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Nanoliposome of black fig Anthocyanins and its application in kombucha beverage

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ABSTRACT

Anthocyanin is one of the bioactive compounds, which is the main pigment of many fruits and vegetables. Since anthocyanins have low thermal stability during food processing, the use of these compounds as natural pigments in foods is associated with challenges. Therefore, microencapsulation of anthocyanin compounds with liposomes is important. Nanoencapsulation of bioactive compounds with liposomes is an effective and efficient way to increase the stability of polyphenolic compounds. Liposomes are polar lipid vesicles that form bilayer structures in polar solvents such as water. In this research, nanoliposomes in ratios of 9-1, 8-2, 7-3 and 6-4 lecithin-cholesterol were prepared using the solvent injection method. Then, the size and zeta potential tests were conducted to determine the characteristics of the produced particles. The average particle size (average hydrodynamic diameter) and particle size distribution for different lecithin-cholesterol ratios were in the range of 132-740 nm and 0.47-0.41, respectively. Zeta potential values were also obtained in the range of –26 to –42 mv. After determining the efficiency of Nanoencapsulation, FTIR test was performed to investigate possible reactions between anthocyanins and nanoliposome wall materials. The morphology of anthocyanin-loaded lecithin-cholesterol nanoliposomes with a ratio of 9-1 was shown by scanning electron microscopy (SEM). The stability of the liposomal sample with a ratio of 9-1 lecithin-cholesterol was evaluated by calculating the amount of release of encapsulated anthocyanin during 60 days of storage at ambient temperature. Samples with 9-1 lecithin-cholesterol ratio were used in Kombucha beverage formulation. Sensory properties of prepared beverages were evaluated which the results showed among the samples, smell, mouthfeel and overall acceptance parameters had no significant difference ($P>0.05$) .The results obtained in this research showed that nanoliposomes are an efficient system for encapsulating of anthocyanins

1. Introduction

Color compounds are crucial additives in the food industry as they significantly influence consumers' acceptance of food products. These compounds not only indicate the quality, freshness, and safety of food but also contribute to its sensory and visual properties. The addition of color to food enhances its appeal and makes it more appetizing [1]. The food industry utilizes both natural and synthetic colors. However, there are growing concerns regarding the health and safety implications of synthetic pigments like quinoline yellow, carmosin, and tartrazine. Additionally, there are limitations on the permissible limit of consumption for these synthetic colors. As a result, consumers are increasingly inclined to opt for natural colors such as carotenoids, chlorophyll, anthocyanins, and betalain in their food choices [2]. Consequently, the utilization of natural pigments derived from plants holds significant importance in the food industry. [3]. Anthocyanins are one of the natural pigments that are known for their physical, chemical and biological properties and are used in various food, pharmaceutical and cosmetic industries as natural colors or for their biological functions. Furthermore, anthocyanins can serve as a natural preservative, flavor enhancer, and safeguard against environmental stress when storing and transporting food products [4]. Anthocyanins are an excellent alternative to synthetic pigments due to their attractive colors and high solubility in water [5, 6]. Anthocyanins, classified as phenolic compounds, are molecules that have a direct association with red, blue, and purple colors [7, 8]. The main sources of anthocyanins are fruits, berries and some vegetables as well as flowers. Black figs contain large amounts of phenolic compounds, flavonoids and anthocyanins, which are the most important natural antioxidants [9]. Anthocyanins in black figs have high antioxidant activity [10]. Due to their high antioxidant properties, these compounds play an important role in preventing neurological, cardiovascular, cancer diseases and inflammation [11, 12, 13, 14]. Despite their important advantages, anthocyanins have no long stability due to their sensitivity to oxidation, epimerization, and polymerization [7,15]. Unsaturated bonds in the molecular structure of anthocyanins make them sensitive to light, heat, pH changes, enzyme activities, metal ions and oxygen [7,15]. During food processing and storage, the stability of anthocyanin molecules may be easily affected by these factors [16]. Consequently, to enhance the stability of these compounds, it is imperative to encase them within various types of wall materials through encapsulation [17, 8]. The microencapsulation process is one of the most useful technologies by which sensitive materials are packaged within a wall [15]. In this method, the inner material is protected against adverse environmental conditions such as the effects of light, moisture, and oxygen, and the wall material creates a barrier between the inner material and the external environment. Due to anthocyanins being very sensitive and unstable, this method preserves them by improving the stability and bioavailability of anthocyanin compounds [18]. The microencapsulation process is performed in various ways, including spray drying, freeze drying, fluid bed coating, extrusion, etc. [19]. Among the various microencapsulation methods, encapsulation inside nanoliposomes is one of the suitable methods for microencapsulation of bioactive substances, including anthocyanin compounds, which are used today [20].The main perspective of nanotechnology applications for food is to improve sensory properties, safety, and enhancement. The objective can be attained through the encapsulation of food constituents or additives, regulation and discharge of core substances, and enhancement of the

bioaccessibility of food constituents. Several encapsulation techniques employed in the food industry rely on biopolymer matrices composed of carbohydrates, proteins, and lipids. Various compounds like antioxidants, enzymes, vitamins, minerals, antimicrobial agents, and fatty acids (omega-3) can be enclosed within nanoliposomes [21]. Nanoliposomes, which are spherical structures made up of polar lipids, have the ability to organize and accumulate into bilayer membranes upon contact with water. These nanoliposomes possess the capability to encapsulate hydrophilic compounds within their core and hydrophobic compounds within the lipid layer. Their compatibility, biodegradability, and uniform distribution of bioactive substances, such as anthocyanins, make them highly versatile and advantageous compared to their free form. As a result, nanoliposomes find extensive applications in various fields [22, 23]. In the encapsulation process, the wall material is a key and important element and should have characteristics such as being edible, biologically degradable, and the ability to create a barrier between the internal phase and its surrounding environment [24]. Hence, opting for phospholipids to constitute the wall of nanoliposomes is a favorable decision with significant implications for nutrition and well-being. Phospholipids, being amphiphilic molecules, play a pivotal role as the primary constituents of the nanoliposome wall. They possess a hydrophilic polar head and a hydrophobic tail, enabling them to transport both hydrophilic substances within the inner core and hydrophobic compounds between the lipid layers. Consequently, they facilitate the gradual release of these substances into food [25]. The encapsulation of anthocyanin compounds within nanoliposomes has the potential to enhance the efficacy of functional foods through improved absorption and stability within the gastrointestinal tract. [26].

The primary objective of this study is to entrap black fig anthocyanin compounds within nanoliposomes to shield them from unfavorable environmental factors and safeguard their stability. Additionally, a secondary goal of this research was to incorporate nanoliposomes generated in kombucha beverages to enhance both their visual appeal and nutritional value.

2. Materials and methods

2.1. Materials

Lecithin with the brand name L-alpha-lecithin with a purity of 99% from Merck, Germany, cholesterol and tween 80% from Merck (Darmstadt, Germany), ethanol 96% from Teb Hamon, and Kombucha beverage and Fig (Ficus carica) were purchased from the market.

2.2. Extraction of black fig extract using ultrasound

First, fresh black figs were purchased from the local market. After cleaning, the figs were peeled and cut into flat sheets and then dried in an oven at 60°C for 24 hours. The dried samples were carefully crushed and powdered by the mill and passed through a sieve for uniformity. The obtained powder was stored in polyethylene bags and at freezing temperature (-18°C) to prevent moisture penetration [27]. 10 grams of the black fig powder was mixed with the solvent ethanol-water (ratio 7:3) in a ratio of 1:10 and sonicated in an ultrasonic bath for 20 minutes at a temperature of 40°C with ultrasonic waves at a frequency of 37 kHz. The supernatant solution was separated by filter paper and the extract containing the solvent was spread on the surface of the glass plates and placed in the 40 °C oven. After evaporation of the solvent, the extracts were placed in a desiccator until a constant weight was reached. The extracts obtained were stored at -18°C until the experiments were carried out [28].

2.3. Determination of extraction yield

The extraction yield was obtained by dividing the weight of dried extract to the raw material [47].

Extraction yield (%) = (weight of dried extract /total weight of figs) * 100

2.4. Determination of anthocyanin concentration

The amount of anthocyanins was measured using the pH change method. In this method, the absorbance of the prepared samples (5, 10, 20, and 25 ppm dilutions) with a buffer of pH=1 and pH=4.5 was measured using a spectrophotometer at a wavelength of 510 nm as cyanidin-3-glycoside pigment [29]. According to Figure 1, the calibration curve of anthocyanin was used to determine the anthocyanin concentration.

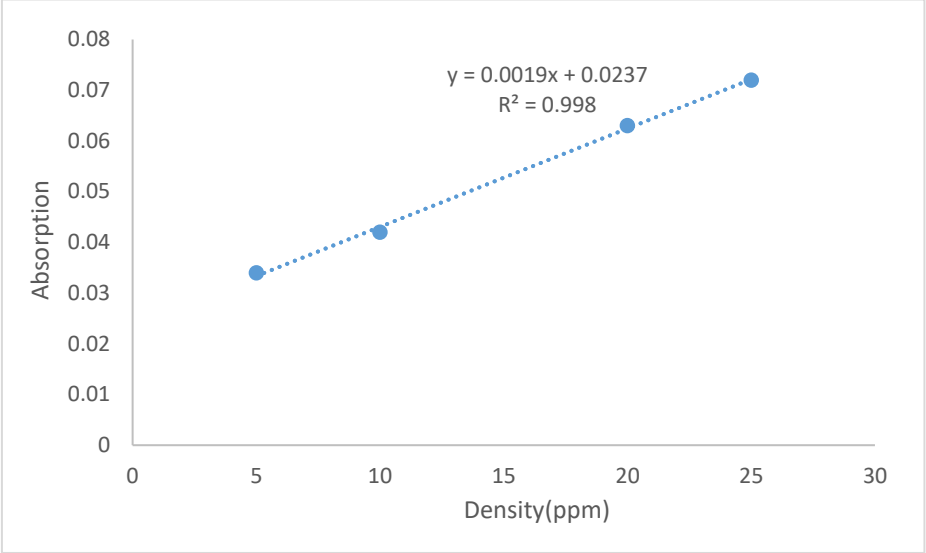

Figure 1: Calibration curve of anthocyanin

2.5. Preparation of nanoliposomes

The nanoliposomes were produced using the ethanol injection method. For this purpose, lecithin at a concentration of 15 mM/ml and anthocyanin extract at different concentrations were dissolved in ethanol. Then, about 10 ml of the ethanol solution was slowly injected into 70 ml of distilled water with a homogenization process at a speed of 2000 rpm and thus the liposomal suspension prepared was kept in a refrigerator at 4 °C for further analysis [30]. To prepare the control sample, the mentioned steps were carried out exactly without the addition of anthocyanin extract.

2.6. Determination of particle size and zeta potential

The particle size and zeta potential of the samples were measured using a DLS, Worcestershire Instruments (ZEN3500, Malvern, UK) in three replicates at a temperature of 25°C [31].

2.7. Determination of encapsulation efficiency

To measure the effect of encapsulation in the prepared nanoliposomes, the method of Homayoonfal et al. (2021) was used. Two samples of anthocyanin-containing nanoliposomes were selected, one sample to measure the concentration of unencapsulated anthocyanin and the other sample to measure the total concentration of anthocyanin in anthocyanin-loaded nanoliposomes. The prepared liposomal samples were centrifuged at 15,000 rpm and 4 °C for 45 minutes, and two separate phases were formed after centrifugation. The non-encapsulated anthocyanin collected in the upper phase and the anthocyanin-containing nanoliposomes collected in the lower phase. To remove the lecithin, which could not be separated in the centrifuge, the upper phase was separated and mixed with chloroform in a 1:1 ratio and centrifuged in a centrifuge at 10,000 rpm and a temperature of 4 °C for 10 minutes. After centrifugation, the upper aqueous phase contained only water and anthocyanins. It was diluted once with potassium chloride buffer and once with

sodium acetate buffer, and finally, the anthocyanin concentration was measured according to the following relationship and considered as non-encapsulated anthocyanins. To calculate the total concentration of anthocyanins, another sample was analyzed and nanoliposomes containing anthocyanins were analyzed by mixing with chloroform at a ratio of 1:1 and other steps were carried out similar to the previous sample, and finally the total concentration of anthocyanins in the sample was calculated [20].

$$\textit{Anthocyanin concentration (mg/liter)} = \frac{(A510-A700)pH1-(A510-A700)pH4.5)\times MW \times DF\times 1000}{\varepsilon \times L}$$

In this equation, A is the absorbance and its index is the wavelength, MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF is the dilution factor, ε is the molar absorbance of cyanidin-3-glucoside (26.900) and L is the cell length (1 cm). The measurements were carried out with a spectrophotometer.

$$\textit{Encapsulationefficiency} = \frac{\textit{Total Anthocyanin concentration} - \textit{non-encapsulated anthocyanin concentration}}{\textit{Total Anthocyanin concentration}}$$

2.8. Infrared spectroscopic analysis (FTIR)

FTIR Spectroscopy RXI model, Perkin Elmer, U.K by License U.S.A was used for infrared spectroscopy analysis. The sample preparation method for measuring with FTIR device is that the components of nanoliposome and the lyophilized powder of nanoliposome are mixed with potassium bromide at a ratio of 1 to 100, and this mixture is analyzed in Fourier transform infrared spectroscopy in the wavelength range of 400 -4000, cm^{-1} was used for sample 1-9 [32].

2.9. Morphological analysis (SEM)

To evaluate the change of surfaces and to examine the morphology of sample 1-9, the field emission scanning electronic microscope (FE-SEM) model LEO1430VP with 50000 magnification was used.

2.10. Stability of anthocyanin-nanoliposome sample

The stability of the samples was determined at room temperature for 60 days. The encapsulation efficiency was evaluated every ten days using a spectrophotometer at a wavelength of 510 nm [20].

2.11. Preparation of beverage

The treatments used in this study included kombucha beverages containing non-encapsulated black fig anthocyanin extract at two levels of 4 and 6% (w/v), kombucha beverages containing nanoliposome of black fig anthocyanin extract at two levels of 4 and 6% (w/v), and the control sample (Table 1).

Among the treatments, the optimal sample, 1-9 (the sample that has 0.09 grams of lecithin and 0.01 grams of cholesterol) was selected and was freely added once to the Kombucha beverage at two levels of 4 and 6% (w/v). Once again, it was added to the beverage in a nano form at two levels of 4 and 6% (w/v) and was subjected to measurement of color, Brix, pH, acidity, hygroscopicity, solubility, and sensory evaluation.

Table 1 Formulations of Kombucha beverage *

Microencapsulated anthocyanin solution	Free anthocyanin solution	Kombucha beverage	Sample
-	-	100	Control
4	-	96	N4
-	4	96	F4
6	-	94	N6
-	6	94	F6

• The units are in milliliters
N4% : nanoliposomed sample at 4% level
N6%: nanoliposome sample at 6% level
F4%: free sample at 4% level
F6%: free sample at 6% level

2.12. Measurement of dissolved solids (brix) of the beverage

The amount of dissolved solids in the beverage was measured by a refractometer (model RA-500 KEM) at ambient temperature in three repetitions (according to the Iranian National Standard No. 2685).

2.13. Determination of the pH of beverages

pH of the samples was measured using a pH meter (Mettler Toledo, Canada) at room temperature for three repetitions (according to the Iranian National Standard No. 2685).

2.14. Sensory evaluation

10 panelists were used for the sensory evaluation of Kombucha beverage samples enriched with nano and free anthocyanin extract. The samples were provided to the panelists and they were asked to taste and evaluate the kombucha samples containing anthocyanin nanoliposomes and express their opinion about its aroma, taste, color and overall acceptability and give a score from 1 to 5 points (score 1 : very bad quality, 2: poor, 3: average, 4: good, 5: excellent) [33]. It should be noted that the ethanol used in the preparation of nanoliposomes was evaporated under vacuum before sensory evaluation.

2.15. Statistical Analysis

In this research, Statistical analysis on a completely randomized design was used. Brix, pH, total acidity, solubility, hygroscopic, color and sensory evaluation tests were performed in three repetitions, and FTIR and SEM tests were carried out, and also concentration, stability, and efficiency tests were performed. Encapsulation and determination of particle size, zeta potential and PDI were carried out in three replicates and the obtained information was analyzed using one-way analysis of variance. Duncan's multiple range test was used to determine the difference among the mean values of the samples at a probability level of 5% ($P<0.05$). All statistical tests were performed using SAS software.

3. Results and discussion

3.1. The rate of extraction efficiency

The efficiency of the extracted anthocyanins was determined to be 0.65% per kilogram of black figs. In a study investigating the use of ultrasound for anthocyanin extraction from purple sweet potatoes, it was found that a short-term, high-temperature method may be beneficial for extracting heat-sensitive anthocyanins. In contrast, longer-term extraction at lower temperatures resulted in decreased yields of anthocyanins [34]. Several factors may contribute to the variation in anthocyanin levels observed in different studies, including genotype, fruit color, and fruiting season [9].

3.2. Determination of particle size

The results related to the evaluation of particle size, zeta potential and particle dispersion index of different treatments (liposomal samples with different ratios of lecithin-cholesterol) are given in Table 2.

Table 2 Particle size, particle size distribution and zeta potential of liposomal nano samples containing black fig anthocyanin*

Particle Dispersion Index (PDI)	Zeta potential	Particle size	Liposomal sample
0.41±0.06[a]	26±7.81[b]	132.6±17.78[c]	1-9
0.36±0.09[a]	36.3 ±.573[a]	260.6±24.58[b]	2-8
0.43±0.05[a]	33.66±1.52[ab]	353±59.57[b]	3-7
0.47±0.15[a]	42.66±6.42[a]	740.03±88.75[a]	4-6

Different lowercase letters in each column indicate significant differences ($P<0.05$).

According to Table 2, it is evident that the experimental treatments exhibited significant differences ($P<0.05$) in terms of particle size and zeta potential. Among the samples, sample 4-6 displayed the largest particle size of 740.03 nm while sample 1-9 was considered to be the optimal sample with the smallest particle size of 132.6 nm.

The particle dispersion index (PDI) serves as an indicator of the uniformity of particle size in a suspension. Consequently, it provides valuable insights into the homogeneous size distribution of vesicles [35]. Notably, no significant difference was detected among the samples with regards to the PDI, indicating a similar level of particle dispersion ($P>0.05$). Nanoliposomes possess another notable attribute known as zeta potential. This parameter, indicative of the surface charge of liposomes, effectively demonstrates the repulsive forces between charged nanoparticles. Moreover, it significantly influences the stability and efficacy of encapsulation processes. In a research, Homayounfal et al. (2021) investigated the average of particle size, PDI and zeta potential in barberry nanoliposome extract, the result showed that the hydrodynamic diameter of the primary nanoliposomes was 140.74 nm and with increasing anthocyanin concentration, finally, the particle size increased to 194.67 nm. The results showed that the encapsulated compound (barberry extract) had a direct effect on the particle size of the colloidal suspension. Also, in the same study, the PDI values for all samples were less than 0.3, and in this sense, there was no significant difference between the samples. In this research, the zeta potential of all samples was less than -42.85 mV, which indicates the high stability of the systems. The negative charge of nanoliposomes in this study was related to anionic phospholipids such as phosphatidylcholine and phosphatidylethanolamine in the structure of rapeseed lecithin [20]. In a study conducted on nanoliposomes containing red cabbage anthocyanin extract, the result showed that the size of nanoliposomes particles varied from 37.12 to 56.1 nm with a zeta potential of about -39 mV [36]. In another study conducted on anthocyanin encapsulation in liposomes, the result showed that with increasing anthocyanin concentration, the size of liposome particles decreased significantly from 1520 nm to 243 nm, while the PDI significantly decreased from 0.241 to 0.342 increased [37]. Also, in another study, Alexander et al. (2012) measured the average particle size in nanoliposomed ascorbic acid between 103 and 136 nm [38]. It has been proven that if the zeta potential value of the samples is more than +30 mV or less than -30 mV, due to the increase of repulsive interactions, including electrostatic, the system is stable and not prone to aggregation [20]. Sample 1-9 was chosen for the next tests due to having the smallest particle size and acceptable dispersion index and zeta potential, and as the sample with the best lecithin-cholesterol ratio.

3.3. Determination of microencapsulation efficiency

Microencapsulation efficiency is one of the important characteristics of nanoliposomes, which shows the amount of trapped active substance. The microencapsulation efficiency for sample 1-9 (optimal sample) and other lecithin-cholesterol ratios was performed to investigate the effect of cholesterol in entrapping the active ingredient. The results related to Microencapsulation efficiency are given in Figure 2.

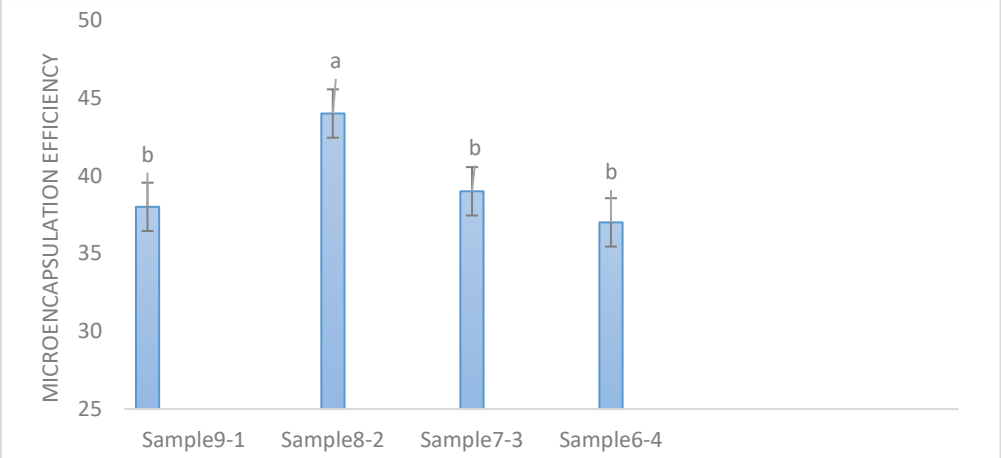

Fig. 2 Measurement of nanoliposome microencapsulation efficiency of black fig anthocyanin compounds in samples 1-9, 2-8, 3-7 and 4-6 lecithin-cholesterol.

Based on the results obtained, it is evident that the examined samples did not exhibit any significant variations in microencapsulation efficiency, except for the 2-8 lecithin-cholesterol sample, which displayed a statistical difference compared to the other samples. The microencapsulation efficiencies for samples 1-9, 2-8, 3-7, and 4-6 were recorded as 38%, 44%, 39%, and 37% respectively. The results showed that sample 2-8 had the highest percentage of microencapsulation, which could probably be due to its optimal amount of cholesterol, and it was also observed that the amount of microencapsulation efficiency decreases with the increase of cholesterol. Cholesterol is introduced into the lipid layer to reduce the permeability of the lipid membrane, stiffen the membrane, and increase stability in the membrane. Therefore, the presence of cholesterol in the liposome membrane probably prevents tearing and changes in the liposome membrane [39]. Therefore, it can be concluded that by increasing the amount of cholesterol, the stability increases and the amount of material leakage from the wall decreases, and as a result, it increases the percentage of encapsulation. In the research conducted by Demirci et al. (2017) on nano liposomal compounds, the result showed that increasing the concentration of liposomal cholesterol decreased the encapsulation rate of hydrophilic substances and increased the release rate. In another study, researchers showed that the encapsulation efficiency in nanoliposomes decreased with the increase of cholesterol [40]. In a study conducted on nanoliposomes containing vitamin A palmitate, the results showed that the encapsulation efficiency increased with the increase of lecithin concentration [39]. Following the result of this research, the results of the research showed that the encapsulation efficiency of nisin Z decreases with the increase of cholesterol levels [41]. Also, Alexander et al. (2012) reported that the encapsulation efficiency of ascorbic acid increased by increasing the amount of soybean phospholipid [38].

3.4. FTIR test

FTIR spectroscopy is a useful method for monitoring changes in the functional groups of molecules. The FTIR spectrum of the anthocyanin extract (Figure 3) reveals several important peaks. The range of 800 cm^{-1} to 1650 cm^{-1} indicates the presence of an aromatic compound, while the peak at 1058 cm^{-1} is indicative of C-H aromatic ring transformation. Additionally, the absorbance observed at 1156 cm^{-1} can be attributed to the vibration of C-O-C esters. The bands present at 1400 cm^{-1}, 1428 cm^{-1}, and 1648 cm^{-1} correspond to C-O, C=C, and C=O groups in the aromatic ring. Furthermore, the wider absorption at 3462 cm-1 is associated with O-H groups found in phenols and sugars. These findings provide valuable insight into the chemical composition of the anthocyanin extract

[42]. As seen in Figure 3, different peaks are observed in the absorbance spectra of AC and liposomes. Some peaks related to liposome and anthocyanin extract remain unchanged, while some of them undergo changes. The position of the carbonyl group of lipids changed after binding to anthocyanin compounds and reached a lower wave number. But the phosphate group of lipids changed to a higher wave number. The peak corresponding to 1740 cm^{-1} shows the stretching vibrations of the carbonyl ester groups of phospholipids (C=O). Absorption bonds belonging to stretching vibrations of carbon-carbon double bonds of alkenes (C=C) at 1650 cm^{-1} were identified. Symmetric and asymmetric vibrations of PO2* were revealed at 1236 cm^{-1} and 1092 cm^{-1}. In addition, the band at 1070 cm^{-1} corresponds to the symmetric and asymmetric CO-O-C stretching bonds. Finally, the 936 cm^{-1} bands represent the asymmetric stretching vibrations of N (CH3)3. In addition, the appearance of the region corresponding to O-H groups also changes. Limitation of O-H bonds is associated with the creation of hydrogen bonds. The AC OH wave in the presence of liposomes was shifted to lower wave numbers by 14 cm^{-1}. This may be due to the breaking of hydrogen bonds and the formation of hydrogen bonds between the OH groups of anthocyanins (phenolic and sugar) and the functional groups of phospholipids (phosphate and carboxyl) [42]. The 1740 cm^{-1} wave is associated with C=O vibrations. Under the influence of the presence of lipids and moving to lower frequencies, it shows that the carbon group in the C ring of phenolic anthocyanins creates a hydrogen bond with lipid molecules. As can be seen, at 1380 cm^{-1}, a new peak with low absorbance intensity is created in the structure of nanoliposomes containing anthocyanin extract. This absorption band may belong to the -N=O stretching vibrations. In fact, the N (CH3)3 group in the lecithin structure is very electrophilic and contains an empty orbital. This group forms a weak dative bond with the free electron pair of the OH group in the structure of anthocyanins. Then, due to the resonance effect of electrons in the benzene ring, it temporarily becomes a weak -N=O bond and creates a new peak at 1380 cm^{-1}[42].

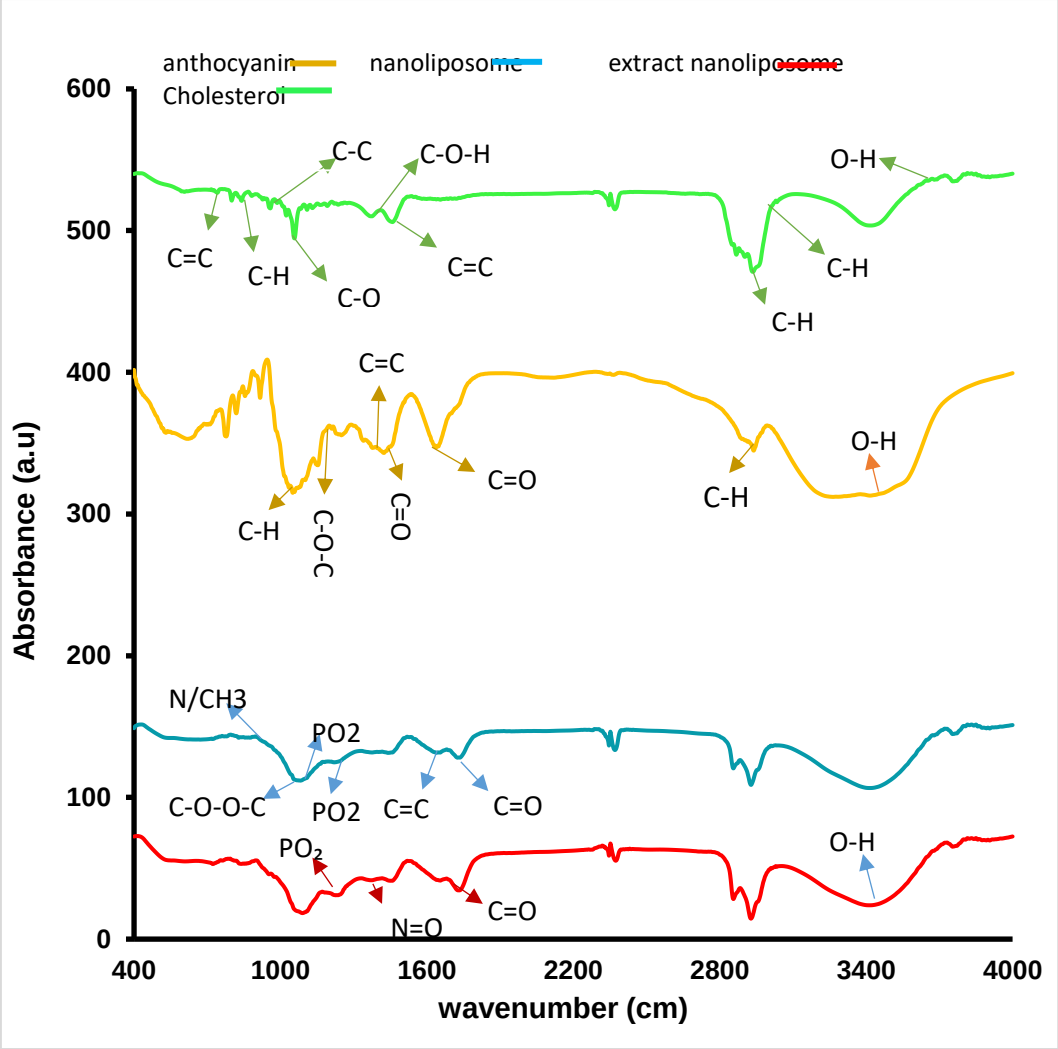

Fig. 3 FTIR analysis results of anthocyanin, nanoliposome, extract nanoliposome and cholesterol

3.5. Morphology analysis by scanning electron microscope (SEM)

The morphological characteristics of microcapsules with 1-9 lecithin-cholesterol ratio have been shown by scanning electron microscope (SEM) (Figure 4). The formation of indentations on some microcapsules has been attributed to rapid water evaporation and consequent shrinkage of the particles during the drying process or to the applied vacuum during SEM analysis. Smooth spheres are desirable for stability of encapsulated materials as well as for controlled release. This concept is one of the main goals of food microencapsulation because it can improve the efficiency of food additives [43].

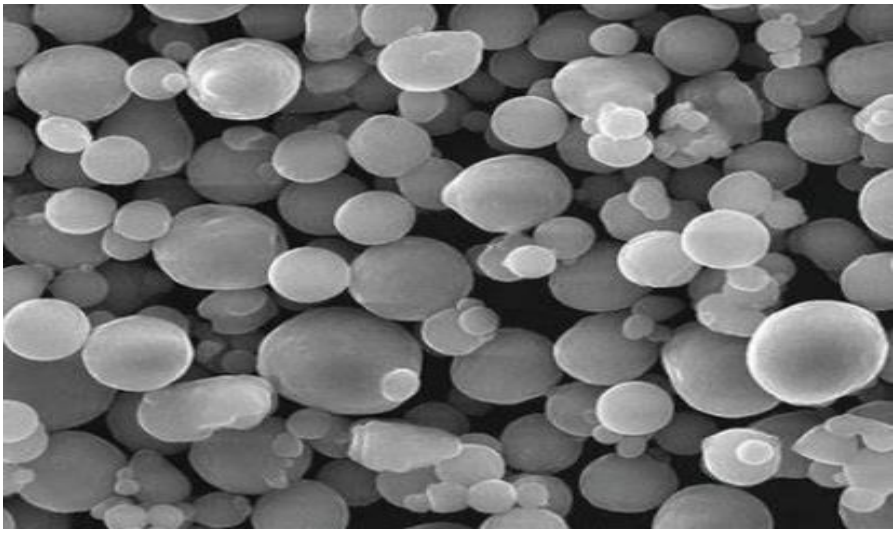

Fig. 4 FESEM image for lecithin-cholesterol sample 1-9

3.6. Determination of stability

The results related to the evaluation of the stability of nanoliposome samples during the 60-day storage period are shown in Figure 5.

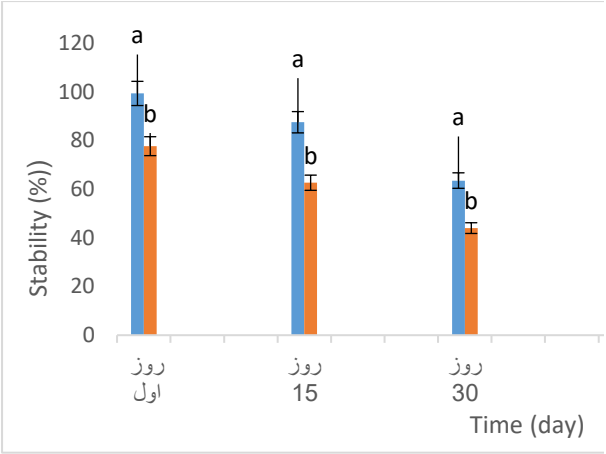

Fig.5 Determination of stability for lecithin-cholesterol sample 1-9
Different lowercase letters in each column indicate significant differences (P<0.05)

According to Figure 5, it can be seen that the stability of the nano-sized samples was significantly different on different days. The level of stability on the 1st, 15th, 30th, 45th and 60th days was: 99.33, 87.5, 63.5, 50.06 and 17.5%, respectively, and gradually the passage of time decreases the level of stability, which indicates that materials have leaked out from the walls of liposomes, and during two months of storage, the stability level has increased from 99.33% to 17.5%, which could be due to the destruction of the walls of liposomes. Adding cholesterol to the liposome formula also reduces the permeability of the liposome membrane. Therefore, the trapped material cannot leak easily. In a research, Mohammadi et al. (2014) investigated the stability of vitamin D3 nanoliposomal compounds for two months at 4 °C and concluded that, based on the lipophilic nature of the entrapped substances, degradation does not occur and the substances showed good stability during the storage period [48]. In a research, the storage stability of curcumin nanoliposomes was investigated by Chen et al. (2015). Curcumin nanoliposomes showed good stability when stored at 4 °C, which was attributed to the fact that low temperature prevented the degradation of nanoliposomes[49]. Chanda et al. (2011) evaluated the stability and strength of liposomes for storing fluconazole during one month in refrigerator (9°C), room (25°C) and high temperature (45°C). The results showed that liposomes are more stable and stronger at lower temperatures (leakage less than 5%) and higher temperatures cause more fluidity of lipid layers and more leakage of fluconazole [50].

3.7. Characteristics of beverages containing anthocyanin extract

The characteristics of beverages enriched with anthocyanin extract (at 4% and 6%

levels as nano-enriched, free form and control sample) were investigated and water-soluble solids (Brix), pH and their sensory properties were evaluated. Also, to measure the solubility and hygroscopic index, the beverages were dried and the powder of the beverages was evaluated.

1.3.7. Water-soluble solids (Brix)

The results of Brix are given in Table3. As can be seen, the beverage containing the Nanoencapsulated extract at the level of 6% has the lowest amount of Brix, and the non-Nanoencapsulated sample has the highest amount of Brix at the level of 6%. This shows that by increasing the amount of liposome, the amount of solids insoluble in water also increases, and in free samples, due to the solubility of anthocyanin in water, the amount of water-soluble solids increases and causes an increase in Brix. In the research that was carried out on Kambocha tea prepared with different herbal teas, the results showed that the Brix at the beginning of fermentation and after that were different in all tea varieties, so that at the beginning of the fermentation process, the lowest amount of Brix in green tea and the highest amount of Brix in Mint tea was observed and after fermentation, Brix values were significantly different in all tea samples, and the lowest Brix was observed in black tea and the highest Brix was observed in sage tea [44].

Table 3 Brix level of Kombucha beverage containing nanoencapsulated and non-nanoencapsulated anthocyanin extract at two levels of 4 and 6 % *

Brix	Sample
5.56±0.20[a]	Control sample
4.43±0.40 [b]	F4%
5.3±0.30 [a]	F6%
4.06±0.11 [b]	N4%
3.23±0.32 [c]	N6%

Different lowercase letters in each column indicate significant differences (P<0.05).

N4%: nanoliposomed sample at 4%

N6%: nanoliposome sample at 6%

F4%: free sample at 4%

F6%: free sample at 6%

2.3.7. pH

The results related to pH values are reported in Table 4. As can be seen, pH of the different treatments had not significant differ. In the research, kombucha tea was prepared using several different tea extracts, the tea extracts used were: black tea, green tea, sage and mint tea. These samples were subjected to fermentation for 14 days, the pH value was measured and the results showed that the lowest pH was in the black tea sample and the highest pH was in the sage tea and the pH of all the samples decreased after fermentation and the lowest and highest pH were observed in Black and sage tea, respectively [44]. In another study, the pH value of kombucha beverage was investigated during the fermentation, the results showed that with the increase in the fermentation time, pH values decreased, but total acidity content (in terms of acetic acid) increased [45].

Table 4 pH value of Kombucha beverage containing nano-encapsulated and non-nanoencapsulated anthocyanin extract at two levels of 4 and 6 % *

pH	Sample
3±0.005[a]	Control sample
3.13±0.11 [a]	F4%
3.20±0.10 [a]	F6%
3.03±0.05 [a]	N4%
3.06±0.05 [a]	N6%

Different lowercase letters in each column indicate significant differences (P<0.05).

N4%: nanoliposomed sample at 4%

N6%: nanoliposome sample at 6%

F4%: free sample at 4%

F6%: free sample at 6%

In a research, the amount of pH changes of kombucha beverage during the fermentation was investigated. The result

showed that the pH of kombucha tea decreased with fermentation time. Within 3 days of fermentation due to increase in concentration of organic acids produced during fermentation process, pH value decreased from 5 to 3 [46]. In another study, pH value of black tea-flavored kombucha beverage was 2.37 and pH of sage-flavored kombucha beverage was 3.25. The reason was related to organic acids produced by fermentation using black tea and sucrose [44].

3.3.7. Sensory evaluation

The results related to sensory evaluation of kombucha beverage samples containing microencapsulated free extract are shown in Table 5. Parameters of color, smell, taste, mouthfeel and overall acceptance were evaluated in sensory evaluation of kombucha samples. There were a significant difference between color and taste of samples. The color of F6% sample and taste of control had the highest score, respectively. However, among the samples, Smell, mouthfeel and overall acceptance parameters had no differ significantly.

In a research conducted on kombucha tea in several varieties of extract, the results showed that taste and smell of mint tea had highest score and black tea had lowest score of smell. In color evaluation as well as smell, mint tea and linden tea had highest and lowest score, respectively. Considering all parameters in sensory evaluation, it was observed that mint tea and linden tea had highest and lowest score, respectively [44].

Table 5 Sensory evaluation of nanoencapsulated and free kombucha beverage samples at 4 and 6%

General acceptance	mouthfeel	taste	Smell	Color	Treatments
3.5±0.52^{a}	3.3±0.67^{a}	3.5±0.84^{b}	3.5±0.70^{a}	4.5±0.52^{a}	F6%
3.6±0.51^{a}	3.2±0.63^{a}	3.6±0.51^{b}	3.5±0.52^{a}	3.6±0.51^{b}	F4%
3.5±0.97^{a}	3.1±0.56^{a}	3.6±0.69^{b}	3.4±0.84^{a}	3.9±0.31^{b}	N6%
3.6±0.51^{a}	3.2±0.63^{a}	3.9±0.31^{b}	3.3±0.94^{a}	3.8±0.63^{b}	N4%
3.4±0.69^{a}	3.2±0.63^{a}	4.9±0.31^{a}	3.3±0.94^{a}	3.9±0.31^{b}	control

Different lowercase letters in each column indicate a significant difference (P<0.05).
N4%: nanoliposomed sample at 4%
N6%: nanoliposome sample at 6%
F4%: free sample at 4%
F6%: free sample at 6%

4. Conclusion

According to the results obtained in this research, the size of nanoencapsulated particles was in the range of 130-740 nm. Also, the distribution index was in the range of 0.36 to 0.47 and the zeta potential was between -26 and -42.66 mv. Increasing cholesterol levels had an adverse effect on particle size and nanoencapsulated efficiency so it increased the particle size and decreased the nanoencapsulated efficiency. FTIR results showed successful loading of anthocyanin extract in liposomes. Also, the results of the FESEM image analysis showed that the size of the particles was in the nano range and the particles had not led to the phenomenon of agglomeration, and the results of the scanning electron microscope were consistent with the results of DLS. Also, the results of sensory evaluation of prepared beverages showed that among the parameters, the taste of control as well as the color of a non-nanoencapsulated sample at a level of 6% had the most acceptance score. This research showed that the loading of anthocyanin inside the nanoliposome was successful in increasing the stability and color of this compound and it can be used on a commercial level.

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نانولیپوزوم ترکیبات آنتوسیانینی انجیر سیاه و کاربرد آن در نوشیدنی کامبوجا

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چکیده

آنتوسیانین‌ها از ترکیبات فعال زیستی هستند که رنگدانه اصلی بسیاری از میوه‌ها و سبزیجات را شامل می شود. از آنجا که آنتوسیانین‌ها پایداری حرارتی پایینی در طی فرآوری مواد غذایی دارند، لذا استفاده از این ترکیبات به عنوان رنگدانه‌های طبیعی در غذاها با چالش‌هایی همراه است. بنابراین، ریزپوشانی ترکیبات آنتوسیانینی با لیپوزوم‌ها دارای اهمیت است. ریزپوشانی ترکیبات زیست فعال با لیپوزوم‌ها، روشی موثر و کارآمد در افزایش پایداری ترکیبات پلی فنولی است. لیپوزوم‌ها وزیکول‌های لیپیدهای قطبی هستند که در حلال‌های قطبی نظیر آب، ساختارهای دولایه تشکیل می دهند. در این پژوهش، نانولیپوزوم‌ها در نسبت های ۹-۱، ۸-۲، ۳-۷ و ٦-٤ درصد وزنی-وزنی لسیتین-کلسترول، با استفاده از روش تزریق حلال، تهیه شدند. سپس آزمون‌های تعیین اندازه و پتانسیل زتا برای تعیین ویژگی‌های ذرات تولید شده صورت گرفت. میانگین اندازه ذرات (میانگین قطر هیدرودینامیکی) و توزیع اندازه ذرات برای نسبت‌های مختلف لسیتین-کلسترول، به ترتیب در محدوده ۷٤۰-۱۳۲ نانومتر و ۰.٤۷-۰.٤۱ قرار گرفتند. مقادیر پتانسیل زتا نیز در محدوده ۲٦- تا ٤۲- میلی ولت بدست آمد. پایداری نمونه لیپوزومی نسبت ۹-۱ لسیتین-کلسترول، از طریق محاسبه ی مقدار آزادسازی آنتوسیانین محصور شده در طول ٦۰ روز نگهداری در دمای محیط مورد بررسی قرار گرفت. نمونه های دارای نسبت ۹-۱ لسیتین-کلسترول در فرمولاسیون نوشیدنی کامبوجا استفاده شدند. نوشیدنی‌های تهیه شده از نظر ویژگی‌های حسی مورد ارزیابی قرار گرفتند که ازنظر بو، احساس دهانی و پذیرش کلی تفاوت معنی‌داری در بین نمونه‌ها مشاهده نشد. نتایج بدست آمده در این پژوهش، نشان داد که نانولیپوزوم‌ها سیستم کارآمدی در درون پوشانی آنتوسیانین‌ها هستند.