



Encapsulation of Essential Oils of *Mentha pulegium* and *Ferula gummosa* Using Nanoliposome Technology as a Safe Botanical Pesticide

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Abstract

Introduction: Recent researches have shown that many plant essential oils (EOs) have a high potential for controlling agricultural pests and can be used as precursors for synthesis of new pesticides. The major limitations for the use of these compounds are rapid evaporation, poor water solubility, and aptitude for oxidation. The aim of this study was to prepare and characterize nanoliposome containing EOs of *Mentha pulegium* and *Ferula gummosa* and fumigant toxicity of nanoliposome containing *M. pulegium* EO against *Tribolium castaneum*.

Materials and Methods: In this study, nanoliposome containing EOs of *M. pulegium* and *F. gummosa* were prepared using heating method and its physicochemical properties were evaluated. Also, the impact of fumigant toxicity of *M. pulegium* EO nanoliposome on *M. castaneum* was investigated.

Results: Results showed that mean (\pm SD) particles of nanoliposome containing *M. pulegium* and *F. gummosa* EOs were 345 ± 3.2 and 309 ± 1.67 nm and their encapsulation efficiency (EE) were $99.38\pm 0.24\%$ and 96.41 ± 0.26 , respectively. The kind of EOs had no significant effect on the physicochemical property of nanoparticles. At the end of 24 hours, the release percentage of EOs of nanoliposomes of *M. Pulegium* and *F. gummosa* were 46% and 33 %, respectively. The estimated LC_{50} values for nanoliposome and crude EOs of *M. Pulegium* against *T. castaneum* were 36.53 and 75.23 μ l/l air, respectively.

Conclusions: The results of the current research showed that release and stability of EOs were significantly affected when change to nanoliposome particles. Also, *M. pulegium* EO nanoliposome showed enhancing fumigant toxicity against *T. castaneum* in comparison with the crude EO of this plant.

Keywords: Essential Oil, Nanoliposome, *Mentha pulegium*, *Ferula gummosa*, Heating Method, Pesticide

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Introduction

The EOs of aromatic plants are composed of a mixture of different volatile chemicals such as terpenoids, alcohols, aldehydes and phenolic compounds which have different biological activities as insecticide, acaricide, fungicide, antibacterial, repellent, attractant, perfumer etc.¹ Several studies have been conducted on the biological activity of EOs and their active compounds and they have significant importance in various fields from food chemistry to pharmaceutical industries.^{2,3}

Currently, the plant secondary metabolites have found wide application as alternatives to conventional pesticides in agricultural products. In most cases, these plant products are safe to human beings and are used as anti-cough, anti-spasm, anesthetic and other human diseases.⁴ Unfortunately, plant EOs are unstable compounds and susceptible to oxidation and photolysis. Also, a high evaporation rate and

low solubility in water restrict the use of these compounds in crop protection programs.⁵ Therefore, it is necessary to formulate these susceptible compounds into usable forms. In many cases, nanoencapsulation can provide a necessary protection for these compounds. Various techniques can be employed to form nanocapsules, including spray drying, spray chilling, extrusion coating, fluidized-bed coating and nanoliposome technology. Among microencapsulation techniques, nanoliposome is the most useful method for the encapsulation of plant EOs. They can improve the stability, solubility and bioavailability of bioactive compounds. Liposomes are colloidal particles consisting of a membranous system formed by lipid bilayers encapsulating aqueous spaces. They can be prepared using completely natural ingredients or indigenous molecules found in our body; thus they are biocompatible and are acceptable for human consumption.^{6,7} Because of specific structural properties, liposomes can

encapsulate hydrophilic, hydrophobic and amphiphilic substances. Moreover, they are biodegradable, non-toxic, non-immunogenic and biocompatible compounds.⁸

Some studies have been conducted on the encapsulation of EOs into liposomes by different methods such as, *Zataria multiflora*⁹ *Allium sativum* L.¹⁰ and *Eucalyptus camaldulensis* leaf¹¹ EOs.

Tribolium castaneum (Herbst) is a very common pest, infesting many flour mills, warehouses and grocery stores. It has a world-wide distribution and is among the most economically important stored-product pests. It has been reported that the EOs from some plants affects egg hatching, growth rate and food consumption of *T. castaneum*.¹² However, there is no study about nanoliposome containing EO properties.

The aim of this study was the encapsulation of Eos of *M. pulegium* and *F. gummosa* that grow in different parts of Iranian nanoliposomes by using the heating method and evaluating their physicochemical characteristics and fumigant toxicity of nanoliposome containing *M. pulegium* EO against *T. castaneum*.

Materials and Methods

The EOs of *M. pulegium* and *F. gummosa* were obtained from Barij Essence Co. in Kashan, Iran. Cholesterol, glycerol, Soybean phosphatidylcholine, chloroform p.a., and methanol p.a. Dialysis bag (cut off 120000 Da) were purchased from Sigma-Aldrich Co. Deionized water was used throughout the experiment.

Preparation of Nanoliposomes

Nanoliposome was prepared using a heating method according to Mozafari's technique.¹³ The liposomal ingredient (lecithin) was hydrated with deionized water. The EO was solved in the glycerol (3% w/v) and was then added to the liposomal ingredients. Then, the mixture was heated while stirring (approx. 1000 rpm) on a hotplate stirrer (HCR2 Gerhardt Germany) (35°C) for 50 minutes. Finally, the obtained solutions were stored at 4°C.

Mean Particle Size, PDI Index and Zeta Potential Measurements

The mean particle diameter (PDI) and distribution size of nanoliposomes were determined by dynamic light scattering (Brookhaven Instruments Ltd., Brookhaven, USA) at 25°C. Samples were scattered at 277 nm (angle of 90 degrees). Before the size measurement, each sample was diluted with deionized water. Zeta potential which is an electrostatic potential at the electrical double layer surrounding a nanoparticle in solution was measured using a Zeta Potential Analyzer (Brookhaven Instruments Ltd., Brookhaven, USA) at 25°C.

Encapsulation Efficiency

The EO content in the nanoparticles was quantified using spectrophotometry at 277 nm. The calibration curve of EOs was constructed at 5 concentrations. For the determination of encapsulation efficiency (EE) of EOs, 100 µL of nanoliposomes of EO was diluted with deionized water. Then, it was filtered

using a 0.45 µm syringe filter, to obtain a clear solution and their absorbance were determined by a spectrophotometer at 277 nm.

The EE% was determined by the difference between the total amount of EO added to the nanoparticles and the amount of free EO, using the following equation:

$$EO\ EE\ \% = (B - A/B) \times 100 \quad (\text{Equation 1})$$

Where A is the amount of free EO in the ultrafiltrate and B the total amount of EO used for.

EO Release from Nanoliposomes

The release of encapsulated EO was determined by the dialysis technique, according to the method described by Varshosaz et al.¹⁴ Briefly, 1 mL of nanoliposome containing EO was deposited in cellulosic dialysis bags and placed in 30 mL of medium containing Tween 80 (1%), ethanol (3%) and deionised water (96%). The system was maintained under magnetic agitation in a thermostatically controlled bath at room temperature (25 ± 2°C). Water sampling was carried out after 0.5, 1, 3, 6, 16, 24 hours and the released EO was determined using ultraviolet spectrophotometer.

The Morphology of Nanoliposomes

The morphological characterization of the nanoliposome was evaluated using an atomic force microscopy (AFM).

Biological Assay

Insect Culture

The red flour beetle, *T. castaneum* was obtained from stock reared in the Department of Plant Protection of Lorestan University, Khoramabad, Iran. Adult insects were reared on wheat flour mixed with yeast (10:1, w/w). The rearing conditions included darkness in 28 ± 1°C and 70% ± 5% (relative humidity). Adult insects, 1-7 days old (both sexes) were used for bioassay test.

Fumigant Toxicity of Nanoencapsulated and Crude EO of *Mentha pulegium*

The fumigant toxicity of nanoliposome containing EO of *M. pulegium* and crude EO of this plant were tested against 1-7 day-old adults of *T. castaneum*. Ten adults (male and female) of *T. castaneum* were placed inside glass vials (volume 100 mL). The first preliminary test was carried out to determine the necessary concentrations for mortality between 25%–75% of adult weevils and then other concentrations were determined using logarithmic method. After determining the concentrations according to Negahban et al,¹⁵ the method paper filter of diameter 2 cm (Whatman No. 1) was placed inside the cap of 100 ml glass jar to deposit nanoliposome on them. Then, 10 adult insects (1-7 days old) were transferred to the jars. With the aid of the sampler, different concentrations of crude EO (41.60, 53.3, 71.6, 93.3 and 125.78 µI/I air) and nanoliposome (18.9, 26.67, 35.28, 47.66 and 66.61 µI/I air) were deposited on the filter paper. Control insects were kept under the same conditions with empty nanoliposome and without EO. Each dose was replicated five times. The number of dead and live insects in each bottle was counted 72 hours after initial exposure to the nanoliposome. Insects were

considered dead if they could not move their appendages.

Statistical Analysis

Results are expressed as mean \pm standard deviation of three simultaneous assays and subjected to one-way analysis of variance (ANOVA). Comparisons were performed by Tukey test. Data were analyzed by one-way ANOVA and mean comparisons were determined by a Tukey test using SAS 9.1., according to the Zabihi et al¹⁰ method. The LC₅₀ values were estimated by probit analysis using Polo-Pc and SAS 9.1 programs. Graphs were plotted using the Excel 2010.

Results

Mean Particle Size and Zeta Potential Measurements

The results showed that there was no significant difference between particle size of nanoliposome encapsulated of EOs ($P>0.05$). The mean particle size, PDI and zeta potential of prepared EO nanoliposomes from *M. pulegium* and *F. gummosa* are shown in Table 1.

Encapsulation Efficiency

The standard curve (concentration diagram) was plotted and the linear equation (it indicates the relationship between concentration and absorption) for EOs of *M. pulegium* and *F. gummosa* was determined (Figure 1). According to the formula and calculations, the EO encapsulation efficiency of *M. pulegium* and *F. gummosa* were estimated $99.38\pm 0.24\%$ and 96.41 ± 0.26 , respectively.

EO Release of Nanoliposomes

The release profile of the EOs from nanoliposomes are shown

Table 1. Physicochemical Characteristics of EOs Nanoliposome of *Mentha pulegium* and *Ferula gummosa*

Characteristics	<i>M. pulegium</i>	<i>F. gummosa</i>
Particle size (nm)	345 \pm 3.2 ^a	309 \pm 1.67 ^a
PDI	0.26 \pm 0.045	0.36 \pm 0.006
Zeta potential	-17.2 \pm 1.34	-14.05 \pm 1.20

^aNo significant difference between particle size of nanoliposome encapsulated of EOs ($P>0.05$).

in Figure 2. At the end of 24 hours, the release percentage of EOs of *M. pulegium* and *F. gummosa* from nanoliposome were recorded 46% and 33%, respectively.

Fumigant Toxicity of Nanoencapsulated and Crude EO of *Mentha pulegium* Against *Tribolium castaneum*

Table 2 shows the results of the fumigation toxicity of crude EO of *M. pulegium* and nanoliposome containing the EO of this medicine plant against adults of *T. castaneum*.

Results showed that after 72 hours fumigation, LC₅₀ and LC₉₀ of *T. castaneum* for crude EO of *M. pulegium* were 75.23 and 181.33 μ l/l air and for nanoliposome containing the EO of this plant were 36.53 and 76.22 μ l/l air, respectively. A result showed that encapsulation of *M. pulegium* EO into nanoliposome had a synergistic effect on fumigant toxicity against *T. castaneum*.

The Morphology of Nanoliposomes

Figure 3 shows a 3D AFM image of f EO nanoliposomes of *M. pulegium* (A) and *F. gummosa* (B) loaded nanoliposomes and shows the formation of nanoliposomes particle for EOS of *M. pulegium* and *F. gummosa*.

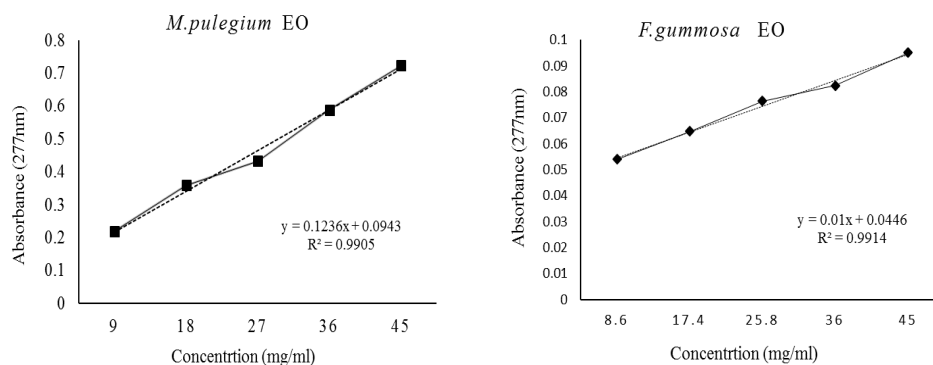


Figure 1. Calibration Curves of *Mentha pulegium* and *Ferula gummosa* EO.

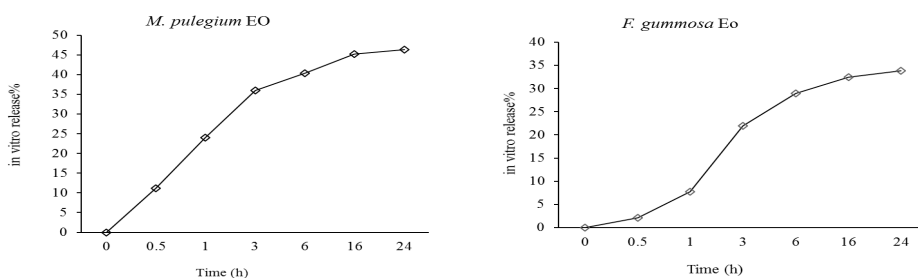


Figure 2. The Release Profiles of the EOs *Mentha pulegium* and *Ferula gummosa* From Nanoliposome Using the Dialysis Pocket Diffusion Technique.

Discussion

Many researchers have reported insecticidal effect of EOs and extracts from *M. pulegium* and *F. gummosa*.¹⁶⁻¹⁹ Some secondary metabolites such as menthone, menthol, pulegone and isomenthone were reported as main components in the EO of *M. pulegium* and α -pinene, β -pinene and β -myrcene as a main active chemical in the EO of *F. gummosa*.^{20,21} These two plants grow in different parts of Iran and can be used as a safe pesticide for controlling pests.

However, there are problems in using the pure EOs and extracts of these plants including sensitive to heat, light and oxidation. Encapsulation of EOs into liposome is a promising approach which prevents their degradation and oxidation.¹³

In the current study, the heating method was used. This is one of the simplest techniques for the preparation of nanoliposomes without employing toxic solvents or detergents. The heating method is economical and capable of preparing nanoliposomes, with a superior monodispersity and storage stability.²²

In present study, mean particle size of nanoliposome containing *M. pulegium* and *F. gummosa* EO were 345 \pm 3.2 and 309 \pm 1.67 nm, and the polydispersity index were 0.26 \pm 0.045 and 0.36 \pm 0.006, respectively.

In a similar experiment, the mean size and polydispersity index of nanoliposome of *Allium sativum* EO were reported 131.73 \pm 14.31 nm and 0.212 \pm 0.013, respectively. Also, the mean size and polydispersity index of encapsulated allicin were reported 145.27 \pm 15.19 nm and 0.204 \pm 0.011, respectively. The entrapment efficiency of the prepared nanoliposome was reported 64.27 \pm 0.78% by this research.¹⁰

In another research, the particle size and PDI of nanoliposomes encapsulated orange EO using the heating

method were reported 100-250 nm and 0.2, respectively. Also, in the mentioned research the encapsulation efficiency was 62%-70%.²²

In another report, the particle size determination for liposomes containing EO of *Eucalyptus camaldulensis* had a wide range from 40.5 to 298 nm for the different formulations.¹¹ Also, in another study by Khatibi et al, the particle size for EO nanoliposomes from *Zataria multiflora* prepared by the sonication method, thin film evaporation-sonication and the ethanol injection were 99.9 \pm 0.4, 395.3 \pm 26.6 and 239.7 \pm 25.2 respectively.⁹ Variations in the results of previous research and the current study may be due to the differences in the nanoencapsulation methods. On the other hand, Moghimipour et al reported that the composition of the lipid vesicle membrane, especially the type of phospholipids, cholesterol content, preparation method and the kind of EO may affect the liposome size and the encapsulation efficiency.¹¹

In a recent research, the zeta potential for nanoliposome containing *M. pulegium* and *F. gummosa* EO were -17.2 \pm 0.134 mV and -14.05 \pm 1.20 mV, respectively. The value of this index was reported -23.20 \pm 0.87 mV by Zabihi et al for nanoliposome of *A. sativum* EO. According to reports, a value between -30 mV and +30 mV would be considered as high and acceptable zeta potentials.¹⁰

In our study, the values of LC₅₀ for crude EO and nanoliposome of *M. pulegium* were estimated 75.23 and 36.3 μ l/l air, respectively. In a similar study, Biddeci et al indicated that a bionanocomposite film containing peppermint EO showed an enhancing antibacterial effect on *E. coli* and *Staphylococcus aureus*, which is in accordance with the present study.²³ Also, Beyki et al found an improved antifungal property of pepper mint EO encapsulated in chitosan-

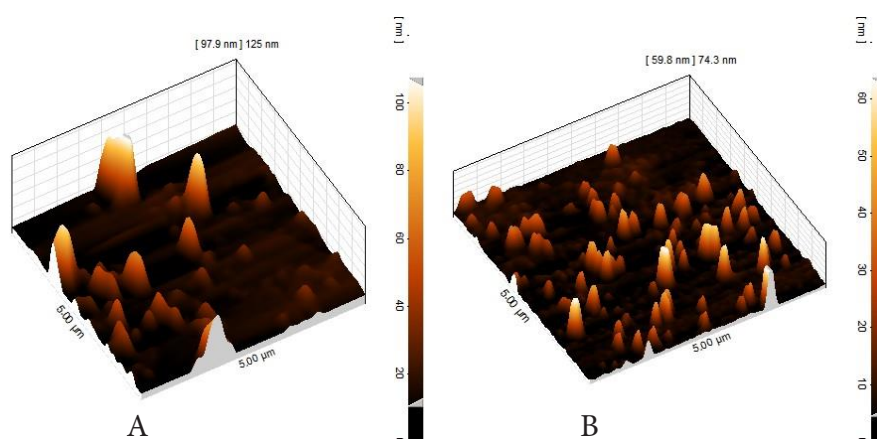


Figure 3. Atomic Force Microscopy (AFM) Image of Nanoliposome of *Mentha pulegium* (A) and *Ferula gummosa* (B).

Table 2. LC₅₀ and LC₉₀ of Nanoliposome and Crude Essential Oil of *Mentha pulegium* Against *Tribolium castaneum*

LC ₉₀ (μ l/l air) (Confidence Limit 95%)	LC ₅₀ (μ l/l air) (Confidence Limit 95%)	Slope \pm SE	Chi-Square χ^2 (DF)	N	Compound
181.33 (142.94-277.65)	75.23 (67.07 -85.20)	4.32 \pm 0.05	4.09(3)	250	Crude essential oil
76.22 (64.31-99.81)	36.3 (33.03-40.53)	3.59 \pm 0.06	3.28(3)	250	Nanoliposome

cinnamic acid nanogels.²⁴ Similar results have been reported by Makwana et al and also Mekkerdchoo et al.^{25,26}

The application of the AFM confirmed successful formation of the EOs-loaded nanoliposome. Also, AFM size measurements of Eos nanoliposomes revealed an average diameter of 500 nm which is larger than this value for the same vesicles obtained through the light scattering measurements. This can be due to vesicle flattening which happens when liposomes are placed on AFM substrates and has been reported by other researchers as well.²⁷

Conclusions

Results showed that encapsulation of EO of *M. pulegium* into Nanoliposome improved stability and controlled the release of volatile components. In the present study, the heating method was used for preparing nanoliposomes because it allows manufacturing of carrier systems in one-step, without employing toxic solvents or detergents. Likewise, the preparation of nanoliposomes is completely natural and safe. Also, encapsulated *M. pulegium* EO showed enhanced fumigant toxicity against *T. castaneum* in comparison with crude EO. The findings of the present study could be recommended to agriculture industries to use encapsulated EOs into nanoliposome. It can as a result lead to a new biopesticide in insect pest management programs.

Authors' Contributions

JSH and ZF designed the study. ZF, JSH and JV performed the experiments (e.g. culture insect, bioassay experiments). JSH, ZF, JV and SHJ analysed the data. ZF wrote the paper. All authors provided final approval of the manuscript.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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