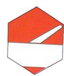

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Effect of Preparation Methods on the Properties of *Zataria multiflora* Boiss. Essential Oil Loaded Nanoliposomes: Characterization of Size, Encapsulation Efficiency and Stability

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Background: Essential oil (EO) of *Zataria multiflora* Bioss., contains components with antibacterial and antifungal properties that can be used as substitutes for synthetic drugs and food preservatives. These applications require appropriate carriers like nanoliposome. The purpose of this study was to encapsulate this EO into nanoliposomes. In addition, the effects of preparation methods were evaluated on the physical properties of nanoliposomes. ***Methods:*** The EO was encapsulated into nanoliposomes by three different methods including thin film evaporation, ethanol injection and sonication methods. Formation of liposomes and their physical properties was studied by means of particle size, polydispersity index, zeta potential and encapsulation efficiency. In addition the changes in EO retention and mean size distribution were studied during one month of storage at 4±1°C. ***Results:*** Liposomal systems prepared by sonication method with about 99 nm displayed the smallest mean size and better dispersivity. The encapsulation efficiency of nanoliposomes containing EO was in the following rank order: thin film evaporation > Ethanol Injection > sonication. Moreover, MLV (multilamellar vesicles) liposomes prepared by the thin layer evaporation method showed better stability during storage. ***Conclusions:*** This study indicated that the preparation method can affect the formation of liposomes and their physical properties and storage stability.

Introduction

Due to increased awareness and concerning of consumers about synthetic chemical additives, food industry experts have become increasingly interested in the use of natural antimicrobial compounds such as plant essential oils in recent years. Many studies have been conducted by researchers about the antimicrobial effects of plant EOs on a various range of microorganisms.[1-5]

EOs are natural and volatile compounds produced by aromatic plants as secondary metabolites. These compounds are composed of various chemicals with remarkable health benefits. Several studies have been conducted about the biological activity of EOs and their active compounds and they have significant importance in various fields from food chemistry to pharmaceutical industries.[6]

The most of EOs are poorly soluble in water, biologically unstable and they distribute defectively to target sites. To improve their stability and bioavailability, new techniques like encapsulation in liposomes[7] have been developed. It can reduce the reaction of these compounds with environmental factors (water, oxygen, light), decrease their evaporation and transfer to the environment and mask their taste. Also, it can help to achieve a uniform distribution in the final product when used in very small amounts.[8,9]

Liposomes are spherical particles of polar fats (such as phosphatidylcholine and phosphatidylethanolamine) or mixture of polar lipids with cholesterol or ergosterol. The size of Liposomes depending on production methods varies from a few micrometers to a few nanometers. In polar solvents such as water, polar lipids tend to self-assemble in the form of bilayer membranes. This bilayer membrane is somewhat flexible and under the shear, it can form particles composed of a thin shell of a polar lipid bilayer and an interior compartment that composed of the initial solution in which the polar lipids have been solved before the application of shear. Depending on the degree of shear force, different structures of liposomes like unilamellar liposomes (with single bilayer membrane shell), multilamellar vesicles (with multiple bilayer membranes) and multivesicular vesicles (liposomes that may contain other randomly sized liposomes in their interior) may be produced.[10]

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Because of specific structural properties, liposomes can encapsulate hydrophilic, hydrophobic and amphiphilic substances. They have many potential applications in food area ranging from the protection of sensitive substances to enhance the efficacy of the food additives.[11] Moreover, they are biodegradable, non-toxic, non-immunogenic and biocompatible compounds [12] which can provide several advantages as carriers for the encapsulated molecules such as enhancing their pharmacokinetic and bio-distribution, decreasing their toxicity and providing target selectivity for them.[13] They can encapsulate antimicrobials and other functional components as separate microenvironments and preserve their activity despite the changes in surrounding aqueous phase.[10] Because of their lipophilic nature, liposomes are considered as a suitable option for encapsulation of EOs in the food industry.[11] For this purpose, several studies have been conducted about various EOs encapsulated within liposomes.[14-18]
Zataria multiflora Boiss. belongs to the Lamiaceae family that geographically grows in Iran, Pakistan and Afghanistan. This plant is known as Avishan-e-Shirazi that are used as a flavor in variety of foods in Iran. The main active components of this EO are phenolic compounds such as thymol and carvacrol that has been approved by the FDA for use as food additives.[19] This plant has a long popularity in traditional medicine for flavoring and preserving foods and drinks,[20,21] treatment of respiratory tract infections, controlling of irritable bowel syndrome[22] and application as an antispasmodic, anesthetic and antinociceptive drug.[21,23] Also, its antibacterial and antifungal activities have been demonstrated.[20,24-26]
The first aim of this study was to encapsulate EO of *Z. multiflora* into nanoliposomes by three different methods including thin-film evaporation, sonication and ethanol injection. Second, effects of these preparation methods were studied on the formation and physical properties of nanoliposomes by measuring the mean particle size, polydispersity index, morphology, zeta potential and encapsulation efficiency. We also determined their storage stability during over four weeks at 4 ± 1 °C.

Materials and Methods

Chemicals

Soybean phosphatidylcholine and cholesterol were purchased from Lipoid GmbH (Ludwigshafen, Germany) and Sigma–Aldrich (Steinheim, Germany) respectively. All of organic solvents were analytical grade and the deionized water was used throughout the experiment.

Plant Material

Z. multiflora Boiss. was collected from plants growing wild in Fars province in Iran at the full flowering stage (July) and identified by the Research Institute of Medicinal Plants, Tehran University of Medical Science, Tehran, Iran.

Extraction of the essential oil

The dried aerial parts (young leaves and stems) of the plant (200 g) were distilled (Steam distillation) in one liter of distilled water over four hours using a Clevenger apparatus. The essential oil was dried over anhydrous sodium sulfate (Sigma-Aldrich, St. Louis, USA) and kept at 4 ± 1° C.

Characterization of the essential oil

The quali-quantitative analysis of the EO was carried out by gas chromatography/mass spectrometer (Agilent technologies, USA). The chromatograph was equipped with a DB-1 capillary column (30 m × 0.25mm ID × 0.25 mm film thickness). Helium was used as the carrier gas at flow rate of 1.5 mL/min. Column temperature was initially programmed at 50˚C as an initial temperature, kept at 50˚C for 6 min, then gradually increased to 240˚C at 3˚C min, followed by a temperature enhancement of 15˚C per minute up to 300˚C, holding at the mentioned temperature for 3 min. Ionization voltage of mass spectrometer in the electron ionization mode was 70 eV and ionization source temperature was 250˚C.

Identification of essential oil compounds

In order to calculate the relative retention indices, n-alkanes (C6-C24) was used as reference. EO components were identified by comparison of their relative retention and mass spectra with those of standards [27] or with available library data of the GC/MS system (Wiley 2009 data software; NIST 2009 data software).

Preparation of nanoliposomes

Nanoliposomes were prepared by soy phosphatidylcholine and cholesterol (in the molar ratio 2.5:1). Three different methods were used for preparation of *Z. multiflora* Boiss. EO loaded nanoliposomes:

Thin film evaporation

Thin film evaporation method was performed according to Bangham's technique.[28] Lipid phase containing soy phosphatidylcholine and cholesterol was dissolved in chloroform/methanol (3:1) (HPLC purity) in a round-bottom flask. EO was dissolved in methanol and then mixed with phosphatidylcholine and cholesterol. Organic solvents were evaporated under reduced pressure by a rotary evaporator at 35˚C until a thin film was formed on the walls. Then the lipid film was hydrated with phosphate buffer saline (PBS) (pH = 7.4) (Sigma Chemicals Company Ltd) for 15 minutes at room temperature.

Ethanol injection method

Liposome preparation by ethanol injection method was performed according to technique described earlier by Chiraz et al.[29] EO and lipids were dissolved in ethanol (99% purity). The ethanolic solution injected by means

of a syringe pump in a defined volume of phosphate buffered saline solution (PBS) under magnetic stirring. Nanoliposomes were formed spontaneously as soon as ethanolic solution was in contact with the aqueous phase. Then the liposomal solution was kept under stirring for 15 minutes at room temperature. Finally, the ethanol were removed by rotary evaporation (IKA, rv 10 digital V, Germany) under reduced pressure.

Sonication method

Liposomal solution obtained by the thin film evaporation technique was sonicated by a probe sonicator (Misonix, USA) at 60% amplifying strength for 10 min.

Mean particle diameter and size distribution measurements

The mean particle diameter and size distribution of nanoliposomes were determined by Dynamic light scattering (Brookhaven Instruments Ltd., Brookhaven, USA) at 25˚C. Samples were scattered at 657 nm (angle of 90 degrees). Before the size measurement, each sample was diluted with PBS (pH = 7.4).

Zeta potential measurements

Zeta potential measurements were done using a Zeta Potential Analyzer (Brookhaven Instruments Ltd., Brookhaven, USA) at 25˚C. All analysis was carried out in triplicate.

Encapsulation efficiency

Encapsulation efficiency was determined by dialysis technique against PBS at 4˚C using a cellulose membrane (with molecular weight cut-off of 10 kD) to separate nanoliposomes and their encapsulated material from the unencapsulated EO. Then, the dialyzed liposomes were disrupted with methanol (Merck, Germany) and the quantity of encapsulated EO was measured using a UV/VIS Spectrophotometer (Beckman, DU 530, Switzerland) at λ = 275 nm.

The morphology of nanoliposomes

Scanning electron microscopy (SEM) of liposome samples was carried out using an AIS2100 (Seron Technology, South Korea). The samples were sputtered with gold.

Stability measurements

liposomal solutions prepared by different methods were stored at 4˚C for four weeks and they were evaluated in terms of changes in the mean size and EO content at specified intervals (days 0, 7, 14, 21 and 28).

Statistical analysis

Results are expressed as mean ± standard deviation of three simultaneous assays and the mean values compared by one-way analysis of variance (ANOVA). Statistical analyses were carried out by SPSS software (version 15.0 for windows).

Results

Chemical composition of the Z. multiflora Boiss. essential oils

Distillation of the aerial part of *Z. multiflora* Boiss. in Clevenger-type apparatus gave a good yield (1.66 ± 0.2%) , yellow-colored and transparent EO. Analysis of EO by gas chromatography mass-spectrometry resulted in the identification of 17 different organic compounds representing 98.06% of oil (Table 1). The main compounds were carvacrol (46.4%) and thymol (23.61%). Other major components were p-cymen (16.37%), α-pinene (3.32%) and Gama-terpinene (2.38%), respectively.

Table 1. Chemical composition of the of *Zataria multiflora* Boiss. essential oils identified by gas chromatography mass-spectrometry.

Compound	Retention index *	%Composition
α-Thujene	924	0.05%
α-Pinene	930	3.32%
Camphene	938	0.11%
β-Pinene	963	0.14%
β-Myrcene	975	0.46%
p-Cymene	1003	16.37%
Eucalyptol	1005	0.26%
Limonene	1009	0.26%
γ-Terpinene	1035	2.38%
Thymol methyl ether	1208	0.17%
Carvacrol methyl ether	1224	1.19%
Thymol	1275	23.61%
Carvacrol	1286	46.40%
Thymol acetate	1336	0.59%
Carvacrol acetate	1348	0.99%
Caryophyllene	1414	1.37%
Aromadendrene	1428	0.39%
Total		98.06%

* Retention index compared to n- alkanes in DB-1 column

Characteristics of Zataria multiflora Boiss. essential oil Loaded nanoliposomes

Mean Particle size

The results showed that prepared nanoliposomes by sonication method gave the smallest size with a mean diameter which was 99.9 ± 0.4 nm. The mean particle size of nanoliposomes containing EO was in the following rank order: thin film evaporation> ethanol Injection> sonication. Also, it was found that the EO can affect the size of nanoliposomes, so that empty nanoliposomes were larger than EO loaded nanoliposomes (Table 2).

Table 2. Effect of three different preparation methods on polydispersity index and zeta potential of *Zataria multiflora* essential oil Loaded nanoliposomes.

Method	Vesicle structure	Mean diameter (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Thin film evaporation	Empty nanoliposome	512.8±39.7 *	0.49±0.04	-29.7±0.7
	Nanoliposome+ essential oil	395.3±26.6	0.41±0.02	-19.1±0.4
Ethanol injection	Empty nanoliposome	256.0±38.5	0.263±0.05	-26.3±0.8
	Nanoliposome+ essential oil	239.7±25.2	0.225±0.02	-21.9±0.2
Sonication	Empty nanoliposome	132.9±2.0	0.155±0.03	-25.2±1.0
	Nanoliposome+ essential oil	99.9±0.4	0.121±0.02	-24.8±0.9

*Mean value ± standard deviation

Polydispersity index

The results of polydispersity index related to each preparation method showed that liposomal system prepared by sonication had better dispersivity (lower polydipersity index) while MLV nanoliposomes prepared by a thin film evaporation showed an irregular distribution (Table 2).

Zeta potential

The zeta potential measurements of all of liposomal samples were between -19.1 mv and -29.7 mv (Table 2).

Encapsulation efficiency

Effect of three different methods on encapsulation efficiency of EO was obtained. Encapsulation efficiency of nanoliposomes prepared by thin film evaporation, ethanol injection and sonication were 22.02, 19.11 and 18.36, respectively (Figure 1).

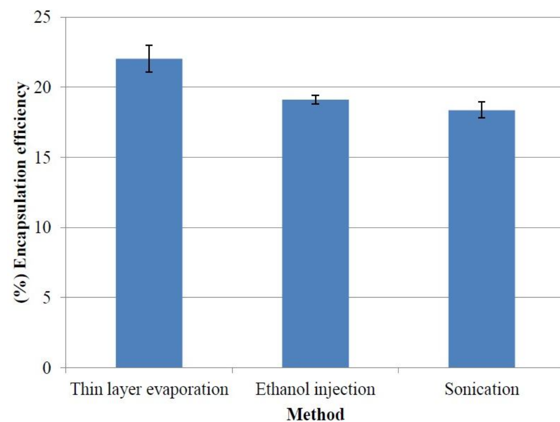

Figure 1. Effect of three different preparation methods on encapsulation efficiency (%) of *Zataria multiflora* essential oil loaded nanoliposomes.

The morphology study of nanoliposomes

SEM images of liposomal systems were shown in **Figure 2**. As it can be observed, the most of particles present a spherical form. In comparison with sonicated nanoliposomes (**Figure 2C**), liposomes obtained by thin layer evaporation (**Figure 2A**) and ethanol injecton methods (**Figure 2B**) were larger and had an irregular morphology.

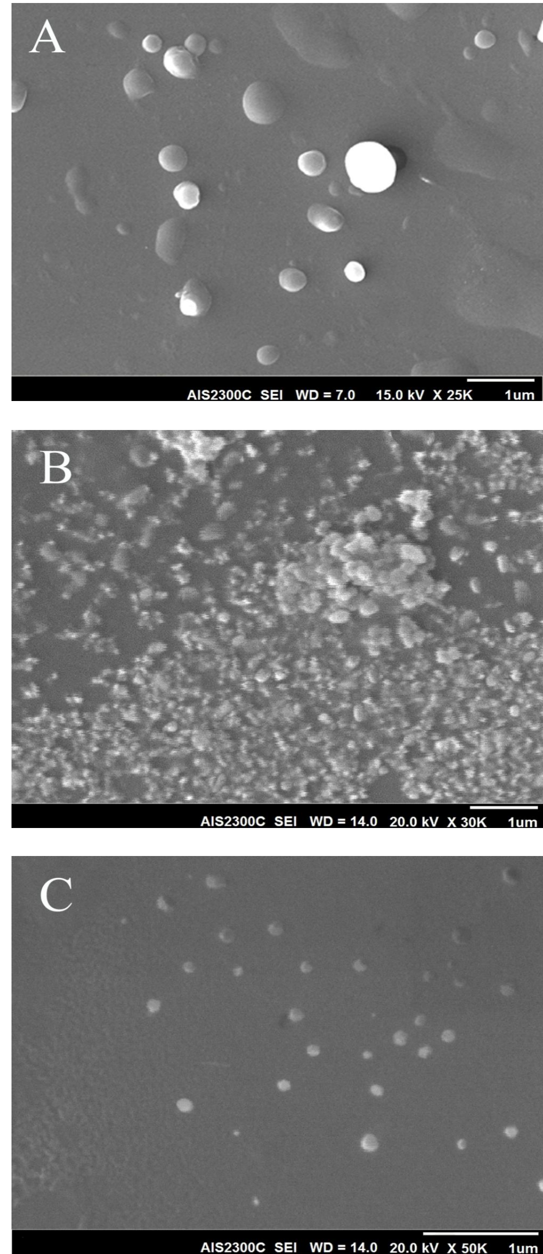

Figure 2. (A) SEM image prepared from *Zataria multiflora* Boiss. essential oil-containing nanoliposomes prepared by thin film evaporation method **(B)** ethanol injection method **(C)** sonication method.

Stability of essential oil loaded nanoliposomes

All of liposomal dispersion showed good stability during four weeks of storage at 4 ± 1˚C (Table 3 and 4). The results showed that the oil leakage was very low after one month of storage. Approximately 95% - 98 % of the EO was remained in nanoliposomes (Table 4) while vesicle sizes increased slightly (2–5%) (Table 3).

Table 3. Stability of *Zataria multiflora* Boiss. essential oil Loaded liposomes produced by the three methods in terms of changes in mean particle size (nm) (Mean value ±SD) over 4 weeks of storage at 4±1° C.

Method	day0	Day 7	Day 14	Day 21	Day 28
Thin film evaporation	395.3±26.6	400.6±22.2	403.1±30.9	401.5±32.5	402.9±43.4
Ethanol injection	239.7±25.2	236.3±28.3	244.6±24.3	242.2±30.0	248.4±34.6
Sonication	99.9±0.4	99.1±0.2	101.8±0.3	105.7±0.4	105.1±0.5

Table 4. Stability of *Zataria multiflora* Boiss., essential oil Loaded liposomes produced by the three methods in terms of essential oil retention (%) (Mean value ± SD) over 4 weeks of storage at 4 ± 1° C.

Method	Day 7	Day 14	Day 21	Day 28
Thin film evaporation	100.59±0.35	98.63±0.24	99.5±0.57	97.72±.87
Ethanol injection	99.52±0.17	97.06±0.49	97.9±0.38	96.12±0.61
Sonication	100.32±0.08	96.02±0.3	97.38±0.26	94.82±0.79

Discussion

EOs and their components are sensitive to heat, light and oxidation[30] that reduce their stability. Encapsulation of EOs into liposome is a promising approach that prevents from their degradation and oxidation.

Since the compound of the EO can affect the physical properties of liposome,[14,31,36] characterization and identification of EO components is important. Analysis of EO by gas chromatography mass-spectrometry confirmed the literature data [40,41] that the oil composition is a complex mixture of organic compounds among which carvacrol and thymol represent more than 70% of the EO (Table 1).

In present study, the results showed that EO can affect the mean size of nanoliposomes, so that EO loaded liposomes were smaller than empty liposomes. These findings correlate with those observed by Sinico et al., which showed that *Artemisia arborescens* L. EO decreased the liposome sizes. The size of EO loaded SUV liposomes were between 78±11 nm and 123±21 nm, while empty vesicles were larger with sizes ranged between 134±21 nm and 207±30 nm. [15] Yoshida et al. also demonstrated that empty MLV liposomes with a mean size of less than 1000 nm were larger than liposomes encapsulating *Eugenia uniflora* L. EO with sizes between 200 nm and 400 nm.[11] Similar findings also have been reported by other researchers about liposomes containing EOs of *Santolina insularis* [14] and *Zanthoxylum tingoassuiba* [18].

Valenti et al. reported that this effect is related to the capability of EOs to cause higher cohesion and packing between the apolar chains in the membrane vesicles.[14] On the other hand, monoterpens can reduce the size of liposomes by increasing the surface curvature of soybean phosphatidylcholine vesicles and it has been suggested that studied monoterpens locate on polar head group of membranes.[31]

Comparison of MLV and SUV nanoliposomes have been showed that the size and structure of nanoliposomes could be affected by the amount of encapsulated EO. Also, the encapsulation of lipophilic molecules in the lipid bilayer of liposomes depends on dimensions of lipid bilayer.[32]

Sonication generally leads to the production of SUV liposomes with a size lower than 100 nm. Due to the smaller size and unilamellar structure, SUV vesicles have lower amount of EO content in comparison with MLVs.[15] It has been reported that following the use of sonication, liposomes may be produced with lower encapsulation efficiency and there is the degradation possibility of phospholipids and the compounds to be encapsulated.[33]

The results of present study confirmed these findings. Different preparation methods resulted in different mean size of nanoliposomes. Sonication method resulted in the smallest size and lowest encapsulation efficiency of nanoliposomes in comparison with other two methods while MLV liposomes prepared by thin film evaporation were larger and had more encapsulated EO on average.

Polydispersity index is usually considered as an indicator of particles diameter distribution in a colloidal system. The lower level of this index, the more likely the particle diameter distribution is narrower, so the diameter of particles is more uniform.[34] The value of less than 0.1 for this index shows a homogeneous population and a value greater than 0.3 indicates a high degree of heterogeneity.[35] In the present study; this value for empty liposomes was greater than EO loaded liposomes.

Moreover, the comparison between preparation methods showed that polydispersity index related to thin film evaporation method was higher and as a result, the nanoliposomes produced by this method were more heterogeneous than nanoliposomes prepared by other two methods. These results correlate with those reported by previous studies. Detoni et al.

reported that empty MLV liposomes had a wider size distribution than EO loaded liposomes.[18] Moreover, *Atractylodes macrocephala Koidz* EO encapsulated into liposomes using a modified RESS technique had a narrow size distribution.[16] There are similar findings that have been reported by Valenti et al. [14] and Sinico et al.[15]

Evaluation of nanoliposomes containing EO of *Z. multiflora* Boiss., showed that a low amount of EO was encapsulated in liposomes (approximately between 18 to 22 percent by different methods). As measured by GC-MS analysis, carvacrol and thymol were major components of *Z. multiflora* EO. Liolios et al. found that encapsulation efficiency of carvacrol in liposomes was about 4.16 percent.[36]

It has been reported that liposomes prepared by the ethanol injection method have high storage stability and this method often predominantly leads to the production of SUV liposomes with a mean diameter of 150 to 200 nm.[37] In the present study, the mean size of the EO loaded nanoliposomes and empty nanoliposomes was about 239 nm and 256 nm respectively. As indicated in other studies about this method, the size of liposomes is influenced by several factors like lipid concentrations[38] and liposome composition.[39]

Measurements of zeta potential are usually used to predict the stability of colloidal systems. If the particles in a suspension have large negative or positive zeta potential values, they will have lesser tendency to aggregate. However, if the zeta potential values for particles were low then there will be no force to prevent the particles flocculating. Particles suspension with zeta potentials between +30 mV and −30 mV are normally considered stable.[43]

The zeta potential of *Z. multiflora* Bioss. EO encapsulating nanoliposomes could be attributed to the interaction between the components of EO and phosphatidylcholine. In the present study, the mean zeta potential of liposomal dispersios was lower than -30 mv that indicated appropriate stability for prepared liposomes. As a result, it was increased with increase in size of liposomes containing EO. This effect can be explained by higher encapsulation of EO in lipid bilayer that causes more interaction between the EO components and phosphatidylcholine.

In this study, preparation method had a significant effect on encapsulation efficiency ($p<0.05$). The MLV liposomes prepared by thin film evaporation method had the higher efficiency than other methods (table 2). Since the MLV liposomes have several lamellas, they can load higher amounts of lipophilic compound.

All of prepared liposomal solution showed a good stability over 4 weeks of storage at 4 ± 1˚C. Stability of nanoliposomes was determined by measuring of the changes in mean size and encapsulation efficiency of liposomal dispersions at specific intervals (Table 3 and 4). The results showed that the oil leakage was very low and the vesicles size was increased about 2 to 5 percent within one month of storage at 4 ± 1˚C.

Valenti et al. found that average size distribution of liposomes did not change appreciably during their storage and the encapsulation of *Santolina insularis* EO in liposome prevent from its degradation. After one year of storage, its composition was very close to that of the freshly prepared EO and the more volatile compounds had a minimal loss and there was not any identifiable degradation product.[14] Ortan et al. also reported that MLV and SUV liposomes encapsulating *Anethum graveolens* EO were stable for 6 mounth at 4–1°C with a slight oil leakage and slight increase in size.[42]

In this study, we observed lower leakage of EO from MLV liposomes. It can be related to the limited transmission of EO from the inner layers to the outer layers of liposomes. So, MLV liposomal system can be used to sustain release of EO.

Conclusion

The EO of *Z. multiflora* Bioss. is a lipophilic compound that is soluble in organic solvents. Since the properties of nanoliposomes containing plant EOs depend on the preparation method; in this study, the EO extracted from the aerial parts of *Z. multiflora* were encapsulated in multilamellar and unilamellar liposomes by three different methods. We observed that the preparation method can affect the physical properties of liposomal dispersion such as mean size, polydispersity index, zeta potential, encapsulation efficiency and storage stability. Liposomal systems prepared by sonication method with about 99 nm displayed the smallest mean size and better dispersivity. However, nanoliposomes prepared by thin layer method showed better encapsulation efficiency and storage stability than other methods. It is known that the size of liposomes, surface properties and their stability have an important effect on biodistribution of these compounds and the possibility of targeting of the encapsulated EO to specific targets. Hence, in order to increase the biological effects of these compounds, optimization of physical properties of EO loaded liposomes is necessary. Furthermore, to evaluate the bioavailability and release of the EO of *Z. multiflora* from liposome, further studies should be conducted under various experimental conditions.

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