Original Article

Comparative Effects of *Elettaria cardamomum* Essential Oil and Its Nanoliposomal State on Mortality of *Anopheles stephensi* Larvae

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Abstract

Background: Malaria has remained the most dreadful vector-borne disease; hence, vector control is the most affordable and achievable approach to mitigate the disease burden. Due to the emergence of resistance and environmental pollution, herbal larvicides are considered an alternative to chemical types. Also, nanotechnology has been proposed as a promising solution to improve the efficiency of plant larvicides. This study aimed to develop an effective herbal larvicide.

Methods: The chemical composition of *Elettaria cardamomum* essential oil (EO) was first investigated. Nanoliposomes containing the EO were then prepared using the ethanol injection method. After that, the larvicidal efficacy of the EO and its liposomal state were compared against *Anopheles stephensi* in laboratory conditions.

Results: Alpha-terpinyl acetate (77.59%), eucalyptol (4.38%), nerolidol (2.96%), linalool (1.77%), and limonene (1.69%) were the five major compounds of the EO. Nanoliposomes containing the EO with a particle size of 73±5 nm and a zeta potential of -16.3±0.8 mV were prepared. Additionally, the ATR-FTIR analysis verified the successful loading of the EO into nanoliposomes. The larvicidal activity of nanoliposomes exhibited remarkable potency, with an LC_{50} value of 14.35 (10–18) µg/mL, significantly more potent than the non-formulated EO, which had an LC_{50} value of 33.47 (28–39) µg/mL against *Anopheles stephensi* larvae.

Conclusion: The nanoliposomes containing *E. cardamomum* EO showed promising efficacy against *An. stephensi* larvae. It could thus be considered for further application against other species of mosquitoes.

Keywords: Mosquito-borne diseases; Malaria; Nanotechnology; Cardamom

Introduction

Despite the advancements in medical and health sciences, a staggering number of malaria cases and deaths were recorded from 2000 to 2021, totaling approximately 2 billion cases and 11.7 million deaths (1). In 2021 alone, 247 million cases and 619,000 deaths were reported across 84 malaria-endemic countries. It is worth noting that nearly 96% of all malaria-related deaths worldwide occurred in just 29 countries. However, in 2022, a significant milestone was achieved as countries and partners raised an unprecedented amount of US$ 15.9 billion for malaria control through the largest replenishment in the history of the Global Fund (1).

Anopheles stephensi, a malaria vector known for its invasive nature, was originally confined to South Asia and certain areas of the Arabian Peninsula. However, there have been document-

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ed reports of its presence in Djibouti, Ethiopia, Sudan, Somalia, and Nigeria (2). Vector control has become a prominent strategy for malaria combat (3). A comprehensive approach known as integrated vector management (IVM) has gained significant attention, which includes various methods like using insecticide-treated mosquito nets (ITN), indoor residual spraying (IRS), repellents, and larviciding. IVM is the central strategy in this endeavor (4, 5). However, *An. stephensi* has become resistant to most insecticides used in public health (6–9). Therefore, herbal insecticides have recently been reconsidered (10, 11). Essential oils (EOs) are liquid oils secreted as secondary metabolites in aromatic plants, with larvicidal properties and other biological traits such as antibacterial, antileishmanial, and antioxidant effects (12, 13). For instance, mosquito larvicidal and repellent effects of *Elettaria cardamomum* (ginger family) as a common flavor and medicinal plant have been reported (14, 15). Furthermore, the loading of EOs in nanoparticles (polymeric or lipid particles) has been proposed as a promising approach for the stability and efficacy improvement of EOs. Nanoliposomes with high drug loading capacity, biocompatibility, and biodegradability have recently received attention as carriers for EO-based larvicides (16, 17). As far as the authors know, no reports on nanoliposomes containing *E. cardamomum* EO have been published.

This study first investigated the larvicidal effects of *E. cardamomum* EO. An attempt was then made to improve efficacy by preparing the nanoliposomal state of *E. cardamomum* EO.

Materials and Methods

Materials

The study acquired the following materials: absolute ethanol, tween 20, fat wool cholesterol, and Egg yolk lecithin from Merck Chemicals Co. (Germany). Besides, *E. cardamomum* EO was purchased from Green Plants of Life Co. Ltd. (Iran). The plant has been cultivated in the fields of Kerman, and the EO was extracted from seeds by hydro-distillation. For the larvicidal bioassays, late 3rd and 4th instar larvae of *An. stephensi* (Kazerun strain) was used. These larvae have been maintained at the insectary of Shiraz University of Medical Sciences (Iran) since 2015 and have not been exposed to any insecticides. They exhibit complete sensitivity to temephos (0.25 mg/L) and Chlorpyriphos (0.025 mg/L). The larvae were reared under controlled conditions at 27±1 ºC with a 12:12 light/dark photoperiod, 70±5 % relative humidity, and provided with powdered fish food daily until they reached the pupation stage.

Chemical composition of *Elettaria cardamomum* EO by GC-MS analysis

The gas chromatography device employed in this study was the Agilent 6890, equipped with a BPX5-type column measuring 30 m in length, 0.25 µm in inner diameter, and 0.25 µm in layer thickness (18). To analyze the compounds present in *E. cardamomum* EO, the sample was diluted using n-hexane and then injected into the GC-MS machine using a 1 µL volume. The initial oven temperature was set at 50 °C and held for 5 minutes, followed by a thermal gradient of 3 degrees per minute, increasing the temperature to 240 °C, and then increasing at a rate of 15 degrees/minute. Finally, the temperature was raised to 300 °C and held at this value for 3 minutes. The injection chamber temperature was set at 250 °C, with a split ratio of 1:35 and He gas was utilized as the carrier gas at a flow rate of 0.5 ml/min. For mass spectrometry, an Agilent 5973N model was used with an ionization voltage of 70 electron volts and an ionization source temperature of 220 °C. The scan range was set from 40 to 500. Data analysis was conducted using Chemstation software, and spectra were identified based on their inhibition index and compared with the spectra available in the ADAMS book, using the mass spectra of standard compounds as references.

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Preparation of nanoliposomes containing *Elettaria cardamomum* EO

The ethanol injection method was utilized to produce nanoliposomes (19). First, a concentrated oil phase was prepared, comprising cholesterol (2.5% w/v), lecithin (10% w/v), tween 20 (0–10% w/v), and *E. cardamomum* EO (10% w/v), which was then dissolved in absolute ethanol and stirred overnight at 2000 rpm at room temperature. Subsequently, 1 mL of the prepared solution was mixed with 4 mL of distilled water and stirred for 60 minutes, resulting in the formation of nanoliposomes containing *E. cardamomum* EO. Likewise, free nanoliposomes were also prepared using a similar process but without including *E. cardamomum* EO.

Characterizations of nanoliposomes containing *Elettaria cardamomum* EO

The prepared samples underwent size Analysis using a DLS-type apparatus to determine particle size and particle size distribution (SPAN). The criteria for appropriate size characteristics were set as particle size less than 200 nm and SPAN value equal to or less than 1. SPAN was calculated based on the formula d90-d10/d50, where d represents the diameter and 90, 10, and 50 are the percentiles of particles with lower diameters than these values. Besides, the zeta potential of the selected nanoliposome sample was investigated using a DLS-type apparatus (SZ-100, Horiba, Japan). For the examination of nanoliposome morphology, TEM analysis was conducted (TEM Philips EM 208S, USA). Furthermore, ATR-FTIR analysis was employed to confirm the successful loading of *E. cardamomum* EO in the nanoliposomes. Spectra of the EO, free nanoliposomes, and nanoliposomes containing *E. cardamomum* EO were recorded within the wavenumber range of 400–3900 cm^{-1}.

Assessment of larvicidal effects

The larvicidal effect of *E. cardamomum* EO and nanoliposomes containing *E. cardamomum* EO was investigated according to a WHO-recommended bioassay (20). Briefly, *E. cardamomum* EO (2.0% w/v) was firstly dissolved in absolute ethanol, equal to the prepared nanoliposomes. Next, glass beakers containing 200 mL de-chlorinated water and 25 larvae *An. stephensi* were ready. Afterward, descending serial dilutions of nanoliposomes containing *E. cardamomum* EO (1000, 800, 600, 400, 200, 100, and 50 µL) were added to each beaker. Subsequently, 1000 µL of absolute ethanol and 1000 µL of free nanoliposomes were injected into separate beakers to serve as the control and negative control groups, respectively. The mugs were then exposed to the recommended conditions, which involved maintaining a temperature of 27±1 ºC with a 12:12 light/ dark photoperiod and relative humidity of 70± 5%. After a 24 h exposure period, larval mortalities were recorded.

Moreover, the experiment was conducted in triplicates (n= 3), and the larval mortality data were presented as the mean ± standard deviation (S.D.). To determine the lethal concentrations at 50% (LC_{50}), Probit analysis, as outlined by Finney, was employed (21).

Results

Chemical compositions of *Elettaria cardamomum* EO

Identified compounds in *E. cardamomum* EO are listed in Table 1. Alpha-terpinyl acetate (77.59%), eucalyptol (4.38%), nerolidol (2.96%), linalool (1.77%), and limonene (1.69%) were the five major constitutive compounds.

Characterizations of prepared nanoliposomes containing *Elettaria cardamomum* EO

Ingredients of the prepared nanoliposomes containing *E. cardamomum* EO and their size analyses are summarized in Table 2. The best size characteristics (particle size 73 nm and SPAN 0.97) were achieved in sample No. 2; it was selected for further characterization and larvicidal bioassays.

DLS and zeta potential diagrams of the selected nanoliposomes containing *E. cardamomum* EO are shown in Fig. 1 A and B. The

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particle size was 73±5 nm, and SPAN was 0.97; meanwhile, the zeta potential was -16.3±0.8 mV. Moreover, as shown in Fig. 2, the nanoliposomes were spherical with a smooth periphery.

Confirmation of loading of the *Elettaria cardamomum* EO in nanoliposomes

The ATR-FTIR spectra of *E. cardamomum* EO, free nanoliposomes, and nanoliposomes containing *E. cardamomum* EO are depicted in Fig. 3. The ATR-FTIR spectrum of the *E. cardamomum* EO indicates the symmetric and asymmetric stretching C-H bonds at 2963 and 2926 cm^{-1} with absorption peaks at around 1438–1365 cm^{-1} showing the presence of methyl and methylene groups in *E. cardamomum* EO. The strong and sharp band at 1729 cm^{-1} is characteristic of the C=O bond, especially in terpineol acetate, and the bands at 1247–1017 cm^{-1} are related to C-O stretching vibrations.

The ATR-FTIR spectrum of free liposome reveals a broad band located at 3367 cm^{-1} related to the hydroxyl groups' stretching vibrations. The spectra of free liposomes have intense absorption bands at 2921 and 2852 cm^{-1}, attributing to CH_3, CH_2, and CH groups, while the bending modes of CH_2 and CH_3 groups at 1465 and 1349 cm^{-1} were detected. The strong band at 1733 cm^{-1} shows the presence of carbonyl groups, and the stretching modes of C-O and PO_2^- groups might also be seen as a strong band with a small shoulder at 1085 cm^{-1}. In addition to these peaks, the spectrum exhibits further peaks at 1295–720 cm^{-1}, typically attributed to vibrations of C-N, PO_2^-, and P-O groups in free liposomes.

Finally, the ATR-FTIR spectrum of the nanoliposomes containing *E. cardamomum* EO was compared with that of free liposomes and *E. cardamomum* EO. Interestingly, the main characteristic signals of both free liposome and *E. cardamomum* EO are presented in nanoliposomes containing *E. cardamomum* EO, which indicates the *E. cardamomum* EO loading into liposomes without significant change in the location of existing bands like OH, C-H, and C=O groups after loading. At 3367 cm^{-1}, the broad-band was credited to the hydroxyl stretching mode of liposome, while the narrow and strong band at 1731 cm^{-1} was assigned to the carbonyl groups of both *E. cardamomum* EO and liposome. The aliphatic C-H group stretching vibrations could be observed along with the aromatic ones at 3059–2851 cm^{-1}. The characteristic bands at 1601-1350 cm^{-1} region attributed to the deformation and bending modes of CH2 and CH3 groups and stretching modes of C=C bonds. Two intense bands at 1084 and 1058 cm^{-1} may be related to the C-O stretching vibration of both free liposome and *E. cardamomum* EO, and the other notable peaks in the region 1216–696 cm^{-1} were assigned to the vibrations of C-N, PO_2^-, P-O groups. According to the identification of bands related to *E. cardamomum* EO in nanoliposomes, it can be concluded that the EO was successfully loaded into nanoliposomes.

Larvicidal effects

Larvicidal effects of *E. cardamomum* EO and nanoliposomes containing *E. cardamomum* EO are depicted in Fig. 4. The dose-dependent effects were observed between larval mortality and sample concentration. About 90–100 % larval mortality was achieved after treatment with 60, 80, and 100 µg/mL of nanoliposomes containing *E. cardamomum*. Meanwhile, free liposomes have a negligible effect on larval viability. In all cases, the control mortality was less than 5 %, therefore not requiring adjustment with Abbott's formula.

The Probit regression lines of *An. stephensi* exposed to different interval concentrations of essential oils of *E. cardamomum* EO and nanoliposomes containing *E. cardamomum* EO is presented in Fig. 5. Besides, equation parameters and the calculated LC_{50} values for the samples are presented in Table 3. It was observed that the LC_{50} value of nanoliposomes containing *E. cardamomum* EO was significantly higher than that of the non-formulated state; the LC_{50} values were found to be 14.35 (ranging from 10 to 18) µg/mL and 33.47 (ranging from 28 to 39) µg/mL, respectively.

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Table 1. Identified compounds in *Elettaria cardamomum* EO by GC-MS analysis

No	RT[a]	%	Compounds	KI[b]	Type
1	11.38	0.17	α-pinene	935	MH[c]
2	13.45	0.68	sabinene	976	MH
3	14.29	0.15	myrcene	993	MH
4	16.27	0.47	para-cymene	1022	MH
5	16.43	1.69	limonene	1031	MH
6	16.62	4.38	eucalyptol	1029	MO[d]
7	20.19	1.77	linalool	1097	MO
8	24.37	0.45	terpinen-4-ol	1179	MO
9	25.16	1.25	α-terpineol	1189	MO
10	27.37	1.54	linalool acetate	1255	MO
11	27.63	0.41	geraniol	1254	MO
12	32.14	77.59	α-terpinyl acetate	1348	MO
13	33.30	1.64	geranyl acetate	1382	MO
14	38.11	0.21	γ-muurolene	1480	SH[e]
15	39.60	0.60	β-selinene	1490	SH
16	40.85	2.96	trans-nerolidol	1532	SO[f]
17	46.76	0.23	cic-farnesol	1700	SO

a: retention time, b: Kovats Index, c: Monoterpene Hydrocarbons, d: Monoterpenes Oxygenated, e: Sesquiterpene Hydrocarbons, f: Oxygenated Sesquiterpene

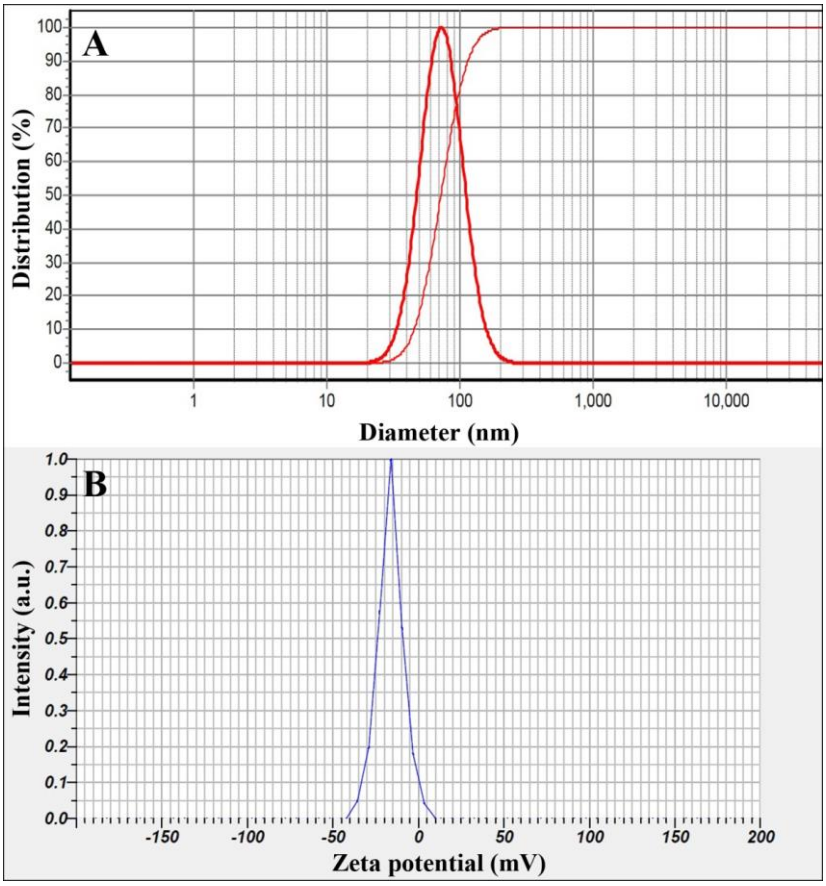

Fig. 1. Dynamic light scattering (A) and zeta potential (B) diagrams of the nanoliposomes containing *Elettaria cardamomum* EO with a particle size of 73±5 nm and a zeta potential of -16.3±0.8 mV

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Table 2. Ingredients of the prepared nanoliposomes containing *Elettaria cardamomum* EO and their size analyses

Ingredient (% w/v)					Size analyses	
No	***E. cardamomum* EO**	**Cholesterol**	**Lecithin**	**Tween 20**	**Particle size**	**SPAN**
1	2.0	0.5	2.0	0.0	217	0.96
2	2.0	0.5	2.0	0.5	73	0.97
3	2.0	0.5	2.0	1.0	35	1.51
4	2.0	0.5	2.0	1.5	23	2.07
5	2.0	0.5	2.0	2.0	26.9	1.55

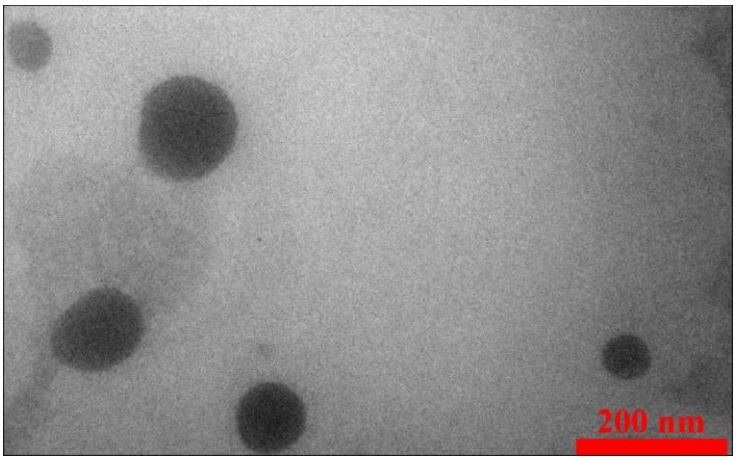

Fig. 2. Transmission electron microscopy (TEM) image of nanoliposomes containing *Elettaria cardamomum* EO

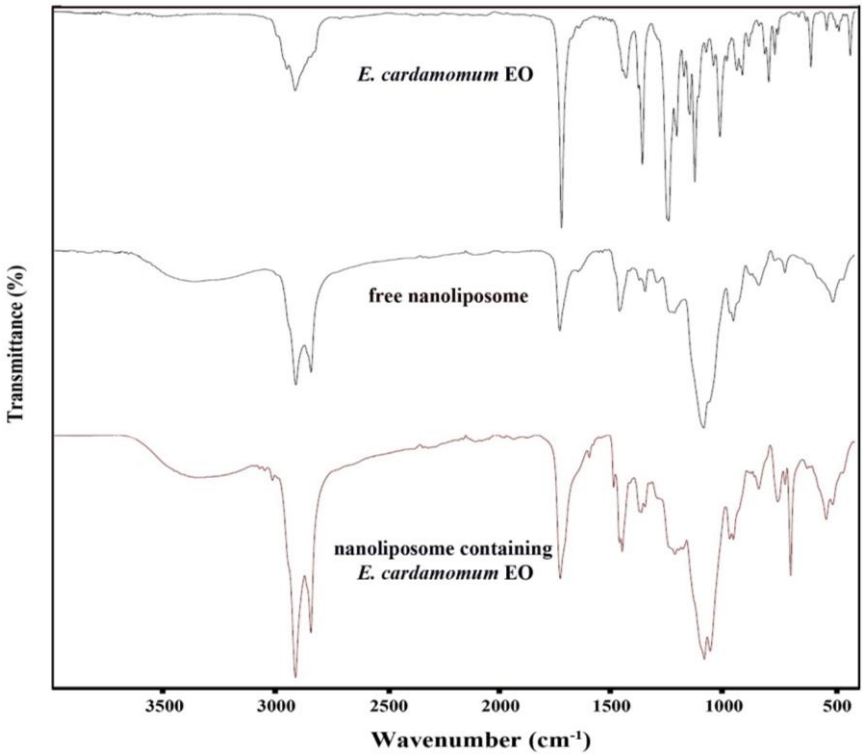

Fig. 3. Attenuated total reflectance-fourier transform infrared (ATR-FTIR) spectra of *Elettaria cardamomum* EO, free nanoliposomes, and nanoliposomes containing *E. cardamomum* EO

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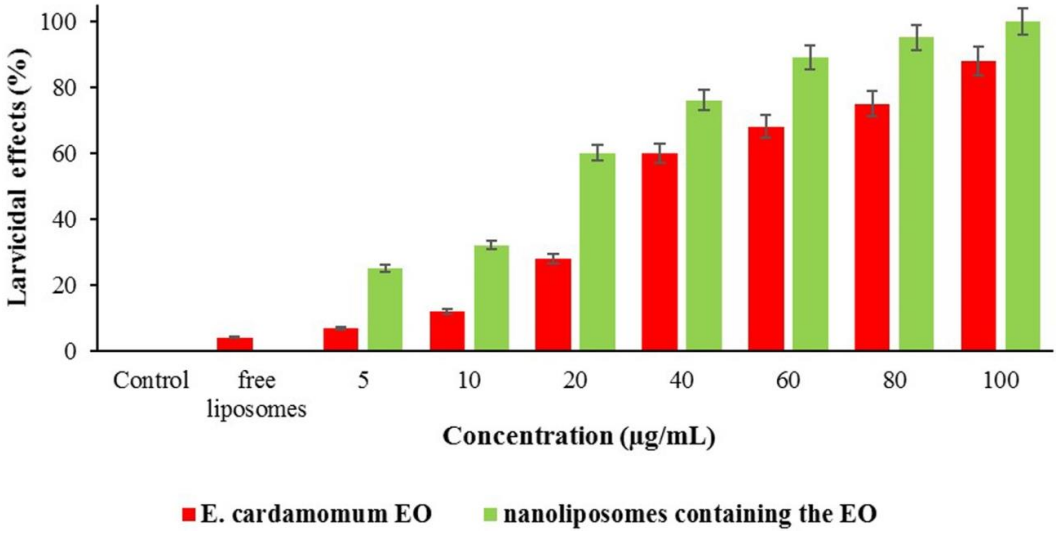

Fig. 4. Larvicidal effects of *Elettaria cardamomum* EO and nanoliposomes containing *E. cardamomum* EO against *Anopheles stephensi*, bars indicate mean ± SE

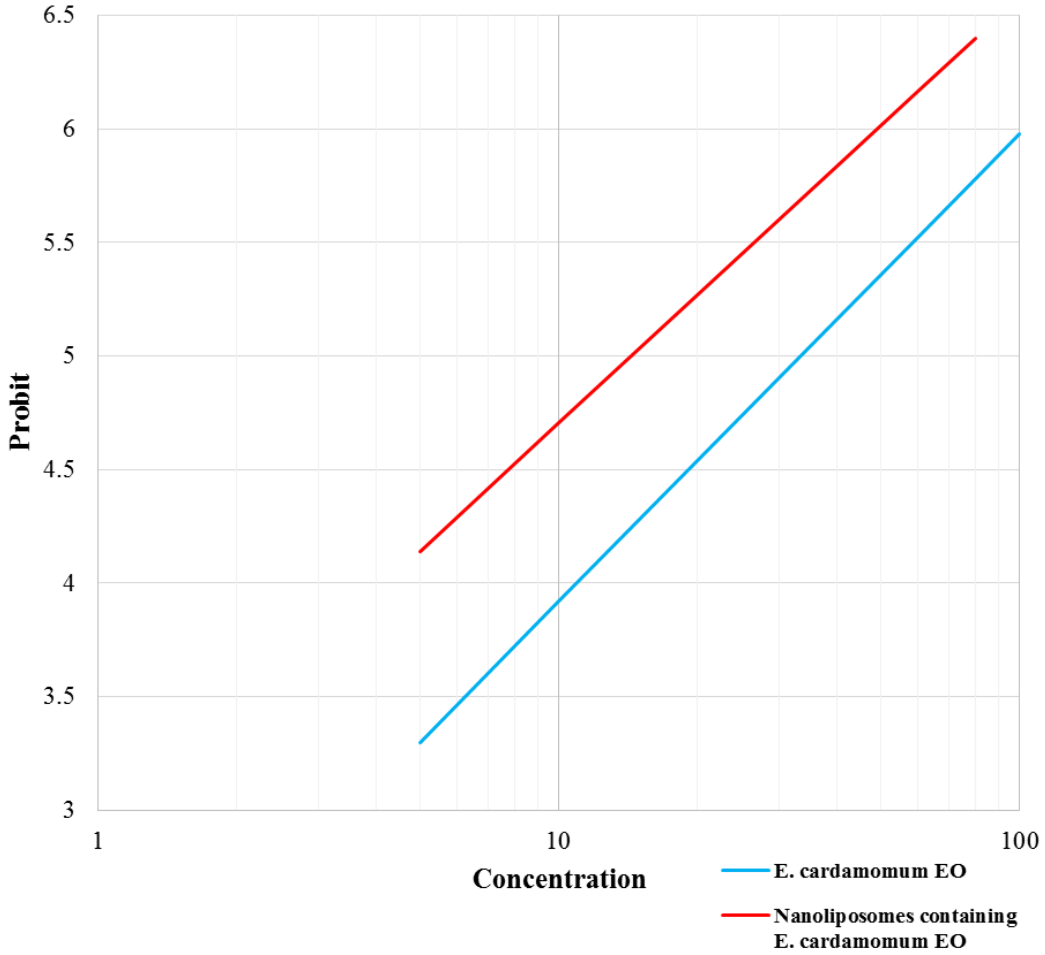

Fig. 5. Probit regression lines of *Anopheles stephensi* larvae exposed to different interval concentrations of *Elettaria cardamomum* EO and nanoliposomes containing *E. cardamomum* EO

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Table 3. Probit regression equation parameters of larvicidal effects of *Elettaria cardamomum* EO and nanoliposomes containing *E. cardamomum* EO against *Anopheles stephensi larvae*

Samples	B±SE	LC_{50} LCL–UCL	$\chi 2$ (df)	*P*-value	Regression equation *Y= A+BX*
***E. cardamomum* EO**	2.06±0.14	33.47 28–39	1.08 (5)	< 0.05	*Y*= − 1.8552+2.0625X
nanoliposomes containing *E. cardamomum* EO	1.87±0.14	14.35 10–18	1.49 (4)	< 0.05	*Y*= − 2.8261+1.8786X

B: The slope of the line; SE: Standard error; LC_{50}= Lethal concentration causing 50% mortality (µg/mL); LCL: Lower Confidence Limit (95%); UCL: Upper Confidence Limit (95%); χ^2: heterogeneity about the regression line; df: degree of freedom; *P*-value: represent homogeneity in the population of tested larvae; A: y-intercept

Discussion

Every mosquito species undergoes immature aquatic stages that breed in stagnant water. As they cannot transmit diseases during this phase, larviciding is thus an effective approach to reducing mosquito populations and alleviating the burden of mosquito-borne diseases (22, 23). This study used *E. cardamomum* EO as a green larvicide. Mechanism actions of larvicidal effects of *E. cardamomum* EO were not mentioned in the literature. However, some modes of action have been proposed for EOs, primarily involving neurotoxic and cytotoxic effects. EOs can disrupt the delicate neurophysiology of mosquito larvae, leading to paralysis and eventual death. Several mechanisms have been proposed for their neurotoxic action. A) EOs can interfere with the activity of acetylcholinesterase, an enzyme crucial for nerve impulse transmission. By inhibiting it, EOs disrupt the normal functioning of the nervous system, leading to paralysis and death of mosquito larvae (24, 25). B) EOs can antagonize octopamine receptors, regulating insect behavior and movement. Octopamine receptor antagonism by EOs can lead to impaired movement, disorientation, and ultimately death of mosquito larvae (26, 27), and C) EOs can interact with GABA receptors responsible for inhibitory neurotransmission. Modulation of receptors by EOs can result in excessive inhibition, causing paralysis and death of mosquito larvae (24, 28).

Furthermore, EOs can also exert cytotoxic effects on mosquito larvae, damaging their cells and tissues. Several mechanisms have been proposed for their cytotoxicity. A) EOs can disrupt the integrity of cellular membranes, leading to leakage of cellular contents and cell death (29, 30). B) EOs can induce the production of ROS, highly reactive molecules that can damage cellular components, including DNA and proteins (31, 32), and C) EOs can interfere with mitochondrial function, disrupting cellular energy production and leading to cell death (33, 34). Moreover, the larvicidal activity of EOs can be further enhanced by synergistic interactions between their components. Minor components in an EO mixture may enhance the activity of major components, leading to increased larvicidal potency (35, 36).

In this study, an attempt was made to efficacy improvement of the EO by preparaing nanoliposomes containing *E. cardamomum* EO. ATR-FTIR analysis was used to confirm the successful loading of the *E. cardamomum* EO in nanoliposomes. ATR-FTIR analysis can detect the characteristic absorption bands of the EO in the nanoliposomes, which indicates that the EO has been successfully incorporated into the nanoliposome structure (37). Interesting-

ly, the obtained LC_{50} in the current study was comparable with previous findings. For instance, EO and nanoliposomes containing *Satureja khuzestanica* had LC_{50} values of 42.99 and 12.38 µg/mL (38). Meanwhile, EO and nanoliposomes containing *Citrus limon* EO exhibited LC_{50} values of 13.87 and 6.8 µg/mL (19). In addition, EO and nanoliposomes containing *Cinnamomum zeylanicum* EO outlined LC_{50} values of 62.2 and 13.7 µg/mL (39). Another study investigated the larvicidal efficacy of EO and solid lipid nanoparticles containing *Z. multiflora* EO with LC_{50} values of 33.33 and 9.19 (40).

As the results of this study and the aforementioned studies showed, there is a significant difference between the efficacy of the nanoformulated and unformulated states EOs. Many reasons have been proposed in previous studies. In summary, the encapsulation process enhances the physicochemical stability of larvicides and safeguards EOs compounds from degradation and oxidation (41, 42). The bioactivity and bioavailability of these compounds are more effective than non-formulated states due to the EO package homogeneously in nanometer; the spreading and permeation of compounds improved on the target sites (43, 44). Considering that nanotechnology has provided a suitable tool for the development of herbal larvicides, it seems that by choosing an EO with potent larvicidal properties, a larvicide with an efficiency comparable to synthetic larvicides can be achieved. However, due to the presence of various compounds, the possibility of resistance to it is low, and due to its biodegradability, it will not cause environmental pollution. Our findings demonstrated the significantly higher larvicidal effects of liposomal formulation of *E. cardamomum* EO against *An. stephensi* compared to the non-formulated state.

Conclusion

In this pioneering study, researchers investigated the chemical composition of *E. cardamomum* EO and prepared nanoliposomes containing the EO. The nanoliposomal formulation exhibited significantly higher larvicidal effects against *An. stephensi* larvae compared to the non-formulated EO. The use of nanoliposomes offers promising potential in vector control and combating malaria by enhancing the stability, bioavailability, and efficacy of herbal insecticides. This research contributes to developing environmentally friendly and efficient strategies to combat malaria and other mosquito-borne diseases.

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Ethical considerations

This study was ethically approved by Shiraz University of Medical Sciences, IR.SUMS.SCHEANUT.REC.1400.051.

Conflict of interest statement

The authors declare there is no conflict of interests.

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