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Preparation and Characterization of Nano-Niosomes Containing Essential Oils of *Thymus caramanicus Jalas* and Evaluation of its Total Phenolic Contents and Antioxidant Properties

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ABSTRACT

Essential oils (EOs) are aromatic oily liquids that have some biological properties such as antioxidant and antimicrobial activities. The nanoencapsulation of these oils in drug delivery systems have been proposed due to their capability of improving the solubility, stability and bioavailability of them by maintenance of therapeutic drug blood levels. In this study encapsulation of thyme essential oil in nano-niosomes and evaluation of its total phenolic contents and antioxidant properties were evaluated. Nano-niosomes containing thyme essential oil were prepared by thin film hydration method using span20 and cholesterol and were characterized for their size, poly dispersity index, zeta potential, encapsulation efficiency and morphology. Then the total phenolic contents and antioxidant activity of free and nano-niosomal essential oil was studied. The results show that particle size was about 83 nm and poly dispersity index was 0.226. The encapsulation efficiency of essential oil was 17% when appropriate formulation is used. We observed that total phenolic contents and antioxidant activity of EO was significantly increased after encapsulation. Nano encapsulation effectively enhanced beneficial properties of thyme essential oils such as total phenolic contents and antioxidant properties.

Keywords: Thyme essential oils, Niosomes, Antioxidant, Total phenolic contents.

INTRODUCTION

Essential oils (EOs) are natural, volatile, aromatic oily liquids as secondary metabolites that can be obtained from several parts of the plants especially the aerial ones as leaves and flowers. They are derived from complex metabolic pathways in order to protect the plant organism from diverse pathogenic microorganisms and repel insects that act as plague vectors [1]. An extensive body of research has demonstrated that essential oils and their main components possess a wide spectrum of biological activity, which may be of great importance in several fields, from food chemistry to pharmaceutics [2].

Different methods of preparation can also provide different EOs. Some of the oldest techniques for EO production are steam and hydro distillation [3]. The first documents describing distillation are dated from the 9th century. *Thymus caramanicus Jalas* is belonging to Lamiaceae family. Among 250 species of this genus with a worldwide distribution, 14 species grow naturally in Iran. *Thymus caramanicus Jalas* is one of the endemic species which has distribution in central Iran.

Generally EOs contain about 20–60 components up to more than 100 single substances, at quite different concentrations; two or three are major components at fairly high concentrations (20–70%) compared to others components present in trace amounts. For example, carvacrol (20.8%) and thymol (52.8%) are the major components of the *Thymus caramanicus* essential oil [4]. The most of EOs are poorly soluble in water, biologically unstable and they distribute defectively to target sites. Currently, some novel methods have been introduced in order to improve their stability and their bioavailability, among which is the use of niosomal encapsulation. Niosomes are microscopic lamellar vesicles containing non-ionic surfactants which may or may not be incorporated with cholesterol. Over the last few decades, the applications of nano technology in medicine have been extensively explored in many medical areas, especially in drug delivery. Many advantages of nano-particle based drug delivery have been recognized, including improving serum solubility of the drugs, prolonging the systemic circulation lifetime, releasing drugs at a sustained and controlled manner, preferentially delivering drugs to the tissues and cells of interest, and concurrently delivering multiple therapeutic agents to the same cells for combination therapy [5-7]. In the present study we investigate the effectiveness and encapsulation of the essential oil of *Thymus caramanicus Jalas* and formulate and characterize a niosomal gel loaded with the essential oil. Some parameters such as vesicle size, entrapment efficacy and the morphology of nano-niosomes are also investigated. The other objective of this study is to evaluate the effect of nano-niosome encapsulation using thin film method, on the antioxidant activities and total phenolic contents of *Thymus caramanicus* essential oils and comparing them to free essential oil.

MATERIALS AND METHODS

Chemicals

Sorbitan monolaurate (Span 20), sorbitan monostearate (Span 60) and cholesterol were purchased from Merck (Germany). All of organic solvents were analytical grade and the deionized water was used throughout the experiment.

Preparation of essential oils

The leaves of *Thymus caramanicus* were collected from kerman (Iran). 200 g of dried samples of the plant was distilled (Steam distillation) in one liter of distilled water over four hours using a clevenger apparatus. The essential oil was extracted and dried over anhydrous sodium sulfate and stored in a refrigerator for future use.

Preparation of niosomes containing essential oil

Nano-niosomes were formulated using thin film hydration technique with a slight modification. Span 20 or Span 60 and cholesterol with various quantities (50:50, 60:40, 70:30 w/w) were mixed in 5ml chloroform as solvent. Then essential oil was dissolved in methanol and was added to the mixture in round flask. Organic solvents were evaporated under reduced pressure by a rotary evaporator at 35°C until a thin film was formed on the walls. The residual solvent removed by nitrogen stream at 25°C. Then 5 ml of distilled water was added to the flask and the lipid film was hydrated in a rotary evaporator (without vacuum) for 45 minutes at 37°C for span 20 (or 55°C for span 60) and 150 rpm to form multi-lamellar vesicles (MLVs). In order to decrease the size, niosomal solution obtained by the mentioned technique was sonicated by a probe sonicator (Misonix, USA) in ice bath at 60% amplifying strength for 10 min (10 sec sonication and 15 sec rest intermittently to allow cooling of the sample). For the separation of impurities (such as titanium particles formed by sonication) of niosomal solution, centrifugation at 5000 RPM for 5 minutes was done. In order to separate the larger particles from smaller particles and homogenization of the resulting suspension, 0.45 micrometer filter was used and finally for sterile filtration, the solution was passed through the 0.22 micrometers filter.

Encapsulation efficiency measurement

Encapsulation efficiency was determined by dialysis technique against distilled water at 4°C using a cellulose membrane (with molecular weight cut-off of 10 kD) to separate encapsulated EO from the unencapsulated material. Then, the dialyzed niosomes were disrupted with methanol and the quantity of encapsulated EO was measured using a UV/VIS spectrophotometer (Beckman, DU 530, Switzerland) at λ_{max}= 274 nm. The essential oil concentration in nano-niosomes was calculated using the following equation.

$$EE\% = C / C_0 \times 100$$

Where C is the amount of (EO) incorporated in nano-niosomes and C_0 is the amount of total essential oil.

Size analysis and zeta potential measurements

The mean particle diameter and size distribution of nano-niosomes were determined by Dynamic Light Scattering technique (DLS) (Brookhaven Instruments Ltd., Brookhaven, USA) at 25°C. Samples were scattered at 657 nm at the angle of 90°. Before the size measurement, distilled water was added to the niosomal suspension for diluting. All analysis was carried out in triplicate. Zeta potential of prepared samples was also assessed by the same DLS-based instrument at 25°C.

The morphology of nano-niosomes

Scanning Electron Microscopy (SEM) was used to determine the morphology of prepared vesicles. Also in order to evaluate multi-layer membrane of multilamellar niosomes, images using a Transmission Electron Microscope (TEM) (with a capacity of 150 kV) were prepared.

Fourier Transform Infrared (FTIR) study

The free essential oil, empty niosome and niosome containing essential oil were assessed by FT-IR instrument. These compounds mixed and pressed with KBr pellets to form tablet and Fourier Transforms Infrared (FTIR) spectra of samples was recorded. The scans were performed over a wave number range of 4000– 400 cm^{-1}.

Total phenolic contents (TPC)

The amount of phenolic compounds in thyme essential oils, before and after niosomal encapsulation was determined using Folin-ciocalteu reagent as described by Velioglu et al. [8].

Two hundred µL of free essential oil (0.97 mg/mL) and noisome containing essential oil was combined with 1.5 mL Folin-Ciocalteu's phenol reagent (previously diluted 10 times with double distilled water) and allowed to stand at 22°C for 5 minutes. Then 1.5 mL sodium bicarbonate solution (6%) was added to the mixture. After 90 minutes at 22°C, absorbance was measured at 725 nm using a UV-Visible spectrophotometer. Standard curve was prepared by using different concentrations of gallic acid and the absorbances were measured at 725 nm. Total phenolic content was expressed as mg gallic acid/g dry essential oil (mg GAE/g EO). Analyses were done in triplicate.

Evaluation of antioxidant activity (FRAP method)

FRAP is a simple direct test of antioxidant capacity. This method was initially developed to assay plasma antioxidant capacity, but can be used for plants too. The total antioxidant potential of sample was determined using a ferric reducing ability of plasma (FRAP) assay that was described initially by Benzie and Strain [9]. The principle of FRAP assay is the formation of a blue colored Fe(II)-tripyridyl triazine compound from colorless oxidized Fe^{III} form by the action of electron donating antioxidants. FRAP reagent was prepared by mixing 5 mL of TPTZ (2,4,6-tripyridyl-s-triazine) solution (10 mmol/L) in HCl (40 mmol/L), 5 ml of $FeCl_3$ (20 mmol/L) and 50 mL of acetate buffer (0.3 mol/L, pH 3.6). it was freshly prepared and warmed up to 37°C. Then this solution mixed with 100 µL of certain concentration of the essential oil in form of free and encapsulated and incubated at 37°C for 10 min. The absorbance of the reaction mixture was measured at 593 nm. For construction of the calibration curve, five concentrations of $FeSO_4.7H_2O$ (1000, 750, 500, 250 and 125 µmol/L) were used and the absorbance values were measured as for sample solutions. The antioxidant activities were expressed as the concentrations of antioxidant having a ferric reducing ability equivalent to that of 1 mM of $FeSO_4$. All the measurements were taken in triplicate and expressed as mean value.

RESULTS

Encapsulation efficiency

Entrapment efficiency is an important parameter for industrial application of the niosomal system. In general, efficiency depends on bilayer forming compounds, encapsulation and interaction between vesicle forming substance and core material.

In this study, some different vesicular formulations were prepared and optimum conditions for preparing niosome containing thyme essential oil were obtained. The results showed that three variables: the ratio of surfactant to cholesterol, the ratio of essential oil to total lipids and hydration temperature can affect the encapsulation efficiency; among them, the hydration temperature and the amount of essential oil are more effective. The results in the Table 1 show that the optimum conditions for the formulation of niosomal essential oil in terms of incorporation yields are using Span 20 as amphiphile and 8% EO by the weight of essential oil to the total lipids and the hydration temperature of 37°C.

Table 1: The components, formulation conditions and encapsulation efficiency percent (EE%) of prepared nano-niosomes containing thyme essential oils.

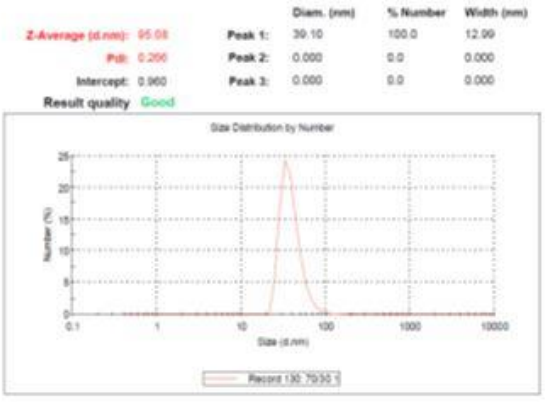
Surfactant	Span/Chol	Essential Oil(%)/total lipid	Hydration Temperature	Encapsulation Efficiency (%)
Span 60	70/30	3	55	4
Span 20	70/30	3	45	6.2
Span 20	70/30	3	37	9
Span 20	60/40	3	37	7
Span 20	80/20	3	37	10.7
Span 20	80/20	5	37	14

Span 20	80/20	8	37	17
Span 20	80/20	10	37	17.9

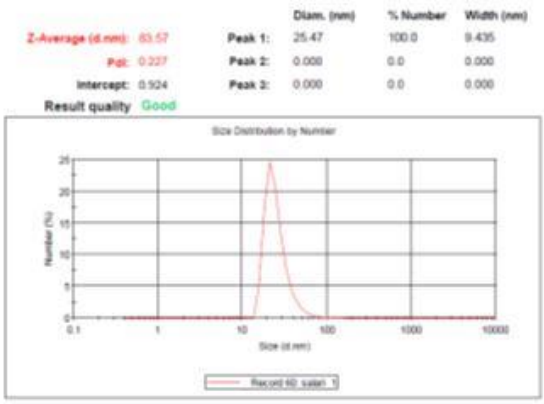
Encapsulation efficiency of nano-niosomes prepared by thin film evaporation under the optimum conditions was 17%.

Particle size analysis

The diameter of prepared nano-niosomes by thin film evaporation method was determined using a particle sizer device in both as volume (VMD) and number (NMD) mean diameters. The size and size distribution of nanoparticles before and after loading EO has been showed in Figure 1. The particle size values given are averages of three measurements. The results showed that the mean size of nano niosomes before and after loading was about 83 nm and 95 nm respectively. Poly dispersity is usually expressed as an index of particle diameters in colloidal systems. When the level of this index is lower, the diameter of particles is more uniform. The value of greater than 0.3 for this index indicates a high degree of heterogeneity [10]. In the present study size distribution of synthesized nanoniosomes was homogeneous and poly dispersity index of them was 0.227.



Before loading EO



After loading EO

Figure 1: Size and size distribution of prepared niosomes before (1) and after (2) loading thyme essential oils.

Zeta potential

Zeta potential of the nano-niosomes containing EO was between -22 mV and -28 mV and this parameter for empty nano-niosomes was -14 mV. Zeta potential shows the surface charge and thereby the stability of the prepared nanoparticles systems. the suspension with greater zeta potential like between ± 30 to 40 mV, which is critical zeta-potential, is more likely to be stable, which is due to the charged particles repelling each other and thus they will have lesser tendency to aggregate [11].

Scanning Electron Microscope (SEM)

In images taken with scanning electronic microscope (with a capacity of 25 kV) (Figure 2), the particles of prepared nano-niosomes containing essential oil has often had a spherical shape and uniform structure. In the picture taken with SEM microscope, nano-sized niosome mean diameter is about 60 to 70 nm, which is consistent with the results of DLS.

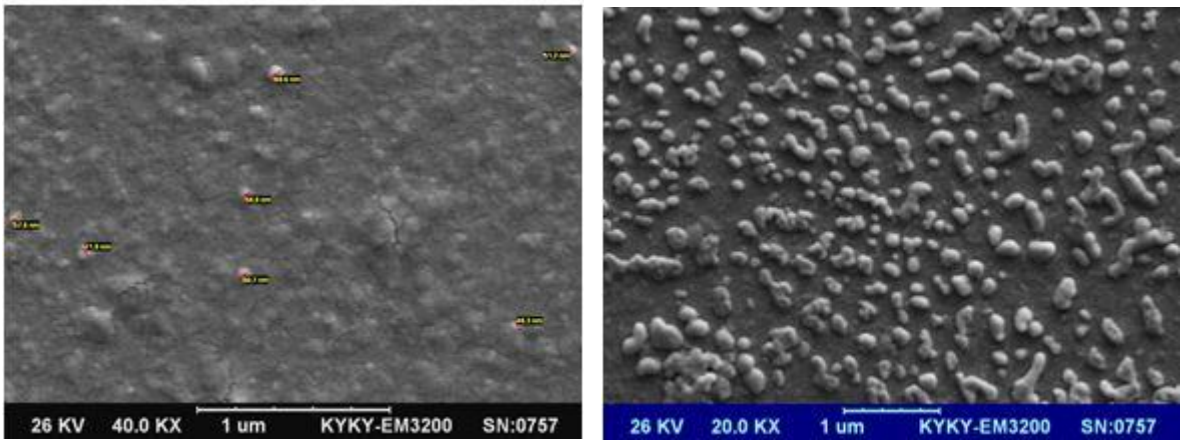


Figure 2: SEM image of nano-niosome containing thyme essential oils with optimum formulation.

Transmission Electron Microscopy (TEM)

Figure 3 is a TEM microscope image from prepared vesicles. Two lipid bi layers and spherical structure of niosomes carrying thyme essential oils in images taken with a transmission electron microscope (TEM) (with a capacity of 150 kV) were observed.

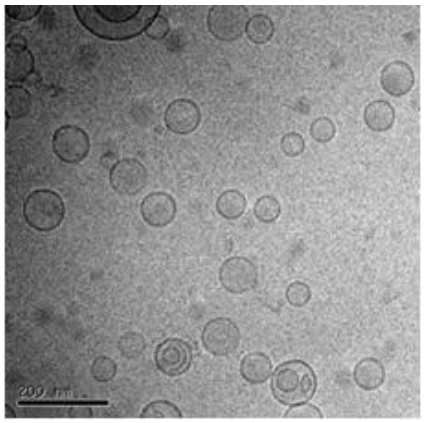


Figure 3: TEM image of nano-niosome containing thyme essential oils.

Analysis by infrared spectroscopy

To determine any possible chemical interaction between the essential oil and nanocarrier, we have used FTIR-spectroscopy, which is one of the most important analyses to describe about the stability of formulation, presence of drug and drug release. Figure 4 shows the infrared spectrum of (a) essential oil, (b) empty niosome and (c) niosome containing essential oil. The peak values of niosome containing essential oil and empty niosome were approximately identical at the wave numbers of 3445, 2570 and 2050 cm^{-1} and there is minor shifting of some peaks. This suggests that there was no chemical interaction between essential oil and carrier compounds.

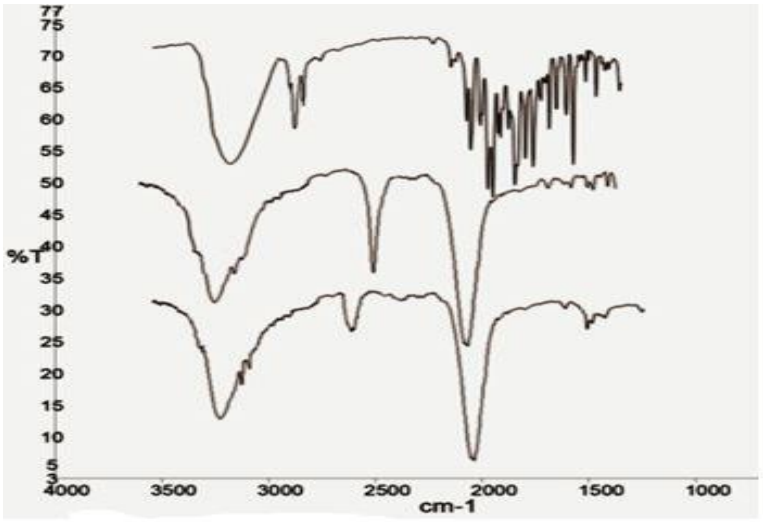


Figure 4: Infrared spectrum of a) essential oil b) empty nano-niosome and c) niosome containing thyme essential oil.

In vitro release studies

In vitro EO release from the niosome was determined using dialysis membrane method. 1 cc of each formulation was put in a dialysis bag. The receptor phase was 10 ml deionized water and using a magnet was continually stirred at 37°C. One ml of sample was withdrawn from each batch at definite time intervals (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 12 h, 24 h and 48 h) and replaced with the same amount of deionized water to maintain sink condition. Then, the concentration of drug released was monitored using a UV spectrophotometer at 274 nm. Figure 5 is a consistent pattern of drug release. This chart shows the maximum amount of drug released from the niosomes for 36 hours at about 37 percent.

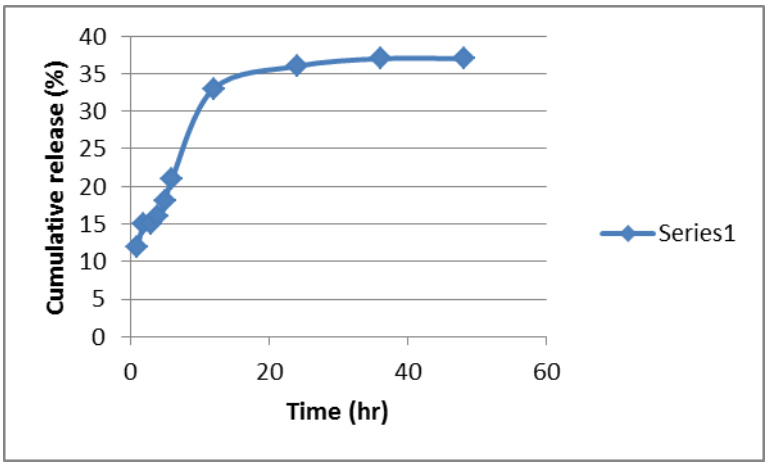


Figure 5: Release profile of EO from nano-niosome.

Total phenolic content and antioxidant activity

Thymus caramanicus Jalas has been used in traditional Iranian medicine according to its medicinal effects [12]. In the literature, the total phenolic content and antioxidant effects of thyme extract have been studied earlier by some authors [13, 14]. In this work, the total phenolic content of free and nano-niosomal essential oil of it was analyzed and is expressed in term of gallic acid equivalent (the standard curve equation: $Y= 0.002x + 0.001$, $r^2 =0.996$). As shown in Figure 6, the total phenol content of free and nano-niosomal EO was 295.4 and 1523 mg gallic acid g^{-1} EO, respectively. The results of our study showed that the total phenol content of nano-niosomal EO was higher than free EO. This could be the reason for the higher antiradical activity of nano-niosomal EO compared to free EO. Also the antioxidant capacity of free and nano-niosomal EO was evaluated as ferric reducing power and expressed in term of Fe^{2+} equivalent. According to the results, antioxidant capacity for free and nano-

niosomal EO is 1.13 and 4.2 mmol Fe^{2+} g^{-1} EO respectively. As it can be seen, the anti-oxidant activity of EO was significantly increased after encapsulation.

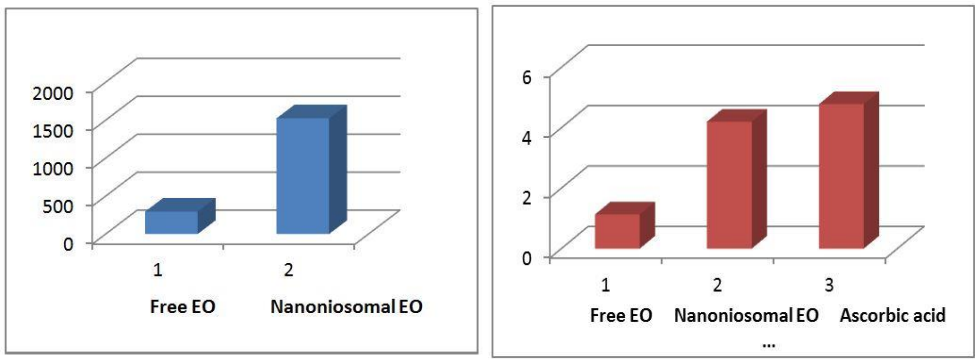


Figure 6: Total phenolic content (mg galic acid/ g of EO) and FRAP antioxidant activity (mmol Fe^{2+}/g of EO) of free and nanoniosomal thyme essential oil.

DISCUSSION

As mentioned before, niosomes are non-ionic surfactant vesicles in aqueous media resulting in closed bilayer structures that can be used as carriers of hydrophilic and hydrophobic compounds. The use of niosomes for encapsulation of essential oils (EOs) is an attractive new approach to overcome their physicochemical stability concerns include sensibility to oxygen, light, temperature, and volatility, and their reduced bioavailability which is due to low solubility in water. The main advantages of niosomes in comparison to liposomes is more chemical stability against both oxidation and temperature and requiring less care in handling and storage, ease of their production in large scale without the use of pharmaceutically unacceptable solvents and greater versatility and lower cost. But a significantly large part of current literature on the encapsulation of EOs deals with liposomes and there are a few researches on encapsulating of EOs in niosomes.

The encapsulation efficiency differs from an EO to another for liposomes or niosomes prepared by the same method. For example, Liolios et al. [15] obtained low encapsulation efficiency (4.16%) for carvacrol while Ortan et al. [16] showed much higher encapsulation efficiency (98%) for *Anethem graveolens* EO. In another study, Noudoost et al. [17] reported entrapment efficiency of nano-liposomal green tea extractunder the optimum conditions was 97%. This difference in the encapsulation efficiency was explained by Detoni et al. [18] by the physico-chemical properties of each EO and/or the variations that may occur in the techniques of preparations of vesicles, such as surfactant concentration, ratio of EO/lipid and cholesterol content.

In the present study characterization and morphology of nano-niosomal EO including particle size, poly dispersity index, zeta potential and entrapment efficiency were assessed. According to the results of DLS, the majority of vesicles were separately

dispersed and PDI was narrow. In this research, the results showed that the mean size of EO loaded nano-niosomes was 83 nm whereas the size of empty niosomes was 95 nm, so that EO loaded niosomes were smaller than empty liposomes. These findings correlate with those observed by valenti et al. [19] which demonstrated empty liposomes had a mean size of around320 - 2200 and 8.7-120 nm for MLV and SUV, respectively, while EO loaded liposomes had a mean size of 45-467 nm and 12-63 nm for MLV and SUV, respectively. Similar findings also have been reported by other researchers about liposomes containing EOs of *Zanthoxy lumtingoassuiba* [20]. Also yoshida et al. [21] showed 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. This effect was explained by the capability of EOs to cause higher cohesion and packing between the non-polar chains in the membrane vesicles [19].

In the last few years, there has been a growing interest in providing natural antioxidants. Natural products such as plant essential oils (EOs) have uses in human health such as functional food, food additives, medicine, nutritional supplements and cosmetic manufacturing. Studies found in the literature have demonstrated that the spices belonging to the lamiaceae family, as well as their extracts and essential oils, are efficient antioxidants [22]. Thyme essential oilis of much attention due to its high content and wide spectrum of phenolic compounds, antimicrobial and anti-oxidant properties. In our study we tried to make niosomes that had been capsulated with antioxidant compounds such as thyme essential oil and then we evaluated the antioxidant activity of these niosomes. Niosomes containing EO, had the highest equivalent antioxidant activity.

CONCLUSION

In this study EO extracted from the aerial parts of *Thymus caramanicus* were encapsulated in multilamellar and unilamellar niosomes by thin film hydration method. We observed that the molar ratios of essential oil, surfactant and cholesterol influence the drug entrapment of the niosomes. Also, the results demonstrated that the nano-niosome encapsulation of EO improved its beneficial properties including antioxidant activities. In agreement with our results, Spigno et al. demonstrated [23] that nano-liposome encapsulation technology improved antioxidant efficiency of phenolic compounds against lipid oxidation by increasing extract dispersibility in the environment.

There is a high correlation between total phenolic content and antioxidant activity. On the other hand, antioxidant activity of plant extracts is not limited to phenolics. Activity may also come from the presence of other antioxidant secondary metabolites, such as volatile oils, carotenoids, and vitamins [24].

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