size. The particle size in this field is in the range of approximately 50 to 160 nm, and most of the particles have a uniform distribution over the image surface. This uniformity and the absence of significant aggregation between the particles indicate that the system has high colloidal stability. The homogeneous dispersion of the particles and their relatively spherical shape are the result of the effective performance of Tween 80 as a nonionic surfactant, which has prevented the aggregation of droplets by reducing the interfacial tension between

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the aqueous and oil phases. This feature creates a stable and uniform nanoemulsion.

Cytotoxicity assay

Statistical analysis by one-way ANOVA, followed by Tukey's post hoc test, revealed no significant differences (P= 0.5299) in cell viability or mortality rate between the control group and any of the treatment groups (nano-eucalyptus, eucalyptus-permethrin nanoemulsion, and permethrin) at any of the assessed time points (24, 48 and 72 hours) (Fig. 5).

Repellent assays

The protection percentages provided by different concentrations of eucalyptus essential oil, permethrin and their combinations applied to cotton fabric against *Ae. aegypti* bites showed that the highest level of protection (93.5%) was achieved with 10% eucalyptus essential oil combined with 3% permethrin (Fig. 6).

Concentrations of 1%, 3%, 5%, 7% and 10% eucalyptus essential oil were used to prepare the optimal eucalyptus nanoemulsion. Finally, the 5% formulation was selected because of its transparent appearance and was used to prepare a eucalyptus-permethrin nanoemulsion.

Figure 7 shows the protection rates provided against adult *Ae. aegypti* mosquitoes by the eucalyptus essential oil-permethrin nanoemulsion.

Stability tests of eucalyptus-permethrin nanoemulsions at 4, 25 and 40 °C over 2 months showed that the eucalyptus-permethrin nanoemulsion 1.5% had greater stability than the other formulations, as indicated by its transparent appearance (Fig. 8).

Table 1. Chemical compounds of *Eucalyptus globulus* leaves essential oil collected from Bandar Abbas County, Hormozgan Province, south of Iran, 2024–2025

No.	Name	Compositions (%)	RI*
1	Alpha-Thujene	0.62	924.56
2	Alpha-pinene	6.92	937.42
3	Beta-myrcene	1.32	985.38
4	Alpha-phellandrene	0.77	1002.76
5	Para-Cymene	2.2	1020.44
6	Eucalyptol	28.33	1052.48
7	Beta-terpinyl acetate	2.95	1076.24
8	Gamma-terpinene	1.71	1085.082
9	Alpha-terpinolene	1.46	1103.100
10	Isoamyl-isovalerate	1.20	1108.91
11	Fenchol	0.55	1121.31
12	Trans-pinocarveol	4.0	1151.55
13	pinocarvone	1.22	1169.76
14	Alpha-terpineol	0.91	1177.51
15	Terpinene-4-ol	4.29	1189.92
16	Para-Cymen-8-ol	1.83	1200.14
17	Trans-Carveol	0.62	1226.78
18	Trans-caren-2-ol	2.54	1238.98
19	Carvacrol	0.68	1288.095
20	Thymol	0.66	1304.18
21	Alpha-terpinyl acetate	1.61	1354.81
22	Aromadendrene	1.77	1447.84
23	Dehydro-aromadendrene	1.30	1467.74
24	Phenylethyl isovalerate	0.95	1493.54
25	Gamma-elemene	1.29	1503.015
26	Epiglobulol	1.05	1565.32

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Table 1. Continued ...

27	Spathulenol	5.07	1598.99
28	Globulol	2.93	1602.14
29	Viridifelorol	2.03	1603.86
30	Beta-Eudesmol	0.96	1611.60
	Total	83.74	

*RI: Retention index. The RI along with the retention time (RT) was used to identify the chemical components of eucalyptus essential oil by comparison with the reference.

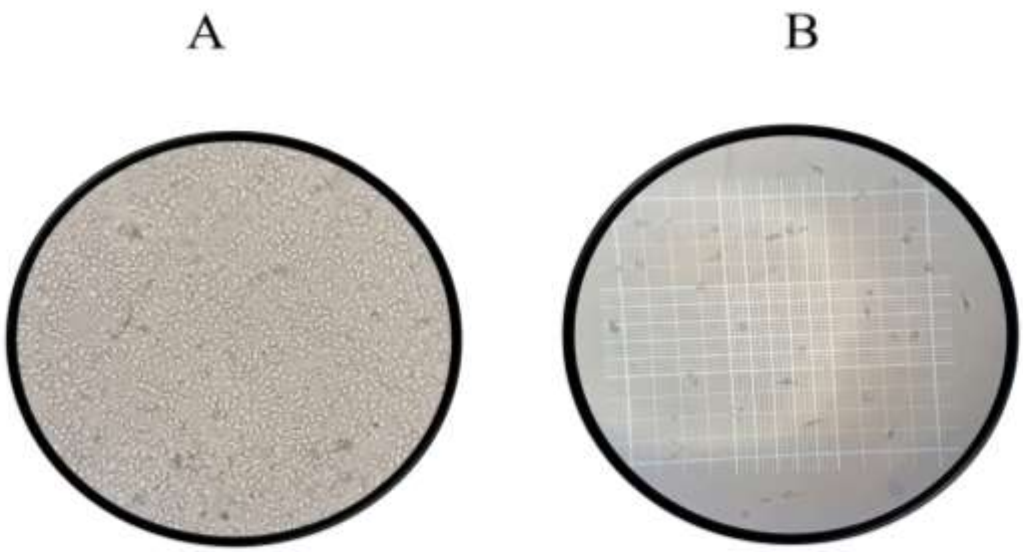

Fig. 1. Cytotoxicity assay conducted at the Central Laboratory of Tehran University of Medical Sciences, Tehran, Iran, in 2025. (A) Cultured L929 fibroblast cells. (B) Counting of L929 cells using a hemocytometer

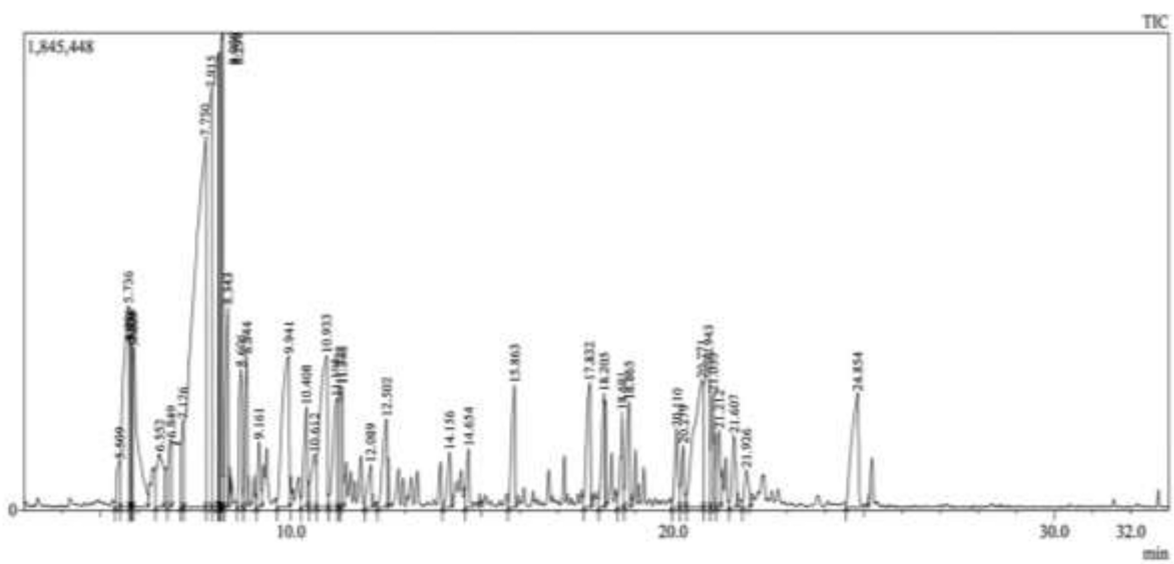

Fig. 2. Gas Chromatography-Mass Spectrometry (GC-MS) fingerprint of *Eucalyptus globulus* essential oil. GC–MS analysis was performed at the Natural Products and Medicinal Plants Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran, 2024–2025

*Retention time (RT): Is defined as the time required for a compound to pass through the chromatographic column and reach the detector after sample injection

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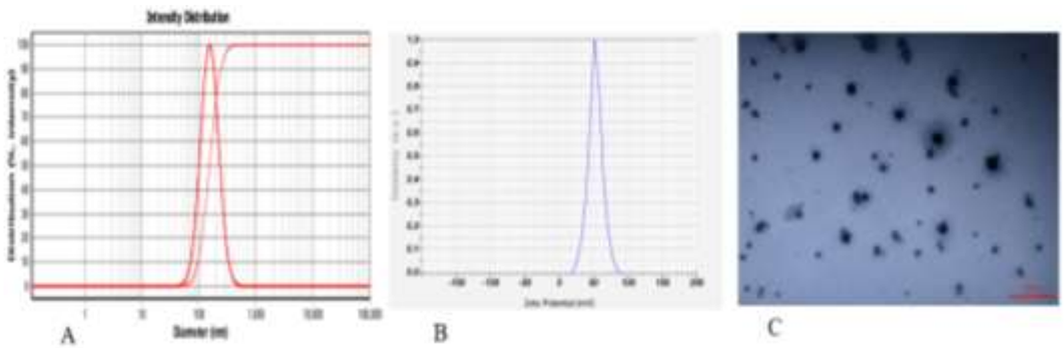

Fig. 3. Characterization of the *Eucalyptus globulus* nanoemulsion. (A) Dynamic light scattering (DLS) profile obtained using the facilities of the Department of Advanced Technologies, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran, 2024–2025. (B) Zeta potential measurement and (C) Transmission electron microscopy (TEM) image obtained at the Comprehensive Research Laboratory of East of Iran, Mashhad University of Medical Sciences, 2024–2025

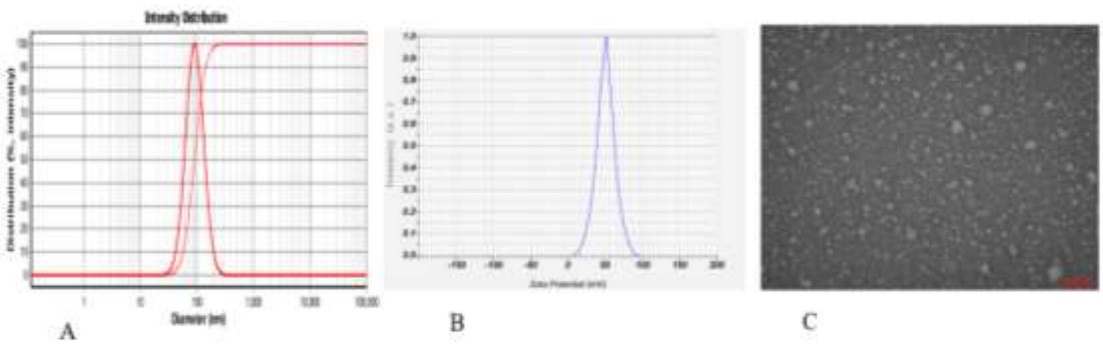

Fig. 4. Characterization of the *Eucalyptus globulus* essential oil-permethrin nanoemulsion. (A) Dynamic light scattering (DLS) profile obtained using the facilities of the Department of Advanced Technologies, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran, 2024–2025. (B) Zeta potential profile and (C) Transmission electron microscopy (TEM) image obtained at the Comprehensive Research Laboratory of East of Iran, Mashhad University of Medical Sciences, 2024–2025

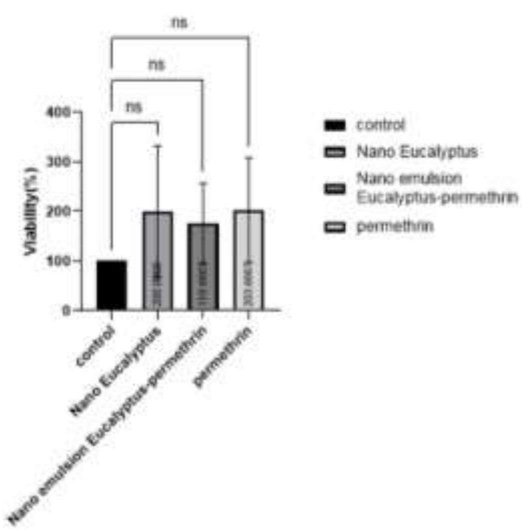

Fig. 5. Cell viability (%) of L929 fibroblast cells after treatment with different formulations. The cytotoxicity assay was performed at the Central Laboratory of Tehran University of Medical Sciences, Tehran, Iran, 2025

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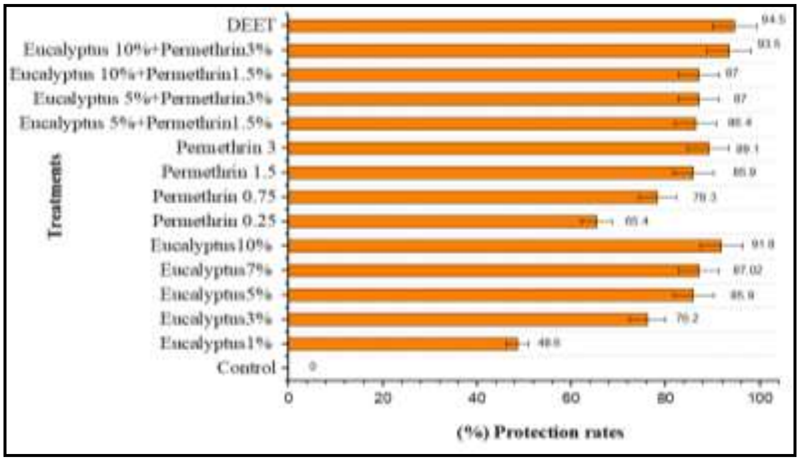

Fig. 6. The protection rate of adult *Aedes aegypti* after exposure to various treatments, in the insectary of the Bandar Abbas Health Education and Research Station, Tehran University of Medical Sciences, Iran, 2025–2026

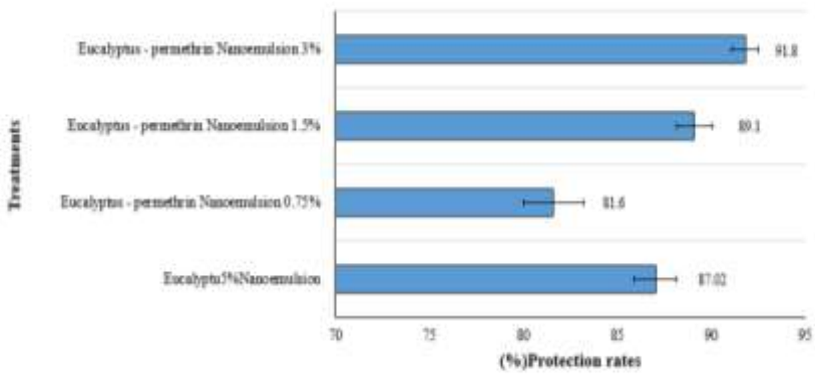

Fig. 7. The protection rates of adult *Aedes aegypti* after exposure to 5% eucalyptus nanoemulsion and permethrin loaded in eucalyptus nanoemulsion in the insectary of the Bandar Abbas Health Education and Research Station, Tehran University of Medical Sciences, Iran, 2025–2026

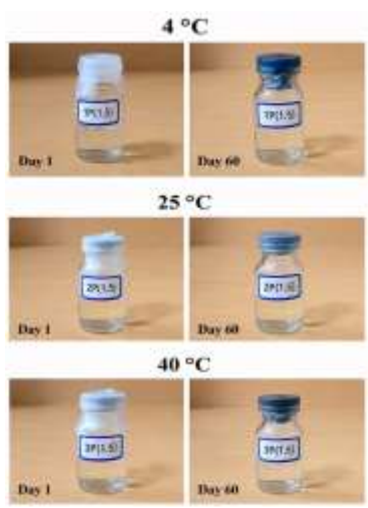

Fig. 8. Stability evaluation of the 1.5% *Eucalyptus globulus* essential oil-permethrin nanoemulsion on days 1 and 60. The evaluation was conducted at the Department of Advanced Technologies, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran, 2024–2025

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Discussion

To reduce environmental impact and improve the effectiveness of insecticides on intended species, experts recommend adopting innovative solutions, such as nano-formulations (28, 29). The current study successfully formulated and characterized stable nanoemulsions containing *E. globulus* essential oil and its combination with permethrin. GC-MS analysis identified 30 compounds, which together accounted for 65.19% of the essential oil's composition. The major compound was eucalyptol (1,8-cineole), representing 28.33% of the total. This finding is in accordance with earlier reports on *E. globulus* oil, which has been widely recognized for its exceptionally high content of eucalyptol (30–32) and its associated activities, such as insecticidal and acaricidal activities (7, 33–35).

In previous studies, the repellent properties of essential oils from *Melissa officinalis*, *Rosmarinus officinalis*, *Lavandula officinalis*, *Citrus limonum*, *E. globulus* and *Ocimum basilicum* were evaluated alongside DEET using animal models against *Anopheles stephensi*. Among the tested essential oils, *E. globulus* exhibited the strongest repellent activity, achieving 97.15% protection at a 3% concentration (36).

DEET is recognized as the gold standard repellent for *Ae. aegypti* mosquitoes; it demonstrates high repellent efficacy under laboratory conditions, with an ED_{90} value of approximately 24 µg/cm² (37). However, its effectiveness can vary across different mosquito populations; this variation is attributed to genetic differences in olfactory receptors that alter behavioral responses to DEET (38). Furthermore, exposure to DEET can lead to habituation and a reduction in its repellent properties, such that nearly 50% of mosquitoes previously exposed to the substance no longer respond to it (39).

Dynamic light scattering (DLS) studies confirmed the nano-sized droplet distribution for both eucalyptus-permethrin and eucalyptus-only nanoemulsions, with a smaller median diameter observed for the eucalyptus-permethrin nanoemulsion (93.5 nm), compared with the eucalyptus-only nanoemulsion (155 nm). This smaller droplet size for the eucalyptus-permethrin nanoemulsion could be attributed to the interfacial interaction between the oil phase, surfactant (Tween 80) and co-surfactant (ethanol), which might be responsible for a higher packing efficiency at the droplet interface. Moreover, the literature suggests that a decrease in nanodroplet size correlates with an increase in larvicidal activity (40, 41).

The physicochemical properties of nanoemulsions, particularly their small droplet size, play a key role in enhancing formulation stability and performance. Therefore, the improved efficacy observed in the present study may be attributed, at least in part, to these structural characteristics (19).

In our study, both eucalyptus-permethrin and eucalyptus-only nanoemulsions were found to possess high absolute values of zeta potential, ranging from -60 mV to -70 mV, which is a significant indicator of high stability for both nanoemulsions. According to the classical DLVO theory, high absolute values of zeta potential indicate high repulsive forces between the droplets, which prevent aggregation and coalescence of the droplets over time. The spherical nature of the droplets, as confirmed through the TEM images, further confirms the formation of stable, well-defined nano-droplets, aligning with the DLS and zeta potential findings.

Plant essential oils have been reported to enhance the toxicity of permethrin against adult *Ae. aegypti* mosquitoes. This likely occurs through facilitated penetration of the insecticide across the cuticle or interference with detoxification pathways. Such synergistic agents can improve insecticidal efficacy while enabling the use of lower permethrin doses, thereby reducing the selection pressure for insecticide resistance (42).

Contrary to previous reports of eucalyptus essential oil toxicity (0.5–5%) in various cell

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lines (for example, HEK293T, HepG2, Vero) (43, 44), our nanoformulations showed no significant cytotoxic effect. It is also interesting that the encapsulation of permethrin in the nanoemulsion did not increase the cytotoxicity of the formulation, which is a positive factor in the development of safe forms of synthetic insecticides.

The findings of this study showed that different concentrations of eucalyptus essential oil, permethrin, and their combinations in cotton fabrics had an acceptable effect in protecting against *Ae. aegypti* bites. The increase in the percentage of protection with increasing concentration indicates a clear dose-response relationship. Such a pattern is expected in repellent compounds, because with increasing the amount of active ingredient, the probability of diffusion of active molecules into the environment and their contact with mosquito chemoreceptors increases. Eucalyptus essential oil is thought to reduce mosquito attraction by interfering with olfactory-mediated host recognition, thereby decreasing landing and biting behavior (45, 46). Permethrin can also enhance this effect with its neurological and behavioral effects. Therefore, the observation of the highest protection in the combination of 10% eucalyptus + 3% permethrin could be due to enhanced activity of the two compounds.

The clarity of a nanoemulsion usually indicates small droplet size, more uniform distribution of the oil phase, and better stability of the system. These characteristics are advantageous for topical and coating applications, especially on fabrics, as such formulations usually have better diffusion, better adhesion and more controlled release of the active ingredient. Stability is one of the main challenges in the development of formulations based on plant essential oils, as volatile compounds in essential oils are easily subject to evaporation, oxidation, or phase separation (47). The retention of clarity over time indicates that the 1.5% formulation was able to better maintain its nanoemulsion structure. Therefore, although the 3% concentration produced a greater protective effect in the bioassay, 1.5 % appears to be a more desirable option in terms of stability and possibly industrial scalability.

The emergence and widespread dissemination of insecticide resistance in *Ae. aegypti* populations have significantly compromised the efficacy of conventional vector control strategies, particularly those relying on synthetic insecticides (10, 11). This growing resistance underscores the urgent need for alternative or complementary control approaches. In this context, plant-derived essential oils, especially when formulated as nanoemulsions, offer a promising strategy. Moreover, their combination with insecticides may provide synergistic or additive effects, potentially reducing the required dose of synthetic insecticides while improving overall repellency and control efficacy. Therefore, nanoemulsified botanical formulations in combination with conventional insecticides represent a sustainable and effective approach for overcoming resistance challenges in *Ae. aegypti* management.

The previous studies showed that nanoemulsion-based formulations of plant-derived compounds can enhance mosquito repellency by improving stability, dispersion and controlled release of volatile active ingredients. For example, nanoemulsion gel formulations based on *Ligusticum sinense* essential oil have been reported to show repellent activity against *Ae. aegypti* and other mosquito species, supporting the notion that nanoscale delivery systems can improve the practical efficacy of botanical repellents (48).

The increased efficacy observed in this study can be attributed to the simultaneous use of permethrin and eucalyptus essential oil within a nano-emulsion system. In such formulations, permethrin exerts insecticidal activity by acting on voltage-gated sodium channels (49), whereas eucalyptus essential oil acts as a repellent through behavioral and olfactory mechanisms. The nanoemulsion acts as an efficient carrier for both active ingredients, enhancing stability, facilitating controlled release and improving their interaction with the target insect. Similar approaches have demonstrated that the co-encapsulation of synthetic insecticides and plant-based repellents improves the formulation's persistence on treated fabrics while enhancing both insecticidal and repellent efficacy (25).

Furthermore, it has been suggested that encapsulated combinations of synthetic and plant-based repellents can reduce the required dosage of individual active ingredients and, by providing multiple modes of action, potentially delay the development of insecticide resistance (50). These findings underscore the potential of nanoemulsion-based combination formulations as an effective strategy for enhancing efficacy, extending the duration of protection and reducing reliance on high doses of synthetic insecticides.

Moreover, the controlled-release characteristics of nanoemulsions can extend the duration of protection for treated textiles by slowing the rapid loss of the volatile repellent, thereby prolonging their effective lifespan and enhancing user safety (50). It has been reported that synergistic interactions between essential oils and permethrin enhance insecticidal efficacy, suggesting that such combination formulations may allow for the use of lower insecticide doses while simultaneously reducing environmental impact (43).

Overall, the results of this study suggest that eucalyptus-permethrin nanoemulsion can be considered an effective and promising formulation for protection against *Ae. aegypti*. Higher concentrations may provide more protection, but formulations with medium concentrations, such as 1.5%, are superior in terms of stability and are more suitable for final product development. Therefore, this study supports the combination of natural and synthetic compounds in the form of nanoemulsion for the control of vector mosquitoes and can be the basis for further semi-field and field studies.

Conclusion

From a practical perspective, these results suggest that eucalyptus essential oil could be considered a natural and acceptable option for developing mosquito repellents, especially when formulated as a nanoemulsion. Combining plant essential oils with low-dose synthetic agents may be an effective approach to designing more stable, potent and potentially safer products. Given the growing concerns about insecticide resistance and the limitations of certain chemical repellents, such formulations could play a significant role in vector control programs. However, it should be noted that the results of laboratory tests are not necessarily fully generalizable to field conditions. Factors such as sweating, temperature, humidity, skin type, duration of contact and environmental conditions can influence the actual performance of the formulation. Therefore, further studies under semi-field and field conditions, along with evaluations of long-term stability, skin safety, and durability of effect, are necessary to confirm the practical application of this nanoemulsion. Overall, the findings of this study indicate that a eucalyptus nanoemulsion combined with permethrin could be a promising formulation for repelling *Ae. aegypti*. These results support the development of novel approaches based on natural compounds and nanoformulations for controlling disease vectors.

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Ethical considerations

The study was approved by the Ethics Committee of Tehran University of Medical

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Sciences under the code IR.TUMS.AEC.1402. 155. All procedures involving animals were performed in accordance with the approved animal welfare guidelines and ethical standards.

Conflicts of interest statement

The authors declare that there is no conflict of interest.

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