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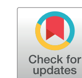
Research article

Entrapment of rosemary extract by liposomes formulated by Mozafari method: physicochemical characterization and optimization



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ABSTRACT

A major obstacle in the utilization of phenolic antioxidant compounds is their sensitivity and as a result stability issue. The current study aimed to encapsulate polyphenolic compounds, extracted from Rosemary, in liposomes prepared by the Mozafari method without the utilization of toxic solvents or detergents. The extract was prepared and converted into a powder by freeze-drying. The process conditions were optimized using response surface analysis, and the optimal parameters were as follows: phosphatidylcholine (PC), 2.5% (25 mg/mL); extract, 0.7% (7 mg/mL); process temperature, 70 °C and process time, 60 min. The entrapment efficiency in optimal sample was 54.59%. Also, optimal glycerosomes formulation were finally physicochemical characterized (permeability, zeta potential, and size distribution). The mean size of empty and containing rosemary extract glycerosome were 265.4 nm and 583.5 nm, respectively, and the Z-potential of optimal glycerosome was -65.1 mV. Total phenolic content was obtained 151.38 mg gallic acid/g extract, in optimal liposomal formulation, which was measured by Folin-Ciocalteu's phenol reagent. Also, the antioxidant activity of rosemary extract by DPPH for the free and optimal liposomal formulation was determined to be 84.57% and 92.5% respectively. It can be concluded that the liposomal rosemary extract formulation prepared in this study, employing a safe, scalable, and green technology, has great promise in food and pharmaceutical applications.

1. Introduction

Oxidation of biomolecules causes serious health issues such as cancer, cardiovascular diseases, cataracts, and diabetes (Berger, 2005). A possible strategy to protect against oxidation is to use antioxidant compounds. Applying natural antioxidants instead of synthetics ones is increasing due to the deleterious effects of synthetic antioxidants, including the possibility of carcinogenesis and liver damage (Li et al., 2021a). The application of herbal extracts as natural antioxidants is vital to protect various food products against oxidation (Komes et al., 2011). Among the most important classes of natural antioxidants are polyphenolic compounds that can be used to prevent oxidation and increase the shelf-life of sensitive food material (Rispail et al., 2005). Rosemary has antioxidant, antibacterial, and anti-cancer properties (Oluwatuyi et al., 2004). Rosemary extract has many polyphenolic compounds such as carnosol, carnosic acid, rosmanol, epi-rosmanol, and iso-rosmanol, which have potent antioxidant properties. These components can prevent of the superoxide production anions (Shah et al., 2014). Rosemary extract had better antioxidant activity than α-tocopherol, due to its content of a caffeic acid ester and rosmaric acid (Tepe, 2008). Polyphenols are sensitive antioxidants. Therefore, various factors such as oxygen level, alkaline pH and even their concentrations can reduce their antioxidant properties (Zou et al., 2014). These compounds can be entrapped in a carrier system to protect them against degradation and oxidation and to increase the stability of these molecules during storage and maintain their antioxidant activity (Dehkharghanian et al., 2009). Among the available entrapment technologies, lipid-based carrier systems possess the most applications in the food and nutraceutical industries. These carrier systems are used to encapsulate and protect materials with different solubility and delivering them to the desired location inside or outside the body.

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Extract entrapped in liposome is enhanced its stability against unfavorable environmental conditions. This phenomenon can be due to control of oxygen level and tea polyphenol concentration which greatly increases stability of polyphenols. Since liposomes have membranes, as a result the reaction between oxygen and polyphenols is limited. Also, due to slow release of entrapped polyphenol in liposome, their degradation is less than free polyphenols. Among the lipid-based entrapment technologies, liposomes have been the most successfully applied systems that have numerous advantages including: made of natural materials, the ability to entrapment bioactive material with different solubility, prevent of ingredient oxidation by free radicals, metal ions and enzymes (Bummer, 2004; Gouin, 2004). Liposomes are colloidal systems, which consist of two phospholipid layers and can incorporate one or more aqueous compartments by self-assemble (Tan et al., 2014). Li et al. (2021b) reported Ascorbyl Palmitate modified liposomes can be applied for encapsulation of different nutraceutics. Chen et al. (2021) prepared liposomes containing soybean protein isolate hydrolysates. The results showed the liposome-containing food systems had the high stability than samples has not been entered in liposomes. One of the limitations of using common methods of liposome production in the food industry is the utilization of volatile solvents and other toxic chemicals including detergents. Mozafari method which is the improved heating method, used in the present study, liposome production is achieved by the agitation of phospholipid compounds in an aqueous medium. Then, these components were heated in the presence glycerol without use any toxic chemical. Glycerol is a food-grade and non-toxic chemical that is used to enhance the stability of liposome. Therefore, it does not need to remove glycerol from the final product (Mozafari, 2005; Silva Malheiros et al., 2010). Rosemary extract is rich in polyphenolic compounds which have potent antioxidant properties and there is no research on the application of glycerosomes for the entrapment of Rosemary extract. The aim of this research was the production of glycerosomes containing rosemary extract by the Mozafari method as well as physicochemical characterization of vesicles.

2. Materials and methods

2.1. Materials and chemicals

Rosemary plant (*Rosmarinus officinalis L.*) was obtained from the farm in Isfahan province (Iran). The phospholipid ingredient of liposomes (99% purity, granular phosphatidylcholine) was procured from Across Company (USA). Ethanol, glycerol, 2-2-diphenyl-1-pycrylhydrazyl (DPPH), and Folin-Ciocalteu's phenol reagent were obtained from Merck Company (Darmstadt, Germany). Particle size and Zeta-Sizer analyzer (Horiba Scientific Instruments, SZ-100 Series, Germany). Ultracentrifuge (Herolab, Hicen, Germany) and spectrophotometer (SpectroFlex, Model UV-6600, Germany) were used in this study.

2.2. Preparation of rosemary extract

In the first stage, air-dried rosemary leaves were grounded into a powder using a domestic electric grinder. The extraction process was then carried out by adding 1000 mL of 70% ethanol in distilled water to 100 g of the grounded rosemary leaves. Ethanol 70%, in water had the best efficiency to extract polyphenolic compounds of rosemary such as for rosmarinic acid, carnosic acid, and carnosol (Oliveira et al., 2016). After 24 h stirring at room temperature (at 500 rpm), the extract was filtered by Whatman No. 1 and rotary evaporator (at 40 °C). After freeze drying of extract at -50 °C, it was kept in air-tight dark bottles in the freezer at -18 °C until usage (Vuong et al., 2011).

2.3. Measurement of phenolic compounds

Total phenolic compounds were measured according to Barros et al. (2007). Absorbance was recorded at 725 nm by a spectrophotometer (SpectroFlex 6600, Germany). Gallic acid was used as the internal standard and the polyphenol amounts are presented as mg/l of gallic acid equivalents (GAE). Base of method is chemical reduction of phosphotungstic acid to blue phosphotungstic under alkaline conditions.

2.4. Free radical scavenging method (DPPH)

The ability of rosemary extract to release a hydrogen atom or an electron before and after the entrapment process was measured. The spectrophotometric evaluation was performed according to a method described by Juhaimi et al. (2012). The absorbance was recorded at 517 nm using a spectrophotometer (SpectroFlex 6600, Germany). The efficiency of scavenging rate of DPPH radical was calculated using Eq. (1):

$$\text{Free radical scavenging rate } (\%) = (\text{Ac}-\text{As})/\text{Ac} \times 100 \qquad (1)$$

where; As and Ac are absorbance of the rosemary and blank, respectively (Adebiyi et al., 2017).

2.5. Liposome preparation

Glycerosomes were prepared using the Mozafari method as green method without using detergents and solvent (Colas et al., 2007; Silva et al., 2010). The basis of method is hydration of phospholipids molecules, as main part of glycerosome structure, in an aqueous medium. Then, these components are heated in the presence of glycerol. Phospholipids are amphiphilic molecules which form aggregated complexes when are placed in an aqueous environment. In the formed structures, hydrophobic sections stay away from the water molecules while hydrophilic head groups are remained in contact with the aqueous phase (Mozafari, 2005). The independent variables are listed in Table 1. The rosemary extract (0.7–2% mg/mL) was first mixed with distilled water. Then phosphatidylcholine (2.5–4.5% equal to 25–45 mg/mL) and glycerol (3% v/v) were added to the rosemary extract solution. The mixture was agitated on a hot plate stirrer at temperatures of 40°–80 °C for different time intervals (15–75 min) at a mixing speed of 1000 rpm. By keeping the mixture at room temperature (1 h), the vesicles anneal and the stability of the liposomes increase. To prevent oxidation of the liposomal phospholipids, all of the stages of glycerosomes preparation were carried out under a nitrogen gas stream. The formulation was stored in a dark bottle at 4 °C under nitrogen until use.

2.6. Entrapment efficiency

To determine the efficiency of the entrapment process, glycerosomes with loaded extract were separated from the free (unentrapped) extract by centrifugation at 36,000 g for half an hour. The supernatant and the sedimented pellet were separated and diluted to a volume of 10 mL using distilled water. In the next stage, the liposomal membrane was fractured using Triton X-100 which was made by mixing 0.02% (w/w) Triton X-100 in distilled water. One milliliter of this solution was added to one milliliter of the glycerosomes, the mixture was shaken for 20 min, using a test-tube shaker, and the entrapped extract was released. Triton X-100 was used for uniformity. The Eq. (2) was used to calculate the entrapment efficiency (Laridi et al., 2003; Vafabakhsh et al., 2013):

Table 1. Independent variables in liposome preparation.

Variable	Variable Levels			References
	+α	0	-α	
Phosphatidylcholine concentration (%)	5.5	3.5	1.5	(Lu et al., 2011; Naghavi et al., 2016)
Extract concentration (%)	2.65	1.35	0.05	(Lu et al., 2011)
Mixing temperature (°C)	80	60	40	(Colas et al., 2007)
Mixing time (min)	75	45	15	(Rasti et al., 2012)

$$\%EE = P/(S + P) \times 100 \tag{2}$$

where P is the content of encapsulated phenolic compounds in glycerosome measured after disruption by Triton X-100 and S is the amount of unentrapped phenolic compounds in the supernatant.

2.7. Particle size characterization

The particle size and polydispersity index were measured by the light scattering method using a Zeta-Sizer analyzer (Horiba Scientific Instruments, SZ-100 Series, Germany). The device can measure particles in the range of 0.3 nm-8 μm (Lu et al., 2011).

2.8. Determination of zeta potential

Z-potential is the main factor to indicate charge density on the surface of the particles, repulsion or attraction forces between the particles, and stability of the produced liposomes (Danaei et al., 2018). In the present research, Z-potential was measured by the Zeta-Sizer analyzer apparatus (Horiba Scientific Instruments, SZ-100 Series, Germany) at 25 °C with an electrode voltage of 3.3 V.

2.9. Statistical analysis

Statistical analysis of the obtained data was performed based on the single factor tests by employing Design expert 10 software (Stat-Ease Inc., Minneapolis, Minnesota, USA). Response surface analysis was adopted to optimize the process conditions including four independent variables: phosphatidylcholine concentration, rosemary extract concentration, mixing temperature, and process time by central composite design (CCD). The experimental design with respect to their values in actual and coded is listed in Table 1. Entrapment efficiency (%) and permeability (%) was measured in duplicate in 30 different experimental runs. In this statistical analysis, after selecting the design, the model equations and their coefficients were determined. Finally, validation experiment was carried out and maximum entrapment efficiency was confirmed using the optimum values for variables predicted by response optimization.

3. Results and discussion

3.1. Total phenolic content

In this study, the total phenolic content of the rosemary extract was determined for the optimal sample which was equal to 151.38 mg gallic acid/g extract. Optimal glycerosome was the formula that had the highest entrapment efficiency. Wang et al. (2018) carried out polyphenol extraction of rosemary (*Rosmarinus officinalis L.*) with 80% ethanol. They stated a total phenolic content of 160.70 ± 2.9 GAE/g dry sample after 60 min of extraction. In other research, the amount of polyphenolic compounds in the rosemary ethanolic extracts is reported 116.7 mg GAE/g dry extract by Turan, (2014).

3.2. Evaluation of the antioxidant activity of rosemary extract

DPPH (1,1-diphenyl-2-picrylhydrazyl) has a spare electron that can move on the whole molecule. The delocalization of the spare electron gives rise to the deep violet color. DPPH is converted to a stable non-free radical when it is mixed with a composition with antioxidant properties. During this process, the violet color changes to yellow color (Almeida et al., 2006). In this research work, the inhibition percentage of rosemary extract in both free and incorporating glycerosome formulation was determined by Expert Design 10 software. The inhibition percentage of rosemary extract for the free and optimal liposomal formulation was determined to be 84.57% and 92.5% respectively. Santos et al. (2012) reported a value of 90.14% for the inhibition percentage of an ethanolic extract of rosemary. Wang et al. (2018) stated an inhibition percentage of 94.79% evaluated by the DPPH method for the rosemary extract. Results of this study showed that the inhibition percentage of rosemary extract entrapped in glycerosomes was higher than that of the free extract. These findings were consistent with the results Gortzi et al. (2006, 2007).

3.3. Entrapment efficiency

Entrapment Efficiency (EE) is an important feature of the entrapment techniques that indicates the liposomal loading capacity. The percentage of EE was ranged from 42.00% to 54.59% for various formulations, which is shown in Table 2. Results were analyzed by Expert Design 10 software.

The regression equation obtained as Eq. (3):

$$EE = +44.71 + 0.17A - 0.49B + 0.20C + 0.087D + 1.10AB - 0.89AC + 1.43A^2 + 2.02B^2 + 1.63C^2 + 0.89D^2 \tag{3}$$

The analysis of the entrapment efficiency variance is shown in Table 3. As seen in Table 3, the effect of AB, AC, C^2, D^2, A^2, and B^2 was significant with consideration of the probability $P < 0.05$. In this research, the impact of phosphatidylcholine amount, temperature, extract concentration, and time on the entrapment efficiency were investigated. The effect of each of the factors is described separately in the following sections.

3.3.1. The effect of phosphatidylcholine concentration on the entrapment efficiency

As we can see in Figure 1(a) the level of entrapment efficiency at the concentration 2.5% (25 mg/mL) phosphatidylcholine was the highest quantity. By increasing the amount of phosphatidylcholine up to 3.5% (35 mg/mL) the amount of EE decreases and then the amount of EE is gradually increased with the increase of phosphatidylcholine concentration. It can be concluded that the phosphatidylcholine concentration has a two-way effect on the entrapment efficiency. Laouini et al. (2013) evaluated the effect of various parameters on the entrapment efficiency of vitamin E. They observed that by increasing the phospholipid concentration from 20 to 50 mg/mL, the average size of vesicles increased from 187 to 237 nm. They found that in higher concentrations of the phospholipid, more phospholipid molecules entered into each vesicle. As a consequence, the size and instability of the glycerosome structure increased. Jaafevr-Maalej et al. (2010) found that by increasing the phospholipid concentration in the preparation process of glycerosomes, the liposomal size increased, and this reduced the entrapment efficiency. Indeed, in higher phosphatidylcholine concentration, more molecules are entered into each vesicle and are increased vesicle's size. In addition, they noted that during the manufacture of glycerosomes, the use of high phospholipid concentrations reduced the solubility of this compound and as a result, there was more possibility of phospholipids aggregation. Because in higher phospholipid concentrations, more consumed energy in the process is used for distribution phospholipids instead of smaller vesicles glycerosome formation. By increasing the size of the glycerosome, the solubility of phospholipids decline and their tendency to aggregation is more likely. Lu, Li and Jiang (2011) prepared liposomes containing green tea polyphenol and assessed their physicochemical properties. They found with increasing phosphatidylcholine concentration, mechanical forces are needed for appropriate distribution and consequently higher energy is requisite for liposome formation.

3.3.2. Effect of rosemary extract concentration on the entrapment efficiency

As indicated in Figure 1(a), the highest amount of EE was observed at the rosemary extract concentration of 0.7%. However, by increasing the extract concentration, the amount of EE decreased. The level of EE decreased to its minimum as rosemary extract concentration reached 1.6% (16 mg/mL) and after this concentration, the amount of EE increased

Table 2. The experimental design and response result.

St. Order	A: Phosphstydil choline (%)	B: Extract (%)	C: Temperature (°C)	D: Time (min)	Response 1: Encapsulation Efficiency (%)	Response 2: Permeability (%)
1	2.5	0.7	50	30	51.17	15.0
2	4.5	0.7	50	30	51.34	4.0
3	2.5	2.0	50	30	45.78	12.5
4	4.5	2.0	50	30	51.62	3.5
5	2.5	0.7	70	30	53.10	13.5
6	4.5	0.7	70	30	46.98	12.8
7	2.5	2.0	70	30	50.79	3.5
8	4.5	2.0	70	30	52.74	3.66
9	2.5	0.7	50	60	50.46	4.2
10	4.5	0.7	50	60	50.71	6.0
11	2.5	2.0	50	60	49.84	2.75
12	4.5	2.0	50	60	51.54	3.05
13	2.5	0.7	70	60	54.59	8.22
14	4.5	0.7	70	60	52.32	9.0
15	2.5	2.0	70	60	50.19	1.56
16	4.5	2.0	70	60	50.35	1.62
17	1.5	1.35	60	45	49.55	9.5
18	5.5	1.35	60	45	50.72	5.47
19	3.5	0.05	60	45	53.42	15.3
20	3.5	2.65	60	45	51.51	6.33
21	3.5	1.35	40	45	51.9	3.33
22	3.5	1.35	80	45	49.96	6.0
23	3.5	1.35	60	15	49.07	4.5
24	3.5	1.35	60	75	46.87	1.0
25	3.5	1.35	60	45	45.82	1.5
26	3.5	1.35	60	45	42.00	3.5
27	3.5	1.35	60	45	45.45	1.5
28	3.5	1.35	60	45	44.28	1.5
29	3.5	1.35	60	45	45.31	2.5
30	3.5	1.35	60	45	45.4	3.5

gradually. An important and effective factor in the entrapment efficiency is the distribution of polyphenols between the core and the membrane phospholipid bilayer (Gü;lseren & Corredig, 2013). The findings demonstrated that increasing the extract concentration decreased the entrapment efficiency. The extract polyphenol components had a negative charge so the higher extract concentration caused to increasing the electrical repulsive forces, particles size, and subsequently developed liposome structural instability. Naghavi et al. (2016) reported that by increasing the amount of green tea extract entrapped in liposomes, the size of the liposomes increased as well, and as a consequence the entrapment efficiency was reduced. In as much as that more polyphenols had a negative charge, they increased the size of lipid vesicles due to their repulsive forces. The findings demonstrated that increasing the extract concentration decreased the entrapment efficiency. The extract polyphenol components had negative charge, therefore the higher extract concentration caused to increasing the electrical repulsive forces, particles size, and subsequently developed liposome structural instability. Lu et al. (2011) explained in liposomes containing green tea polyphenol, when the ratio of tea polyphenol to lecithin was changed from 0.333: 1 to 0.111: 1, the entrapment efficiency increased too. This increased ratio caused the useable capacity of lipid vesicles to entrap tea polyphenols decreased. Therefore, with decreasing volume of liposome core, the number of polyphenols were located interior bilayer and accordingly the size of liposome increased. Manafi Dizajyekan et al. (2018) achieved 74–78% entrapment efficiency for the olive leaf extract in liposomes prepared by the ethanol injection method. By increasing the ratio of extracts to lipid vesicles, the entrapment efficiency decreased. Indeed, with increasing extract concentration to a special amount, the negative charge of the system decreased. Because polyphenols were located in liposome surface in addition to core and repulsive forces declined. By increasing extract amount, system negative charge again increased that its reason was relative ionization of polyphenols and then EE again increased.

3.3.3. *Effect of temperature on the entrapment efficiency*

As can be seen in Figure 1(b), increasing the process temperature had a positive effect on the entrapment efficiency. As a result, the EE value was maximum at the temperature of 70 °C (maximum temperature tested). Mixing temperature plays an important role in the glycerosome preparation process, choosing the right temperature is one of the extract conditions for glycerosome production. Phospholipids in the glycerosome structure had a phase transition temperature (TC). The phase transition temperature of soy lecithin was 50–60 °C. Soy lecithin dispersion generated a gel-like phase at temperatures less than 50 °C, while at the temperatures higher than the transition temperature, lecithin formed a crystal-liquid phase. In the liquid phase, each molecule could move more freely and formed lipid layers to generation glycerosome. This stage in the formation of glycerosomes was critical (Mozafari, 2005). Putri et al. (2017) reported that increasing the mixing temperature reduced the size of the liposomes and subsequently increased the entrapment efficiency. Corrêa-Filho et al. (2019) investigated the effect of temperature and concentration of constituent materials of microparticles consisted of beta-carotene on the entrapment efficiency. They found out that increasing temperature had a positive effect on entrapment efficiency.

3.3.4. *Effect of process time on the entrapment efficiency*

The effect of process time on the efficiency of entrapment of rosemary extract by glycerosomes can be seen in Figure 1 (c), Increasing the mixing

Table 3. Analysis of variance for encapsulation efficiency and permeability.

Source	D f	Mean Square	F-Value	P- value
Encapsulation Efficiency[1]				
Model	10	23.21	9.31	<0.0001* significant
A-Phosphatidylcholine	1	0.67	0.27	0.6093
B-Extract	1	5.65	2.26	0.1489
C-Temperature	1	0.93	0.37	0.5490
D-Time	1	0.18	0.072	0.7909
AB	1	19.40	7.78	0.0117*
AC	1	12.67	5.08	0.0362*
A^2	1	56.45	22.63	0.0001**
B^2	1	111.60	44.75	<0.0001**
C^2	1	73.17	29.34	<0.0001**
D^2	1	21.89	8.78	0.0080**
Residual	19	2.49		
Lack of Fit	14	2.66	1.31	0.4086 not significant
Pure Error	5	2.03		
Core Total	29			
Permeability[2]				
Model	10	49.06	19.29	>0.0001* significant
A-Phosphatidyl choline	1	27.43	10.79	0.0039
B-Extract	1	142.69	56.10	<0.0001**
C-Temperature	1	2.80	1.10	0.3071
D-Time	1	63.57	24.99	<0.0001**
AC	1	20.70	8.14	0.0102*
AD	1	34.46	13.55	0.0016 **
BC	1	41.54	16.33	0.0007**
A^2	1	46.66	18.34	0.0004 **
B^2	1	126.25	49.63	<0.0001*8
C^2	1	9.61	3.78	0.0609
Residual	19	2.54		
Lack of Fit	14	3.11	3.21	0.1019 not significant
Pure Error	5	0.97		
Core Total	29			

[1] R-Adjusted = 0.74%, R-Square = 0.83%, R- Prediction = 0.53%. (**) highly significant, $p < 0.01$; (*) significant, $p < 0.05$.

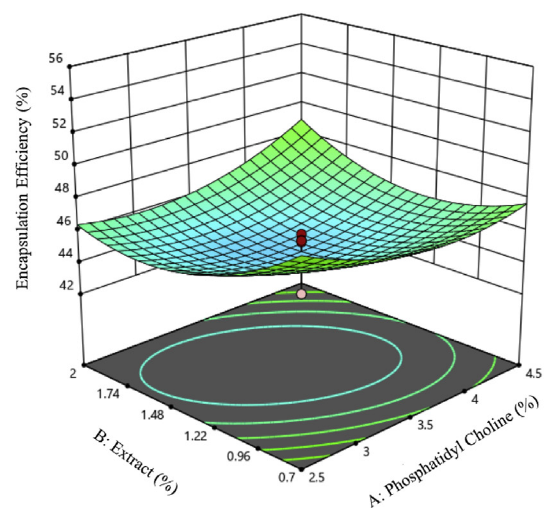
[2] R-Adjusted = 0.86%, R-Square = 0.91%, R- Prediction = 0.77%. (**) highly significant, $p < 0.01$; (*) significant, $p < 0.05$.

time caused an increase in the entrapment efficiency. It seems that by increasing the processing time there is more time for the glycerol molecules to be incorporated in the structure of the glycerosomes and this will optimize the formation of phospholipid bilayers around the extract molecules and, as a result, improved EE. Selecting a suitable time provided it possible to form the glycerosome structure and glycerol molecules have enough time to enter the structure and then increased stability of glycerosome 's structure.

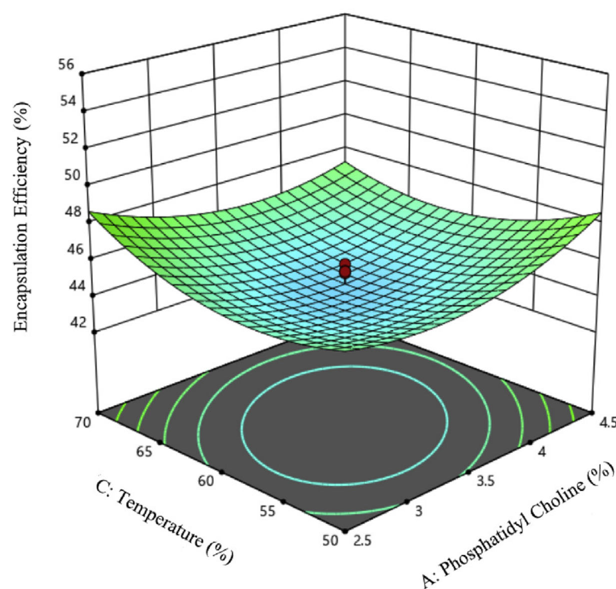
3.4. Permeability

In the entrapment process, liposomes protect the entrapped compounds from light, oxygen, moisture, and extreme pH and also provide controlled release of the entrapped material. One main parameter that affects the mentioned protection and controlled release characteristics of liposomes is their permeability, which is also a determinant of their stability. The percentage of permeability is shown in Table 3. The results obtained in the present study were analyzed through Design Expert 10 software and the regression equation obtained as Eq. (4):

$$\text{Permeability} = +2.48 - 1.07\,A - 2.44B + 0.34\,C - 1.63\,D + 1.14\,AC + 1.47\,AD - 1.61\,BC + 1.29\,A^2 + 2.12\,B^2 + 0.59\,C^2 \quad (4)$$



(a)



(b)

Figure 1. Response Level Charts on the Encapsulation Efficiency of Liposomes; (a) Effects of Phosphatidylcholine and green tea extract Concentrations; (b) Effects of Phosphatidylcholine Concentration and Process Temperature.

3.4.1. *Effect of phosphatidylcholine concentration on the permeability of glycerosomes*

As indicated in Figure 2(a), by increasing the phosphatidylcholine concentration, the permeability was reduced initially and then increased. This was due to the two-side effect of phosphatidylcholine on permeability. The results showed, with increasing concentrations of phosphatidylcholine, the permeability was initially reduced and then increased. This can be explained by the fact that increasing the amount of phosphatidylcholine to a specific concentration initially led to faster formation of the glycerosome wall and the entrapment of the polyphenols. While phosphatidylcholine was partially decomposed through exposure to oxygen and moisture for a long time that the most important product of this destruction was lysophosphatidylcholine. This component was obtained as a result of the hydrolysis of the steric bond on carbon,

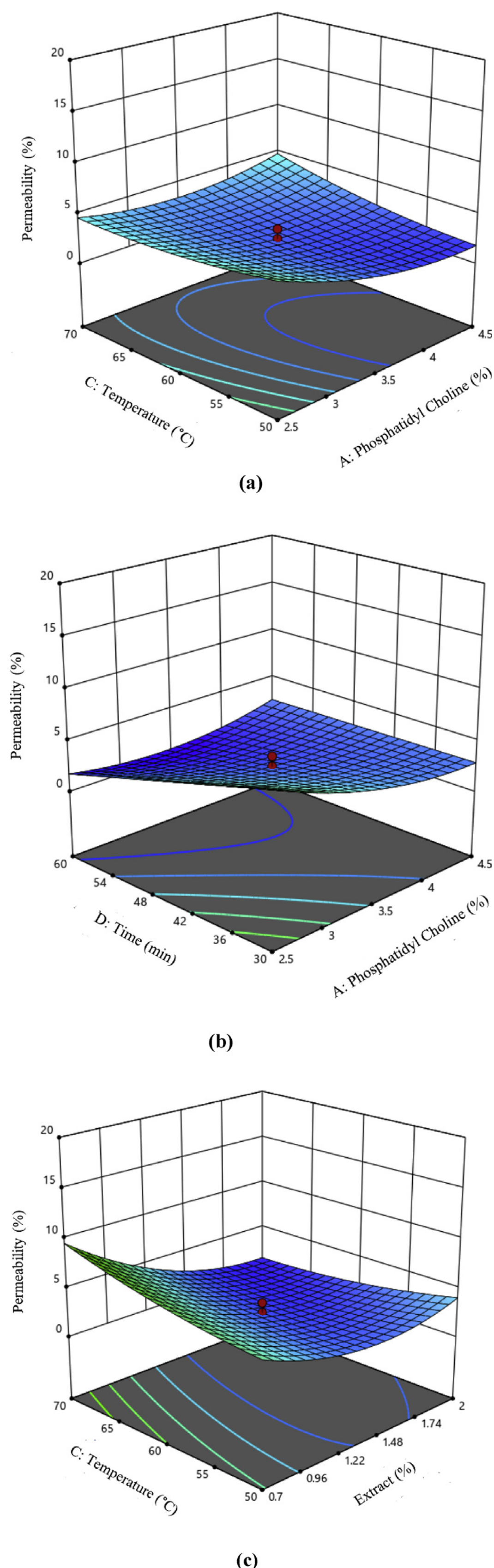


Figure 2. Response level charts on the permeability of liposomes; (a) effects of phosphatidylcholine concentration and temperature; (b) effects of phosphatidylcholine concentration and process time; (c) effects of extract concentration and process temperature.

position 1 and 2 of glycerol generated phospholipid molecules with only one non-polar chain (Machado et al., 2014). Increasing the presence of lysophosphatidylcholine increased the permeability capacity of the liposomal membranes and decreased the capacity of the entrapped substances over time (Lutz et al., 1994). The main reason for increasing glycerosome membrane permeability was the creation of irregularity and disturbance in the membrane structure (Putri et al., 2017). Yeagle et al. (1975) demonstrated that on the surface of lecithin layers, there are areas where intra-molecular electrostatic bonds between positively charged N-methyl groups and negatively charged phosphate groups of adjacent phospholipid molecules are formed. This chemical interaction influences the permeability of lipid vesicles. Studies showed that the polar head of the phospholipid molecules plays an important role in maintaining the structure and function of the glycerosomes.

3.4.2. Temperature effect on the permeability of glycerosomes

As shown in Figure 2 (b), with increasing temperature, the permeability was reduced and then slightly increased. This was due to the phase transition temperature of phospholipids and its effect on the structure of the glycerosomes. One of the most important factors in phospholipids was the phase transition temperature. By increasing of temperature to phase transition point phospholipids will shift from a crystalline structure to a liquid and chaotic form. The results distinguished high temperature of the process increased the permeability. This was due to the effect of temperature on the structure of the glycerosome (Liang et al., 2007).

3.4.3. Effect of rosemary extract concentration on the permeability of glycerosomes

The permeability level decreased by increasing extract concentration to 1.6% (16 mg/mL) and then increased. This was due to the bonding of polyphenols with phospholipid bilayer, it is apparent from in Figure 2 (c). This phenomenon was happened by two reasons: (1) the placement of non-polar compounds in the membrane bilayer which can also cause the increase size and rupture in glycersome membrane, as a result the permeability was enhanced, and (2) the formation of hydrogen bonds between the more hydrophilic polyphenols and the polar heads of the lipids at the membrane interface, which increase the stability of the glycerosome structure and permeability is decreased (Oteiza et al., 2005). These links influenced both the permeability and entrapment efficiency (Mignet et al., 2013).

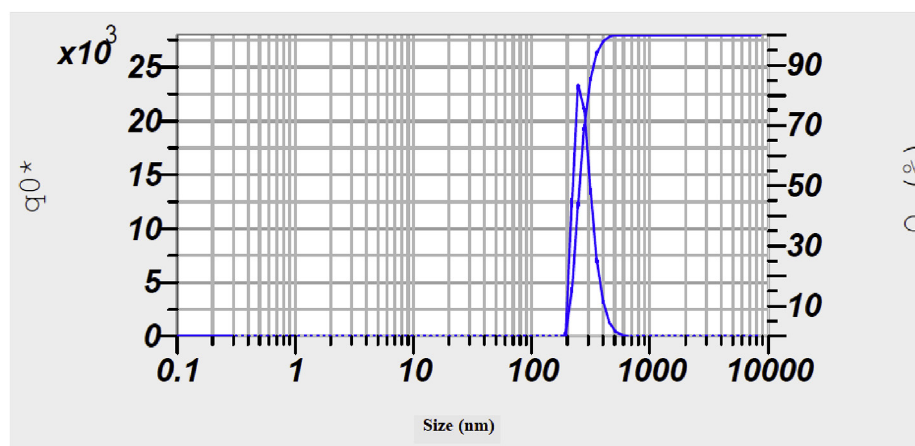
3.4.4. Effect of process duration on the permeability of glycerosomes

In Figure 2 (b), the effect of process time (duration) on liposomal permeability is evident, which reflected the fact that by increasing mixing time during the preparation of glycerosomes, there was sufficient chance to import glycerol molecules to the glycerosome structure, accordingly improved stability of glycerosome and prevented from polyphenol leakage of the inner glycerosome. The results demonstrated that increasing the processing time reduced the permeability during the storage. Indeed, increasing the processing time along with the mixing with 1000 rpm provided enough time to converted phospholipids to the stable glycerosome structure.

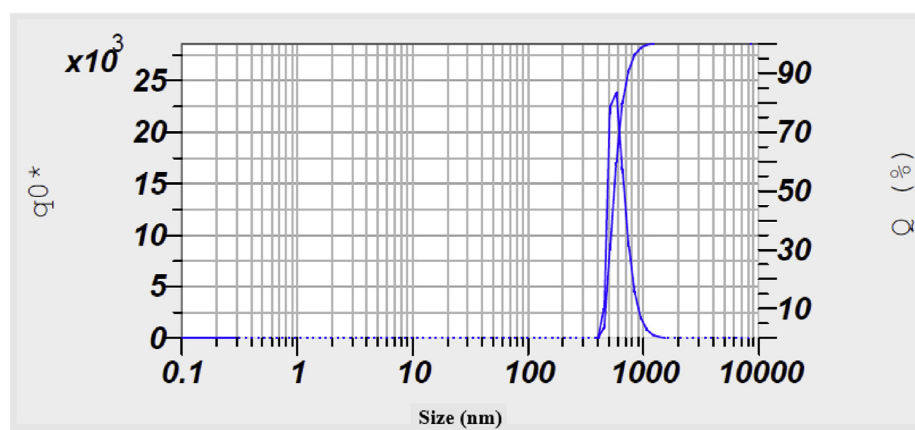
3.5. Distribution of glycerosome particles sizes

Particle size distribution is an important factor in the formulation of glycerosomes. This is because it affects the stability of glycerosomes and the release of its entrapped active compounds (Naghavi et al., 2016).

As can be seen in Figure 3, the average size of the empty glycerosomes was 265.4 nm (Figure 3a) while the average size of glycerosomes containing rosemary extract was 583.5 nm (Figure 3b). This result is in agreement with the study conducted by Gibis et al. (2012) and Rashidinejad et al. (2014). Also, González-Ortega et al., (2020) reported liposomes containing olive leaf extract had the greater size than empty liposomes. In this study, no additional procedure was employed to reduce the particle size of glycerosomes (e.g. sonication, homogenization, or extrusion) and



(a)



(b)

Figure 3. Particle size in (a) empty liposomes; (b) liposomes encapsulating rosemary extract.

the required energy for reducing the particle size of the vesicles was provided by agitating the system using a magnet stirrer with 1000 rpm force and at the same time thermal energy. The polydispersity index (PDI) of glycerosomes containing rosemary extract was 0.449. PDI more than 0.7 shows a broad particle size distribution (Danaei et al., 2018). DPI equal and less than 0.2 are acceptable for polymer-based particle materials. In applications of glycerosomes for drug delivery, PDI of 0.3 and below is acceptable (Grazia Calvagno et al., 2007). Consequently, the PDI value obtained in this study needs to be lowered in future optimizations of the formulation. Laridi et al. (2003) reported size of nisin Z incorporating-glycerosomes was in the range of 140–2400 nm with a mean diameter of 740 nm Putri et al. (2017) stated that the Mozafari method can produce glycerosomes with a mean particle size of 600 nm without using particle size reduction procedures. Corrêa-Filho et al. (2019) entrapped beta-carotene and reported the size of their manufactured liposomal particles to be between 1.82 to 1.90 μm. The obtained results proved, there is no direct relationship between the entrapment efficiency and glycerosome permeability during the storage. These findings conformed to the final results obtained by Gulsern and Corredig (2013).

3.6. Z- potential

Zeta potential is one of the main parameters to determine the stability of liposomal formulations (Heurtault et al., 2003). High values of Z-potential (i.e. $> +30$ mV and < -30 mV) prevent or minimize aggregation and fusion of liposomal particles (Lu et al., 2011; Danaei et al., 2018). Z-potential of glycerosomes containing extract was -65.1 mV that is illustrative of good stability. This is similar to report of Rashidinejad et al. (2014), whose data of Z potential of glycerosomes encapsulating catechin and epigallocatechin gallate was -45.1 mV. Hydrogen interaction or hydrophobic bonding may occur in phospholipid molecules (Gibis et al., 2012). In this research, Z-potential for rosemary extract incorporating glycerosome achieved -65.1 mV an indicator of optimal electrostatic stability between liposomal particles. This phenomenon cause to reducing the natural tendency of particles to aggregation (Heurtault et al., 2003). Lu, Li and Jiang (2011) reported Z-potential of liposomes containing green tea polyphenol was -67.2 mV. Higher Z-potential demonstrates higher stability due to more repulsive forces which prevent particles aggregation. The different values of Z-potential in various studies dependents on different phospholipids properties useable in glycerosome structure. Also, circumference temperature and ionic power effects on Z-potential amount. When circumference ionic power is low, phosphatidyl groups of phospholipids locate in the outer section of the lipid polar head and cause to particles negative charge. While, with increasing ionic power, in constant temperature, choline groups close to exterior part lipidic bilayer and phosphatidyl groups hide in molecule back.

4. Conclusion

The effect of phosphatidylcholine, rosemary extract, temperature, and time was studied on the entrapment efficiency and stability of glycerosomes formula. All the independent variables are optimized by

Expert Design 10. Optimal values were obtained for independent variables including phosphatidylcholine equal to 2.5% (25 mg/mL) percent, rosemary extract concentration equal to 0.7 mg/mL, at a temperature of 70 °C and 60 min. The innovation aspect of this study was the use of a solvent-free preparation method for glycerosome production. Also, the Mozafari method will be able to produce glycerosomes containing rosemary extract with nanometer dimension without the use of reduced methods such as ultrasonic or homogenization. Because these methods with high shear or pressure are caused instability and wall destruction of the liposome. As a result, entrapment efficiency is reduced. In addition, the extract rosemary possessing a strong antioxidant which can be able to use as a synthetic antioxidant alternative in food oils. The results showed that liposomal entrapped rosemary extract possesses more antioxidant activity in compare to free extract. This phenomenon caused the better stability of extract polyphenolic components during storage.

Declarations

Author contribution statement

Shima Jahanfar: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mehrdad Gahavami, Mahshid Jahadi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kianoush Khosravi-Darani: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

M. R. Mozafari: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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