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Encapsulation of *Rosa damascena* Mill. Essential Oil in Nanoliposomes

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ABSTRACT

***Rosa damascena* Mill. Essential Oil (EO) was encapsulated in nanoliposomes to overcome its low stability and limited solubility. Preparation of EO-Loaded Nanoliposomes (EO-LNLs) was optimized based on the Response Surface Methodology (RSM) with a Central Composite Face-centred (CCF) design. Different concentrations of EO (500, 1,000, and 1,500 ppm) and lecithin (0.5, 1.25, and 2% w/v) were applied for preparing nanoliposomes. The produced nanoliposomes had a particle size of 82-124 nm, a zeta potential of -55 to -30 mV, and a Polydispersity Index (PDI) of 0.270- 0.342. The nanoliposomes prepared with 1.56% lecithin and 500 ppm of EO had the best properties with the encapsulation efficiency of 84.02±2.23%. The results obtained from different instrumental methods (DSC, FT-IR, and TEM) verified the encapsulation of EO in nanoliposomes. According to the antioxidant activity evaluations, free EO had higher radical scavenging activity and lower EC_{50} than encapsulated EO. The highest *in vitro* release of EO from nanoliposomes occurred at pH= 3. During the storage of nanoliposomes for seven weeks at 4°C, their particle size was increased by 7.0%. Finally, *Rosa damascena* Mill. EO was successfully encapsulated in nanoliposomes and, therefore, its use as a natural preservative in different products such as foodstuffs, medicines, and cosmetics can be suggested.**

Keywords: Bioactive compounds, Foodstuffs preservative, Liposome, Nanoencapsulation, Natural preservative.

INTRODUCTION

Essential oils are secondary metabolites of aromatic plants. They contain various volatile and complex compounds (Bakkali *et al*., 2008). The major components of EOs are hydrocarbons (including sesquiterpenes and terpenes) and various oxygenated components such as aldehydes, phenols, esters, lactones, ketones, and alcohols (Rezaei *et al*., 2019).

Rosa damascena Mill., also known as Damask rose, is one of the most important members of the *Rosa* genus from the Rosaceae family. Iran has a long history of the cultivation and consumption of *Rosa damascena* Mill. (Jalali-Heravi *et al*., 2008). *Rosa damascena* Mill. EO is a safe natural oil with numerous beneficial activities like antioxidant (Mileva *et al*., 2014; Nikolova *et al*., 2016), antibacterial (Basim and Basim, 2003; Ulusoy *et al*., 2009; Mahboubi *et al*., 2011; Ghavam *et al*., 2021), antifungal (Mahboubi *et al*., 2011; Mileva *et al*., 2014; Ghavam *et al*., 2021), and anticancer (Zu *et al*., 2010; Rezaie-Tavirani *et al*., 2013) properties. Because of its aromatic and favourable properties, it has applications in the perfumery, cosmetic, pharmaceutical, and food industries. Typically, citronellol, nonadecane, geraniol, and heneicosane are the dominant components in *Rosa*

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damascena Mill. EO (Mileva *et al*., 2014; Ghavam *et al*., 2021).

Contrary to the advantages of essential oils, they have some drawbacks, such as less stability, intensive odour, heat sensitivity (Ajeeshkumar *et al*., 2021), and low solubility in aqueous media. Therefore, they cannot be used directly in foodstuffs. Essential oils lose their beneficial properties during the processing and storage of EO-containing products.

Encapsulation is one of the novel technologies that can be used to overcome these limitations of EOs. Encapsulation can protect the EO components from undesirable conditions such as heat, light, oxygen, and inappropriate pH. By encapsulation, their stability and bioavailability will improve, causing their gradual release during storage. Although a few studies reported the rose EO encapsulation, those methods were complicated and applied uncommon and non-economical wall materials like mung bean protein and isolate-apricot peel pectin (Xiao *et al*., 2019; Heydari *et al*., 2021; Qiu *et al*., 2022). In this research, a more straightforward method is introduced and a common and inexpensive wall material is used.

Liposomes, lipid-based carrier systems, are the promising carriers stabilizing the encapsulated ingredients against environmental changes during processing and storage (Đorđević *et al*., 2016). During the production of liposomes in an aqueous solution, a phospholipid that is amphipathic forms vesicles with a bilayer membrane; as a result, the produced liposomes have a hydrophilic center with a phospholipid bilayer around them. Hence, they can load hydrophilic and lipophilic ingredients in their core and phospholipid bilayer, respectively (Ajeeshkumar *et al*., 2021). Nanoliposomes have higher surface areas with diameters lower than 100 nm that can enhance the solubility, controlled release, and bioavailability of the encapsulated components. Overall, since liposomes can be manufactured using completely natural ingredients, they are biocompatible and safe for human consumption (Khorasani *et al*., 2018). Several studies have shown the successful encapsulation of EOs in liposomes (Detoni *et al*., 2012; Sebaaly *et al*., 2015; Wu *et al*., 2015; Cui *et al*., 2020; Aguilar-Pérez *et al*., 2021). Encapsulation of bioactive compounds in liposomes is simple, efficient, and inexpensive; so, it can be used on a large scale. Furthermore, liposomes have a polar surface and can easily dissolve in aqueous media. Therefore, the lipophilic components of rose EO inside the lipid bilayer can quickly disperse in an aqueous medium.

Accordingly, the present study aimed to produce *Rosa damascena* Mill. EO-Loaded Nanoliposomes (EO-LNLs) at optimal conditions using the Response Surface Methodology (RSM). In addition, the physicochemical properties of the nanoliposomes, the efficiency of the encapsulation, the stability of the nanoliposomes during storage, the release rate of the EO from nanoliposomes, and the antioxidant activity of EO-LNLs were investigated.

MATERIALS AND METHODS

Rosa damascena Mill. EO was obtained from Golab Zahra Co. (Kerman, Iran). Soybean lecithin (99%) was purchased from Acros Organics Chemical Co. (Geel, Belgium). All other analytical grade chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, USA), Rankem (New Delhi, India), or Merck Chemical Co. (Darmstadt, Germany).

Determination of EO Chemical Components

The EO chemical components were determined using GC/MS-Solid Phase Microextraction (SPME) based on the method of Siroli *et al*. (2014). About 0.5 mL of each sample was poured into sterile closed glass containers with a volume of 10

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mL, and their lids were completely closed with a silicone/Teflon cap. The samples were then equilibrated at room temperature for 30 min. The volatile constituents of EO were absorbed for 20 min on fiber coated with divinyl benzene/carboxen/polydimethylsiloxane (65 mm) (Supelco Inc., Bellefonte, PA, USA). The adsorbed volatile constituents were desorbed in the injector for 10 min. Agilent gas chromatograph equipped with an FID detector and 5973 MSD mass spectrometer was used to detect the peaks. HP1 non-polar capillary column (30 m×0.25 mm×0.25 µm) was used as the stationary phase. Helium gas at a flow rate of 1 mL/min was applied as the carrier gas. The temperature programing was applied for improving the resolution (starting from 40°C and increased to 250°C at 3°C min^{-1}). The temperature of the injection port and detector was set at 320 and 310°C, respectively. Mass spectra were prepared at 70eV, and their range was selected from 40-350 amu with a 1-second scan time. The EO components were identified using the information of the library of GC/MS and the Wiley mass spectral database (ver. 6, 2017).

Preparation of Nanoliposomes

The Essential Oil-Loaded Nanoliposomes (EO-LNLs) were prepared by different concentrations of EO (500, 1,000, and 1,500 ppm) and soy lecithin (0.5, 1.25, and 2% w/v) based on Colas *et al*. (2007) method with some modifications. First, the required amounts of EO and lecithin were weighed and then hydrated by adding 20 mL of deionized water and 3% v/v of glycerol. The mixtures were stirred for 30 minutes at 30°C (1,000 rpm). Then, the liposomal suspensions were subjected to sonication (1 minute, 1 second on and 1 second off) using the probe sonicator (S-4000, 20 kHz, maximum nominal power 600 W, Misonix, USA) at 80% of total power. During sonication, temperature controlled in a double-wall circulation cell at 30±1.0°C. The Empty Nanoliposomes (ENLs) were also prepared under optimal conditions.

Particle Size, Zeta Potential and Polydispersity Index (PDI) of Nanoliposomes

The particle size, zeta potential, and PDI of nanoliposomes were measured by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS instrument (Malvern Instruments) as described by Bouarab *et al*. (2014).

Transmission Electron Microscopy (TEM)

Morphological characteristics of all nanoliposomes (ENLs and EO-LNLs) were determined by TEM (Zeiss-EM10C-100kV, Germany) according to the method of Lu *et al*. (2014).

Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra of EO, ENLs, and EO-LNLs were recorded using the FT-IR (PerkinElmer, Frontier, USA) in the range of 400-4,000 cm^{-1} (Zou *et al*., 2014).

Differential Scanning Calorimetry (DSC)

The phase transition Temperature (Tc) of nanoliposomes and the oxidative stability of free and encapsulated EOs were analyzed by DSC (Mettler Toledo, Switzerland) according to Gortzi *et al*. (2006) method. The Tc was determined by heating the samples from -30 to 50°C with a scan rate of 0.5°C min^{-1}. For oxidative stability determinations, the samples were placed under the oxygen flow of 50 mL min^{-1} and were heated from 30 to 300°C according to the mentioned reference.

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Determination of Encapsulation Efficiency (EE)

To evaluate EE, the EO-LNLs were centrifuged at 41,858 g for 45 minutes (3-30k; Sigma) at 4°C. Then, the supernatant was collected and the amount of unentrapped EO was determined based on the EO`s absorbance calibration curve (y= 0.003298x+0.2691, n= 6) at λ_{max}= 225 nm. Finally, its EE was determined by Equation (1):

$$EE\% = ((C_i - C_s)/ C_i) \times 100 \quad (1)$$

Where, C_i is the initial amount of EO and C_s represents free EO in the supernatant (Lu *et al*., 2014).

In vitro Release Behaviour

In vitro release of EO from nanoliposomes in various buffer media, including citrate (pH= 3), acetate (pH= 5), and phosphate-buffered saline (pH= 7) at room temperature was studied according to Madrigal-Carballo *et al*. (2010) method with minor modifications. EO-LNLs were isolated by centrifugation from the suspension, submerged in each of the above buffers in sealed tubes, and kept at room temperature under continuous stirring (200 rpm). In the defined time intervals, sampling was done every two hours in the first 10 hours, and after that, release behavior was studied up to 121 h. The amounts of EO were determined by UV-Vis spectrophotometer (by linear equation, y= 0.003298x+0.2691, n= 6 and λ_{max}= 225 nm). The Cumulative Release (CR) percent of EO-LNLs was calculated using Equation (2):

$$CP\ (\%) = \sum_{t=0}^{t} \frac{Ct}{Co} . 100 \quad (2)$$

Where, C_o and C_t are the initial amount of EO and released EO at time *t,* respectively.

Stability of Nanoliposomes during Storage

To investigate the stability of nanoliposomes, the samples were stored at 4°C for seven weeks. Then, the particle size, zeta potential, and PDI of samples were determined at specific times (Rafiee *et al*., 2017).

Antioxidant Activity Assay

Antioxidant activities of free and encapsulated EOs were evaluated by three different methods: Diphenyl-1-picrylhydrazyl (DPPH radical scavenging activity (Sebaaly *et al*., 2015), 2,2′-Azino-bis(3-Ethylbenzothiazoline-6- Sulfonic Acid) ABTS radical cation scavenging activity (Re *et al*., 1999), and Ferric Reducing Antioxidant Power (FRAP) (Benzie and Strain, 1996). Before the experiments, defined amount of nanoliposomes were suspended in ethanol to obtain an equal EO concentration of 100-1,500 ppm. Afterward, they were placed in a shaker and agitated at 200 rpm for two hours to release the EO. Finally, the results of antioxidant activities were expressed as EC_{50} value

Statistical Analysis

RSM with a Central Composite Face-centred (CCF) design with two factors and three levels was used to obtain the optimum conditions for encapsulation of EO in nanoliposomes. CCF designs provide relatively high quality predictions over the entire design space and do not require points outside the original factor range. However, CCF gives poor precision for estimating pure quadratic coefficients.

Before the development of the RSM, preliminary tests were performed to select the main factors (EO and lecithin concentrations). These factors are effective in the nanoencapsulation process. The effect of two independent variables, EO concentration (500, 1,000, and 1,500 ppm) and lecithin concentration (0.5, 1.25, and 2.0% w/v), on particle size, zeta potential, and PDI, as responses, were studied (Table 1). The data were analysed by multiple

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regressions to fit the following second-order (quadratic) polynomial model (Equation 3):

$$Y_k = \beta_{k0} + \sum_{i=1}^{3} \beta_{ki} X_i + \sum_{i=1}^{3} \beta_{kii} X_i^2 + \sum_{i=1}^{3} \beta_{kij} X_i X_j \quad (3)$$

Where, β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant regression coefficients of the model, Xi and Xj are the independent factors. MODDE 7 program was used for the polynomial regression and visualizing the relationships between responses and independent factors and the results were expressed graphically (graphs are not shown). In addition, analysis of variance was carried out by JMP 10 using Tukey's test at $P< 0.05$. All the runs were carried out in duplicate and a center point was repeated six times. Particle size (Y_1), PDI (Y_2), and zeta potential (Y_3) for each set of variable combinations were obtained (Eqn. 4-6), where X_1 is the EO concentration (ppm) and X_2 is the lecithin concentration (%). Multiple regression coefficients were calculated by employing the least square technique to predict quadratic polynomial models for Y_1, Y_2, and Y_3 (Equations 4-6):

$Y_1= 89.7226 + 13.3808\ X_1 – 7.1042\ X_2 + 5.8109\ X_1^2 + 2.3059\ X_2^2 – 7.7512\ X_1X_2$ (4)

$Y_2= 0.3080 + 0.0004\ X_1 + 0.0127\ X_2 – 0.0094\ X_1^2 + 0.0056\ X_2^2 + 0.0248\ X_1X_2$ (5)

$Y_3= - 47.0711+ 0.4499\ X_1 + 3.6167\ X_2 – 5.6026\ X_1^2 + 4.0026\ X_2^2 + 4.5000\ X_1X_2$ (6)

In all experiments, free EO was used as the control.

RESULTS AND DISCUSSION

GC/MS Analysis of *Rosa damascena* Mill. EO

Overall, 67 compounds were identified in *Rosa damascena* Mill. EO, which accounted for 96.4% of the total constituents of the EO. Nonadecane, with 26.7%, was the most dominant constituent of the EO. Followed by citronellol (18.2%), geraniol (16.9%), heptadecane (6%), germacrene D (5.1%), heneicosane (4.6%), α-pinene (2.8%), geranyl acetate (2.7%), dimethyl-2,6-octadiene (1.6%), α-guanine (1.3%), EE-farnesol (1.3%), β-caryophyllene (1.1%), eicosane (1.1%), and β-myrcene (1.1%) (See Table 2).

Several studies have investigated the componennts of *Rosa damascena* Mill. EO. For example, Ulusoy *et al*. (2009) introduced citronellol (35.23%), geraniol (22.19%), nonadecane (13.85%), nerol (10.26%), heneicosane (4.85%), and phenyl ethyl alcohol (2.30%) as the predominant constituents of this EO. In another study, n-nonacosane (26.31%) and bisabolol oxide (12.18%) were identified as the main constituents, and unlike other studies, geraniol, nerol, and phenyl ethyl alcohol were not observed (Naquvi *et al*., 2014). Overall, the differences observed in the EO components can be due to factors such as plant genotype, cultivation conditions, harvesting time, post-harvest storage conditions, and extraction methods (Toluei *et al*., 2019).

Physical Properties of Nanoliposomes

The results of all designed experiments are presented in Table 1. The particle size of EO-LNLs was in the range of 82-124 nm. As shown, by increasing the EO concentration from 500 to 1,500 ppm and fixed amount of lecithin, the particle size of nanoliposomes increased. However, this observation is insignificant in 2% lecithin ($P< 0.05$). The enhancement in particle size by increasing EO content can be attributed to the interactions between the hydrophobic molecules of EO and acyl chains of the phospholipid bilayer. This event alters the order of the acyl chain, increases the membrane`s fluidity, and increases the particle size of nanoliposomes (Bouarab *et al*., 2014; Keivani Nahr *et al*., 2019).

Another main dependent response of the produced liposomes is zeta potential, which determines their surface charge. During storage, the electrostatic stability and the release rate of EO are affected by zeta potential (Keivani Nahr *et al*., 2019). As

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Table 1. Experimental design and data related to size, zeta potential, and Polydispersity Index (PDI).

Treatment	EO [a] conc (ppm)	Lecithin conc. (%w/v)	Size (nm)	Zeta potential (mV)	PDI
1[b]	1000	1.25	87.0 ± 1.4[c]	-44.14 ± 2.76[a]	0.296 ±0.004[ab]
2	1500	2.00	93.0 ± 1.0[bc]	-30.50 ± 13.51[a]	0.342 ± 0.035[a]
3	1000	2.00	89.0 ± 2.2[bc]	-35.90 ± 9.19[a]	0.324 ±0.042[ab]
4	1000	0.50	99.7 ± 3.6[b]	-55.30 ± 0.28[a]	0.293 ±0.013[ab]
5	1500	0.50	124.4 ± 1.1[a]	-40.70 ± 9.48[a]	0.270 ± 0.006[b]
6	500	2.00	84.4 ± 0.2[c]	-40.70 ± 3.68[a]	0.294 ±0.004[ab]
7	500	1.25	81.8 ± 1.0[c]	-44.20 ± 1.98[a]	0.290 ± 0.011[ab]
8	500	0.50	84.8 ± 8.4[c]	-32.85 ± 3.89[a]	0.322 ±0.015[ab]
9	1500	1.25	113.9 ± 5.5[a]	-43.80 ± 11.46[a]	0.298 ±0.001[ab]

[a] EO: Essential Oil. [b] Central point. The mean values of six replicates reported for central point and two replicates for other values±standard deviations. Different lower case letters in the same column show significant differences (P< 0.05).

Table 2. Kovats retention indexes and the percentage of the main *Rosa damascena* Mill. essential oil constituents[a].

Constituent	Kovats index	Percentage	Constituent	Kovats index	Percentage
α-Pinene	855	2.8	Germacrene D	1357	5.1
β-Myrcene	912	1.1	β-Caryophyllene	1372	1.1
Citronellol	1138	18.2	(E, E)-Farnesol	1556	1.3
Geraniol	1156	16.9	Heptadecane	1559	6.0
Dimethyl-2,6-octadiene	1249	1.6	Nonadecane	1752	26.7
Geranyl acetate	1290	2.7	Eicosane	1823	1.1
α-Guaiene	1333	1.3	Heneicosane	1895	4.6

[a] Minor components (< 1%) are not presented in the Table.

shown in Table 1, the zeta potential of nanoliposomes ranges from -55.30 to -30.50 mV. This high zeta potential causes sufficient repulsive forces between the nanoliposomes, so, leading to a stable system. Generally, zeta potentials less than -30 and above +30 mV can make stable systems (Lu *et al*., 2014). The negative charges of nanoliposomes can be due to the presence of phosphatidic acid in soy lecithin (Caddeo *et al*., 2008; Machado *et al*., 2019). On the other, the surface charges of all samples did not change significantly (P< 0.05). These observations indicate that the *Rosa damascena* Mill. EO used in this experiment mostly contained non-ionic molecules.

PDI is an essential parameter for determining the physical stability of nano-dispersions. PDI values less than, and above, 0.3 indicate the narrow and broad distribution of particle size, respectively (Yen *et al*., 2008). Our findings revealed that the PDI of nanoliposomes was in the range of 0.270-0.342, indicating the narrow range, homogeneity, and more stability of nanoliposomes during storage.

Determination of Optimized Conditions

Independent variables for the preparation of nanoliposomes were optimized by RSM based on minimum PDI, particle size, and maximum zeta potential. These parameters were measured under optimized conditions to confirm the predictive capability of the model (Table 3). The results confirmed the predictability of the model for production of

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Table 3. Particle size, surface charge, and PDI achieved from the experiment and predicted by the model under the optimized conditions.[a]

Independent variables		Predicted values			Observed values		
EO (ppm)	L (%w/v)	Size (nm)	Zeta potential (mV)	PDI	Size (nm)	Zeta potential (mV)	PDI
500	1.56	82.8	-41.600	0.294	84.5±1.2	-47.950±1.344	0.296±0.005

[a] Mean of the observed values (n= 2) under optimized conditions. EO: Essential Oil; L: Lecithin, PDI: Polydispersity Index.

nanoliposomes. Finally, 500 ppm of EO and 1.56% w/v of lecithin were selected as the optimal conditions. ENLs (with the particle size, PDI, and zeta potential of 93.0±11.9 nm, 0.397±0.106, and -40.800±1.411mV (n= 3), respectively) were also prepared under optimal conditions. The results demonstrated that the particle size of ENLs after loading decreased in comparison to EO-LNLs with the same amount of lecithin. It may be due to the interactions between the hydrophobic constituents of EO and the acyl chains of lipid bilayers (Rafiee *et al*., 2017). Besides, EO-LNLs had more uniformity and homogeneity than ENLs (0.296 vs. 0.397).

Morphological Evaluation of the Nanoliposomes Produced by TEM

TEM is one of the excellent techniques to obtain useful information about the shape, particle size, and lamellarity of liposomes. Figure 1 illustrates the TEM images of ENLs and EO-LNLs. As shown, the vesicles have a spherical shape and a bilayer structure, indicating that the formed vesicles are liposomes. Furthermore, the size of liposomes is ~ 100 nm, which is almost similar to the DLS results. It indicates that nanoliposomes are Small Unilamellar Vesicles (SUVs).

Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR spectroscopy was used to evaluate the functional groups and confirm the encapsulation of EO in nanoliposomes. Figure 2 indicates the IR spectrum of free EO, ENLs and EO-LNLs. The peak that appeared at 3,335.95 cm^{-1} belongs to the ENLs and it can be originated from OH stretching of the hydrogen bond between water and lecithin. Free EO showed some characteristic peaks at the wavenumbers of 3,323.90 (stretching vibration of the

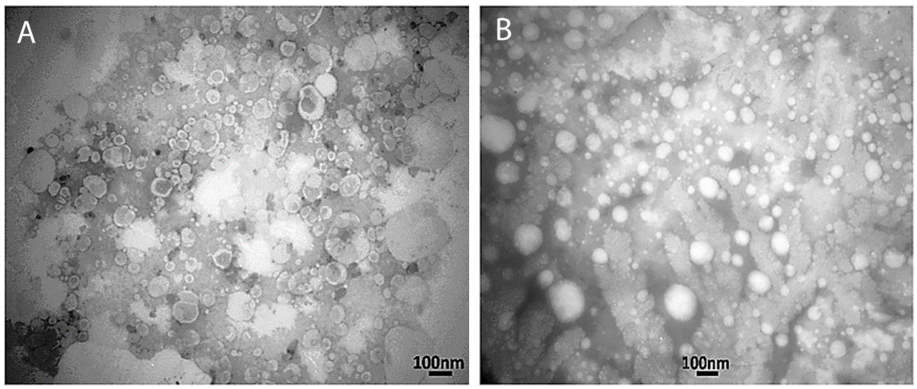

Figure 1. TEM images of Empty Nanoliposomes (ENLs) (A) and EO-Loaded Nanoliposomes (EO-LNLs) (B). EO: Essential Oil.

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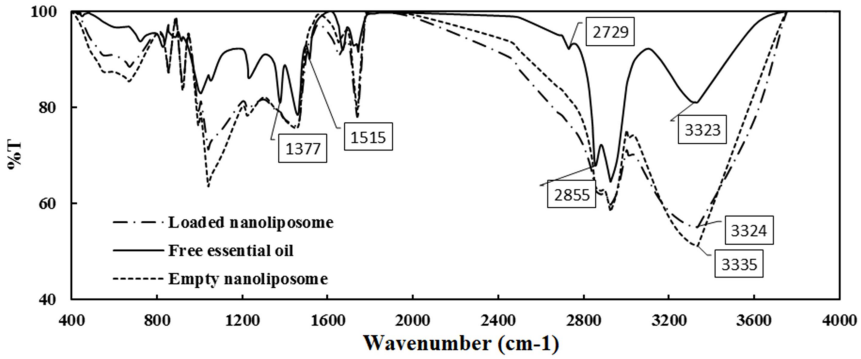

Figure 2. FT-IR spectrum of free EO (A), ENLs (B), and EO-LNLs (C). EO: Free Essential Oil; ENLs: Empty Nanoliposomes; EO-LNLs: Essential Oil-Loaded Nanoliposomes.

hydroxyl groups), 2,959.49 (asymmetric stretching vibration of the CH_3 groups of alkyl chains), 2,855.09, and 2,925.24 (symmetric and asymmetric stretching vibration of the CH_2 groups of alkyl chains, respectively). Other bands at 2,729.42 (CH group of aldehydes), 1,744.30 (stretching vibration of C=O), 1,670.85, 1,515.37, and 1,455.45 (C=C aromatic ring), and 1,235.28, 1,056.66, and 1,007.23 cm^{-1} (C-O alcohols) confirm the presence of the mentioned functional groups of EO (Rafiee *et al*., 2017; Luo *et al*., 2020).

Compared to the free EO spectrum, most of the peaks have moved to higher or lower frequencies with a slight displacement after EO encapsulation in nanoliposomes. Also, some bands of EO at 2,855.09, 2,729.42, 1,515.37, and 1,377.35 cm^{-1} disappeared. Moreover, there was a significant spectral change in the O-H stretching bond of free EO compared with EO-LNLs, which could be due to the formation of hydrogen bonds between the OH groups of EO components and the polar head of phospholipid. These results confirm the successful loading of EO in nanoliposomes.

Differential Scanning Calorimetry (DSC)

The peaks obtained under the nitrogen gas are displayed in Figure 3A. As shown, there is an endothermic peak in the DSC curves of ENLs and EO-LNLs that belongs to Tc [Figure 3 (A2 and A3)]. The Tc of liposomes is related to the temperature at which the lipid bilayers transform from gel state to liquid crystal. As illustrated in Figure 3A, the Tc of EO-LNLs and ENLs is -2.61 and -2.70°C, respectively.

Loading of EO constituents in changeable phospholipid bilayers increases the order of acyl chains and increases Tc. In the present study, Tc of EO-LNLs were slightly increased in comparison with ENLs. It may be due to the interactions between the hydrophobic constituents of EO and unsaturated phospholipid bilayers. Moreover, the increase of Tc can be due to the formation of hydrogen bonds between the EO constituents and the polar head of phospholipid, as well as the increase of van der Waals interactions between the lipid bilayers. Therefore, it may be concluded that after EO encapsulation, the liposome size could decrease and formed a rigid, ordered, and compact structures (Rafiee *et al*., 2017).

Phospholipids of lecithin have a low Tc due to their high amounts of unsaturated fatty acids. Meanwhile, low Tc can be attributed to the presence of phospholipids with a negative charge in the lecithin used in this study. Moreover, there is no peak related to the EO in the DSC curve of EO-LNLs. This observation confirms the encapsulation of EO [Figure 3 (A3)]. Phase

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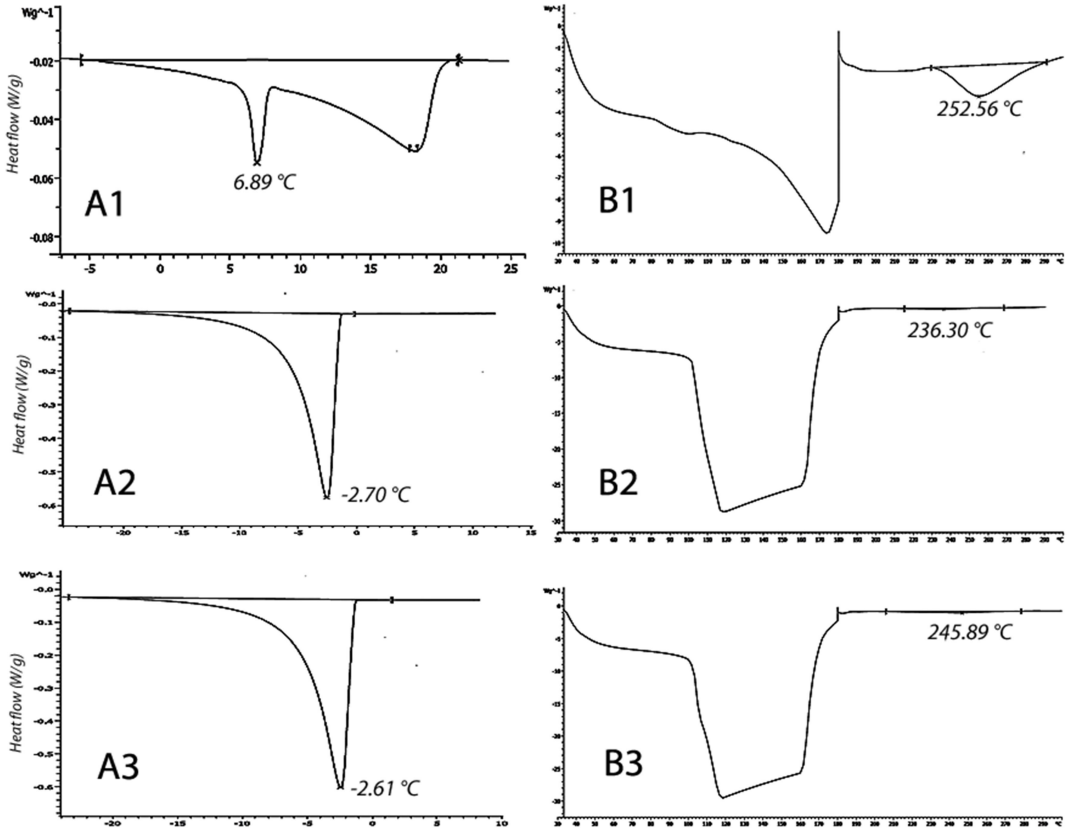

Figure 3. Differential scanning calorimetry; Oxidative stability test; 1-3 belong to free EO, ENLs and EO-LNLs, respectively. For abbreviations see Figure 2 caption.

transition temperature significantly affects the properties of liposomes. The low phase transition temperature is an advantage for liposomes as a carrier system for bioactive constituents. High phase transition temperature can lead to a slower release of EO (Maherani *et al*., 2012).

The peaks obtained under the oxygen gas are displayed in Figure 3B. As shown, the starting Temperature of oxidation (T_{Onset}) for free EO is 252.56°C [Figure 3 (B1)], and there is no EO-related oxidation peak in EO-LNLs [Figure 3 (B3)], which could be due to the encapsulation of EO in the nanoliposomes. A comparison of ENLs and LNLs peaks showed that T_{Onset} for EO-LNLs was higher than for ENLs (245.89 vs. 236.30°C).

The loading of EO in the phospholipid bilayers causes a delay in oxidation process of lecithin and, therefore, shifts the oxidative peak as compared to ENLs. The incorporation of EO in lipid bilayers can prevent free radical access, preserve nanoliposomes, and shift the peak to a higher temperature. In other words, loading of EO in nanoliposomes, improves the oxidative stability of the liposomal membranes during storage. Liolios *et al*. (2009) observed a similar effect for encapsulated carvacrol and thymol. T_{Onset} was higher for EO-LNLs compared to ENLs. Detoni *et al*. (2012) also reported that the thermal-oxidative stability of EO-LNLs was higher than that of ENLs.

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Encapsulation Efficiency

Under the optimized conditions, the encapsulation efficiency (EE) of EO in nanoliposomes was 84.02±2.23% (n= 3). This high EE indicates interaction between the phospholipid bilayers and a large part of the nonpolar components of EO, as well as the interaction between the polar head of phospholipids and most of the polar ingredients of EO.

Lin *et al.* (2019) encapsulated *Eucalyptus citriodora* EO in nanoliposomes. The maximum Encapsulation Efficiency (EE) of this EO was about 22.47%. In another study, the EE of *Sargassum boveanum* algae`s phenolic compounds was reported to be about 45.5±1.2% (Savaghebi *et al.*, 2020). Encapsulation of olive pomace extract was carried out in a liposomal system and EE was up to 58% (Trucillo *et al.*, 2018). Based on the literature review, two papers reported the encapsulation of rose essential oil in mung bean protein isolate-apricot peel pectin (Qiu *et al.*, 2022) and in liposomes by a supercritical process (Wen *et al.*, 2011) that their EE was 89.91 and 89.46%, respectively. It must be noted that present encapsulation method is simple, user-friendly, and inexpensive and can be used on an industrial scale. In addition, lecithin is a safe compound, produces on a large-scale, and is available worldwide.

In vitro Release of EO from Nanoliposomes

The *in vitro* release of EO from nanoliposomes was studied at 4°C and different pH values (Figure 4). As shown, the release occurred rapidly at the first two h. At this time, 31.6 and 42.7% of the total encapsulated EO were released at pH 7.0 and 5.0, respectively. In the early minutes, all essential oil was released from nanoparticles (at pH 3.0).

The initial burst release may be related to the separation of EO compounds from the surface of nanoliposomes. Still, the slow release could be due to the diffusion of EO compounds from the inner layers. Consequently, the nanoliposomes need more time to release core compounds (Nounou *et al.*, 2006). After the burst release, the gradual release continued until 70 hours. At this time, the cumulative releases of EO were 38.5 and 56.8% at pH 7.0 and 5.0, respectively. Moreover, the nanoliposomes were not capable of maintaining EO at pH 3.0, because the release was completed at the initial minutes (data not shown). This

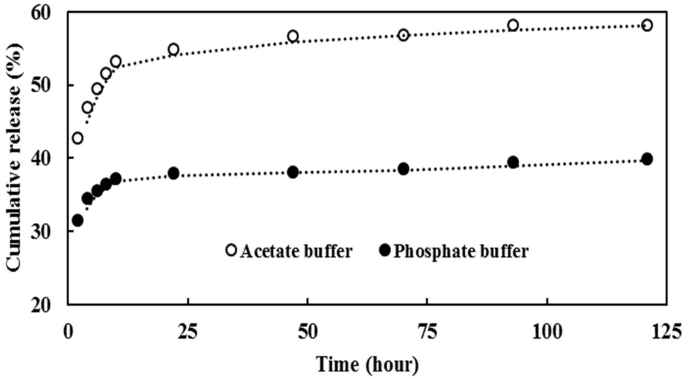

Figure 4. Cumulative release of EO from nanoliposomes in phosphate buffer (pH= 7.0) and acetate buffer (pH= 5.0). EO: Essential Oil.

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extremely fast release can be attributed to low pH, which can control the structure and fluidity of lipid bilayers. Acidic pH reduces the surface charges of liposomes and the repulsion between them and, therefore, increases their size. Hence, by decreasing the pH, the uniformity of phospholipid bilayers decreases, which leads to instability and increased release rates. A similar study showed that the release rate of ellagic acid was faster at pH= 3.0 than pH=5.0. Authors attributed this result to the decrease of surface charge and stability of liposomes (Madrigal-Carballo *et al*., 2010).

Stability of EO-LNLs

The stability of EO-LNLs was evaluated by measuring their particle size, zeta potential, and PDI during seven weeks of storage at 4°C. The results showed that the particle size slightly increased (by 7.11%) to 87.17 nm during storage. However, no sedimentation or significant change in the zeta potential and charge of nanoliposomes was observed. The negative charges of vesicles create sufficient repulsion force, leading to suspension stability. The PDI values increased (by 36.9%), which is due to the adhesion of nanoliposomes during the storage. Therefore, the homogeneity of the liposomal system reduced to 0.4 after seven weeks of storage.

Antioxidant Activity Evaluation

In this study, the antioxidant activities of the free and encapsulated EOs were also investigated. The EC_{50} obtained by the $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and FRAP assays were as follows: 1216.0±0.7, 943.6±0.8, and 324.0±1.0 (for free EO) and 1441.9±0.3, 1302.1±0.2, and 346.7±0.3 ppm (for encapsulated EO). According to the results, free EO had higher activity than encapsulated EO. Moreover, lower concentrations of free and encapsulated EOs were required for ferric reduction compared to others. The *Rosa damascena* Mill. essential oil`s components can scavenge the released radicals and have a high reducing potential. An acidic solution is used in the FRAP assay. As previously mentioned, the EO is released quickly from the nanoliposomes in the acidic pH; this can be a reason for the higher reduction ability of nanoliposomes in the FRAP assay compared to the other two assays.

EO-LNLs had less antioxidant activity, in all of the mentioned assays, compared to the free EO. The EO compounds are encapsulated into the liposomes in different manners depending on the polarity, chemical structure, and concentration. Some EO compounds are absorbed on the membrane surface, while others diffuse into the hydrophobic part of the lipid bilayers or the hydrophilic cores. As a result, the antioxidant compounds have different antioxidant efficiency in various locations of liposomes (Zhong and Shahidi, 2012). Accordingly, the lower antioxidant activity of EO-LNLs can be due to a large proportion of the EO compounds placed in the lipid bilayers.

CONCLUSIONS

In this study, *Rosa damascena* Mill. EO was successfully encapsulated in nanoliposomes. The prepared nanoliposomes, under optimum conditions, had a particle size of 84.5 nm, a zeta potential of -47.950mV, and a PDI value of 0.296. The morphological evaluation showed that the vesicles had a spherical shape and a bilayer structure. The FTIR, DSC, and oxidative stability test results confirmed the successful encapsulation of EO in nanoliposomes and the enhancement of EO`s stability after encapsulation. The EO encapsulation efficiency under optimum conditions was 84.02±2.23%, and the highest release rate occurred at pH= 3.0. During seven weeks of storage at 4°C, a slight increase in the nanoliposomes' particle size and PDI was observed, with no

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significant change in their zeta potential. Concerning the antioxidant activity, free EO showed higher activity than the encapsulated EO. Accordingly, it can be concluded that encapsulation of *Rosa damascena* Mill. EO in nanoliposomes can protect it against undesirable conditions while keeping its properties. Hence, it can be used in different products like cosmetics, foodstuffs, nutraceuticals, and alike as a natural preservative.

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ریزپوشانی اسانس گل محمدی در نانولیپوزوم‌ها

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چکیده

اسانس گل محمدی در نانولیپوزوم‌ها برای غلبه بر پایداری و حلالیت پائین، ریزپوشانی شد. تهیه نانولیپوزوم‌های حاوی اسانس (EO-LNLs) با روش تجزیه آماری سطح پاسخ با طرح مرکب مرکزی نوع مرکز وجه (CCF) بهینه سازی شد. غلظت‌های مختلف اسانس (۵۰۰، ۱۰۰۰ و ۱۵۰۰ ppm) و لسیتین (۰/۵، ۱/۲۵ و ۲%) برای تهیه نانولیپوزوم‌ها به‌کار برده شدند. نانولیپوزوم‌های حاصل شده دارای اندازه ذرات ۸۲-۱۲۴ نانومتر، پتانسیل زتا ۵۵- تا ۳۰- mV و شاخص پراکندگی (PDI) ۰/۲۷۰ - ۰/۳۴۲ بودند. نانولیپوزوم‌های تهیه شده با ۱/۵۶ درصد لسیتین و ۵۰۰ ppm اسانس، بهترین ویژگی‌ها را دارا بودند و در این شرایط کارایی ریزپوشانی ۲/۲۳ ± ۸۴/۰۲ درصد حاصل شد. نتایج به‌دست آمده از روش‌های دستگاهی مختلف (DSC، FT-IR و TEM) ریزپوشانی اسانس را در نانولیپوزوم‌ها تایید کردند. طبق ارزیابی‌های فعالیت ضداکسایشی بر اساس روش‌های DPPH°، $ABTS^{\circ+}$و FRAP، اسانس آزاد دارای توانایی مهار رادیکال بالاتر و EC50 پائین‌تری نسبت به اسانس ریزپوشانی شده بود. بالاترین میزان رهایش برون‌تنی اسانس از نانولیپوزوم‌ها در pH برابر ۳ رخ داد. در طی نگهداری نانولیپوزوم‌ها به‌مدت ۷ هفته در ۴ درجه سانتی‌گراد، اندازه ذرات به‌میزان ۷ درصد افزایش یافت. بر این اساس می‌توان استنباط کرد که ریزپوشانی اسانس گل محمدی در نانولیپوزوم‌ها می‌تواند از آن در برابر شرایط نامطلوب حفاظت کند و ویژگی‌های آن را حفظ نماید. بنابراین می‌توان آن را برای استفاده به‌عنوان نگهدارنده طبیعی در صنایع غذایی، دارویی و آرایشی پیشنهاد کرد.

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