Formulation, characterization, and physical stability of encapsulated walnut green husk (*Juglans regia* L.) extract in phosphatidylcholine liposomes

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Footnotes

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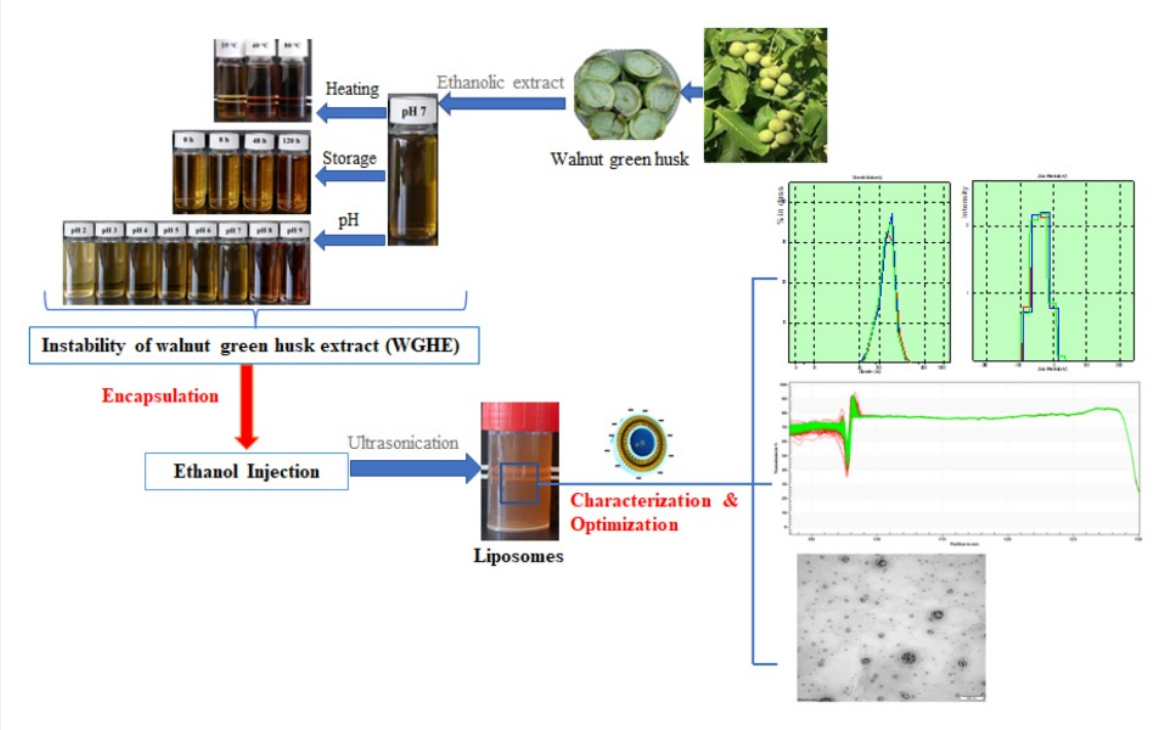
Abstract

This study aimed to optimize a liposomal formulation to enhance the stability of a phenolic-rich extract from green walnut husk. Liposomes were prepared using varying concentrations of phosphatidylcholine (0.15–2% w/v), extract (0–1.3% v/v), and sodium laurate (0–0.2% w/v) via ethanol injection and sonication. Characterization included visual appearance, particle size, polydispersity index, surface charge, encapsulation efficiency, and morphology. Stable

liposomes were achieved at 0.15% and 0.3% w/v phosphatidylcholine, although with low encapsulation efficiency (<40%). The addition of 0.2% (w/v) sodium laurate improved the stability, especially at higher phosphatidylcholine concentrations, enhancing the electrostatic repulsion. Optimal concentrations of 2% w/v phosphatidylcholine, 0.2% w/v sodium laurate, and 0.6% v/v extract were determined. The liposomes exhibited a spherical unilamellar morphology with a size of 97.5 ± 0.9 nm and a negative surface charge of –39.8 ± 0.9 mV. These nanoliposomes showed 79.7 ± 0.7% encapsulation efficiency and remained stable under pH, temperature, ionic strength, and storage time variations. Overall, the liposomes proved effective in preserving the natural phenolics of walnut husks under challenging environmental conditions.

GRAPHICAL ABSTRACT



KEYWORDS

Liposomes; walnut green husk extract; phosphatidylcholine; sodium laurate; encapsulation efficiency

Introduction

Phenolic compounds, which are widely found in plants, offer various health benefits and applications in foods due to their antioxidant and flavoring properties. Therefore, there is a growing interest in finding new natural and cost-effective

sources of phenolics. Walnut trees, known for their valuable agricultural products, yield fleshy green husks that are discarded as waste despite being rich in polyphenols, naphthoquinones, and flavonoids with anticancer, antidiabetic, hepatoprotective, and anti-inflammatory properties. Valorizing green walnut husks not only contributes to environmental preservation but also offers an economical source of phenolic compounds.[1–4]

However, the sensitivity of phenolic compounds to pH, temperature, light, and oxygen largely restricts their use in the food and pharmaceutical industry due to their poor stability in natural conditions or during processing. Encapsulation may provide a suitable solution by preserving the phenolics and enabling their incorporation into food products.[5–7] Most encapsulation systems rely on synthetic compounds (e.g., surfactants, carboxymethylcellulose), which have raised concerns due to potential negative health effects, including disruptions to the gut microbiota and chronic intestinal inflammation.[8] In contrast, phospholipids offer health-promoting effects and are used as food supplements for regulating the blood lipid profile, reducing cardiovascular risks, inhibiting tumors and metastasis, supporting neurological development, and improving cognitive functions.[9] Liposomal dispersions, composed of biocompatible, biodegradable, and non-immunogenic phospholipid bilayer membranes, have emerged as a promising option for encapsulating phenolics. These liposomes, formed through self-assembly in an aqueous phase, utilize hydrophobic interactions and van der Waals forces to create spherical vesicles capable of effectively entrapping phenolics.[7,10–12]

Different methods have been proposed to produce small and uniform particles.[10,13] The choice of fabrication method and delivery system depends on the nature of the compounds and the intended purpose of encapsulation. Ethanol injection, a non-hazardous technique, is commonly used for the formation of liposomal dispersions. In this method, the phenolic compounds and phospholipids are dissolved in ethanol and injected into an aqueous phase. The appearance and stability of the liposomes are influenced by their size and charge, often requiring the incorporation of additional techniques like ultrasonication, extrusion, or microfluidization to achieve smaller vesicles with a uniform size distribution.[7,10,13]

Several studies have focused on encapsulating individual phenolics such as curcumin,[14,15] quercetin,[16] gallic acid,[17] taxifolin,[18] or catechin,[19] but few have explored the encapsulation of phenolic mixtures, particularly the extract of green husks. Liposomes, while effective for encapsulation, can face challenges such as aggregation, fusion, and hydrolysis, leading to potential leakage of the encapsulated compound. Therefore, enhancing the stability of liposomes is a growing area of interest.[20] Chitosan enhances the stability of liposomes through steric and electrostatic repulsion. Studies by Hao et al.[21] used chitosan to stabilize soy lecithin liposomes containing quercetin. However, chitosan also increases the dispersion viscosity and requires negatively charged liposomes.[21] Alternative charged molecules have been used widely. Sanchez et al.[22] achieved stable liposomes using dicetyl phosphate (DCP) or stearylamine (SA). The current study introduces the novel use of sodium laurate to impart surface charge to liposomes made from zwitterionic phosphatidylcholine.

The stability of the green husk extract was first evaluated under various environmental stress conditions such as variations in heat treatment, and pH, revealing the need for encapsulation. The chosen approach involved a combination of ethanol injection and ultrasonication. Given the presence of a phenolic mixture in the extract, formulating the liposomal system posed a significant challenge.

Liposomes were fabricated using different concentrations of phosphatidylcholine (PC), sodium laurate (SL), and

walnut green husk extract. PC was chosen for its high ethanol solubility, unlike anionic phospholipids such as phosphatidylinositol (PI) and phosphatidic acid (PA) which are barely soluble in ethanol.[9] The challenge of physical instability, particularly sedimentation in aqueous dispersions, was addressed using centrifugal photosedimentometry. This method allows for a quick prediction of the gravity-related effects during long-term storage by considering the g-force.[23,24] The characteristics of the vesicles, including appearance, morphology, size, polydispersity index, charge, encapsulation efficiency, and stability during centrifugal photosedimentation were analyzed to achieve the optimum formulation with a high stability. This study provides practical and theoretical insights into the design of a liposomal formulation for the efficient encapsulation of an ethanolic phenolic-rich green walnut husk extract, laying the groundwork for the development of a stable encapsulation system for phenolic mixtures or plant extracts.

Materials and methods

Materials

Walnut (*Juglans regia* L.) fruits from the Saman variety were collected after their full ripening in September 2021. The green husks were separated by hand, immediately frozen, and completely freeze-dried (48 hours) using a freeze dryer (Dena Vacuum, Iran). The dry biomass was milled and sieved to obtain a fine and homogeneous powder (<1 mm). Finally, the prepared powder was packed in a container, purged with nitrogen gas, sealed, and stored at –80 °C until extraction.

Epikuron 200 (purified wax-like phosphatidylcholine of soybean origin, produced by column chromatography) was kindly donated by Cargill (Cargill Texturizing Solutions Deutschland GmbH & Co KG, Hamburg). The product consists of phosphatidylcholine (minimum 92%), a small amount of the accompanying phospholipids (maximum 5%), and oil (maximum 2%). Sodium dodecanoate (also known as sodium laurate) was purchased from Tokyo Chemical Industry Co., Ltd. (TCI-Europe, Belgium).

Ethanol, monosodium phosphate, disodium phosphate, gallic acid, Folin–Ciocalteu reagent, and sodium chloride were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and purchased from Merck Co. (Darmstadt, Germany). For all analyses, ~~bi-~~distilled and purified water was prepared using a Milli-Q system (Merck Millipore, Burlington, MA, USA).

Preparation of Phenolic-Rich Walnut Green Husk Extract

Briefly, 500 mg of walnut green husk powder was extracted with 20 g of 80% ethanol. The powder was mixed well using a vortex mixer (60 minutes, 25 °C) and to separate the soluble compounds, centrifugation (13540 ×g, 10 minutes) and filtration were performed through 0.2 µm polyamide filters. Finally, the organic solvent was recovered using a vacuum evaporator. The extract was redissolved in ethanol and the polyphenol-rich walnut green husk extract (WGHE) with a concentration of 60% was prepared. The phenolic composition of the WGHE was characterized by UPLC/MS (according to our previous study) and is given in Table 1.[25]

Table 1. Phenolic composition of the ethanolic walnut green husk extract (WGHE) used

Compound	Concentration (%)
1-Salicylate glucuronide	35.2 ± 0.1
Quercetin 4′-glucoside	17.5 ± 0.0
(+) Catechin	13.2 ± 0.0
Taxifolin	11.4 ± 0.0
Quercetin pentoside	8.1 ± 0.0
Gallic acid	6.9 ± 0.1
Neochlorogenic acid	5.2 ± 0.0
Taxifolin 7-glucoside	2.5 ± 0.0
Total phenolic content (mg gallic acid per mL)	17.8 ± 1.3
Total antioxidant activity IC_{50} (µg per mL)	48.8 ± 1.8

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Solubility and Stability of Walnut Green Husk Phenolic-Rich Extract

To investigate the solubility of the walnut green husk extract in different solvents, the prepared extract was dried for 24 hours using a freeze dryer. Then, the dried extract was dispersed at a concentration of 0.1% w/v in absolute ethanol, phosphate buffer (10 mM, pH 7), and a mixture of ethanol and phosphate buffer (4:1 v/v, pH 7), covered by aluminum foil and left on a stirrer for 60 minutes. Then, the appearance of each sample was evaluated.[26]

The effect of storage time, temperature, and pH on the stability of the extract was evaluated. For the stability measurement as a function of storage time, a dispersion of fresh extract in a mixture of ethanol and 10 mM phosphate buffer (4:1 v/v, pH 7) at a concentration of 0.6% v/w was prepared and stored for 0, 8, 48, and 120 hours. For thermal stability, samples were heated at 40 and 80 °C for 30 minutes, immediately cooled to room temperature (25 °C) and stored for 0 and 120 hours. For pH evaluation, the pH of a freshly prepared dispersion of extract in ethanol and 10 mM phosphate buffer (4:1 v/v) at pH 7 was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 using hydrochloric acid and sodium hydroxide (1–5 mM). The prepared samples were stored for 120 hours. The visual appearance, spectrophotometric profile, and total content of phenolic compounds of all samples were investigated. The prepared dispersions were placed in a quartz cuvette and the absorption spectra across the 200–450 nm wavelength range were scanned. The total phenolic content of the dispersions was measured using the Folin–Ciocalteu method. Briefly, 20 µL of the dispersion, standard (gallic acid with concentrations of 0–500 ppm), or blank was mixed with 100 µL of Folin-Ciocalteu's phenol reagent and 1.58 mL of water for 8.50 minutes. Then, 300 µL of sodium carbonate solution (20% w/v) was added. The mixture was incubated at 40 °C for 30 minutes in a water bath and the absorbance was measured

using a spectrophotometer (PowerWave XS, BioTek, Inc., Winooski, VT, USA) at 765 nm.[27] The content of phenolics in the samples was determined using a linear regression equation of gallic acid solutions (y = 0.000098x, R2 = 0.9970).

Preparation and Formulation of Liposomal System

Liposomes formulated with different percentages of PC, SL, and WGHE were fabricated by the ethanol injection method with sonication treatment (Supplementary Information, Table S.1). First, PC, WGHE, and SL were dissolved in 2 mL ethanol, and then were injected manually by means of a needle syringe (21Ga-1 inch/25 mm) at a constant speed of 2.1 mL/min into 50 mL of sodium phosphate buffer (10 mM, pH 7) while the mixture was stirred at 1000rpmat room temperature. After continuous stirring for 60 minutes in the next stage, the samples were sonicated inside a 100mL beaker (inner diameter=46mm) using an ultrasonicator (Branson, Sonifier S-250A, USA) and a horn probe (Disruptor horn, 13mm diameter) dipped into the sample (1cm) at a power of 50 W for 10 minutes (at 70% duty cycles). The percentage of the constituent components of the liposomes was based on the weight or volume (w or v) in the total volume of liposomes. The unloaded liposomes are samples formulated without walnut husk extract. The prepared liposomes were stored in a refrigerator for further analyses (4 °C).

Characterization of Liposomes

Determination of Particle Size, Polydispersity Index and ζ-Potential

Liposomes were characterized in terms of their so-called z-average particle size (d_z) and polydispersity index (PDI) using dynamic light scattering (Zetasizer 3000HS, Malvern, UK) at 25 °C. The particle size distribution was estimated by multimodal data analysis and measurements were made in triplicate.[26]

The zeta-potential was determined at 25 °C by electrophoretic light scattering (Zetasizer 3000HS, Malvern, UK). To avoid multiple scattering effects, liposomes were diluted at a 1:1 (v/v) ratio in 10 mM sodium phosphate buffer (with a similar pH). The measurements were made in triplicate.[26]

Encapsulation Efficiency

The encapsulation efficiency was determined by calculating the difference between the total phenolic content in the dispersion and the total non-encapsulated phenolic content in the permeate of the liposomal dispersions after ultrafiltration, according to Muhammad et al. [28] with minor modifications. Briefly, 2.5 mL of liposomal dispersion was placed in Amicon centrifugal ultrafiltration tubes (Amicon altracel-3K 15ml, Millipore Cork Ireland) and centrifuged (Sigma 4K15 Sartorius AG, Germany) at 13102 g for 90 minutes. The Folin-Ciocalteu's method was used to measure the total phenolic content of the permeate.

For measuring the total phenolic content, green husk extract (0–1.3% v/v) was dissolved in 52 ml of ethanol and buffer solution at pH 7 and sonicated at 50 W for 10 minutes. The encapsulation efficiency (EE, in %) was calculated according to the following equation:

$$\mathrm{EE}\ (\%) = (\text{total phenolic content} - \text{phenolic content in permeate}) \times 100/\text{total phenolic content}$$

Transmission Electron Microscopy

The morphology of the liposomes was visualized using a JEM 1010 (JEOL, Tokyo, Japan) transmission electron microscope (TEM) equipped with a Veleta side-mounted CCD camera (EMSIS GmbH, Muenster, Germany). First, sample~~s~~ w~~ere~~as diluted ~~50 and~~ 100 times with milli-Q water and 2μL of ~~them were~~it was blotted on a Formvar film-coated copper single slot grid (Agar Scientific, Standard, UK). After air-drying under the fume hood at ambient temperature, the copper grid was covered by negative staining and imaging was performed at magnifications of 80,000 ×.[29]

Stability of Liposomes

Stability of Liposomes Against pH, Temperature, and Ionic Strength Variations

The pH of the freshly prepared liposomes with optimum formulation was adjusted to acidic or alkaline pH (2–9). The thermal stability of the liposomes was evaluated after 30 minutes heating in a water bath at a temperature of 40 and 80 °C; the tubes were cooled immediately afterwards. The ionic strength was adjusted by adding an aqueous 3 M NaCl stock solution to give NaCl concentrations of 10, 50, 100, 200, and 400 mM.

Physical Stability Analyzed by Centrifugal Photosedimentometry

The physical stability of fresh liposomes was assessed using the LUMiSizer dispersion analyzer (LUM, Burg-Haamstede, The Netherlands). This centrifugal method eliminates the requirement for extended sample storage and instead enables accelerated storage stability analysis, allowing for predictions of the sample stability in the gravity field.[30] Rectangular polycarbonate cells (LUM 10 mm, PC, Rect. Synthetic Cell (110-132xx)) were filled with 0.8 mL of liposomes and were subjected to a centrifugal rotor speed of 4000 rpm for 42,000 seconds at 25 °C. Transmission profiles and trace instability phenomena of each sample were recorded in intervals of 60 seconds. The data were analyzed by LUM SEP view V6.4. software.

Statistical Analysis

The data were analyzed in a completely randomized design. Analysis of variance was performed on the data using the general linear model. The confidence interval was set for a level of significance at $p < .05$ using Statistical Analysis System (SAS) version 13.1 to evaluate the significance level of difference between the mean values. All experiments were performed in three independent replicates and the reported values were the mean ± standard deviation of the repetitions.

Results and discussion

Solubility and Stability of Walnut Green Husk Phenolic-Rich Extract

The dried walnut green husk extract was soluble in ethanol, phosphate buffer, and a mixture of ethanol and phosphate buffer. Therefore, in terms of solubility, there was no problem for using the extract directly. The stability of the phenolic compounds over time, upon heating, and at different pH values was studied based on spectrophotometric scans and measurements of the total phenolics content. The obtained results are shown in Figures 1, 2 and Table 2.

Figure 1. The total phenolic content (TPC) of walnut green husk extract (0.6% v/v) in ethanol and 10 mM phosphate buffer solution (4:1 v/v, pH 7) as a function of storage time at different pH. Different letters for each series show significant differences at $p < .05$.

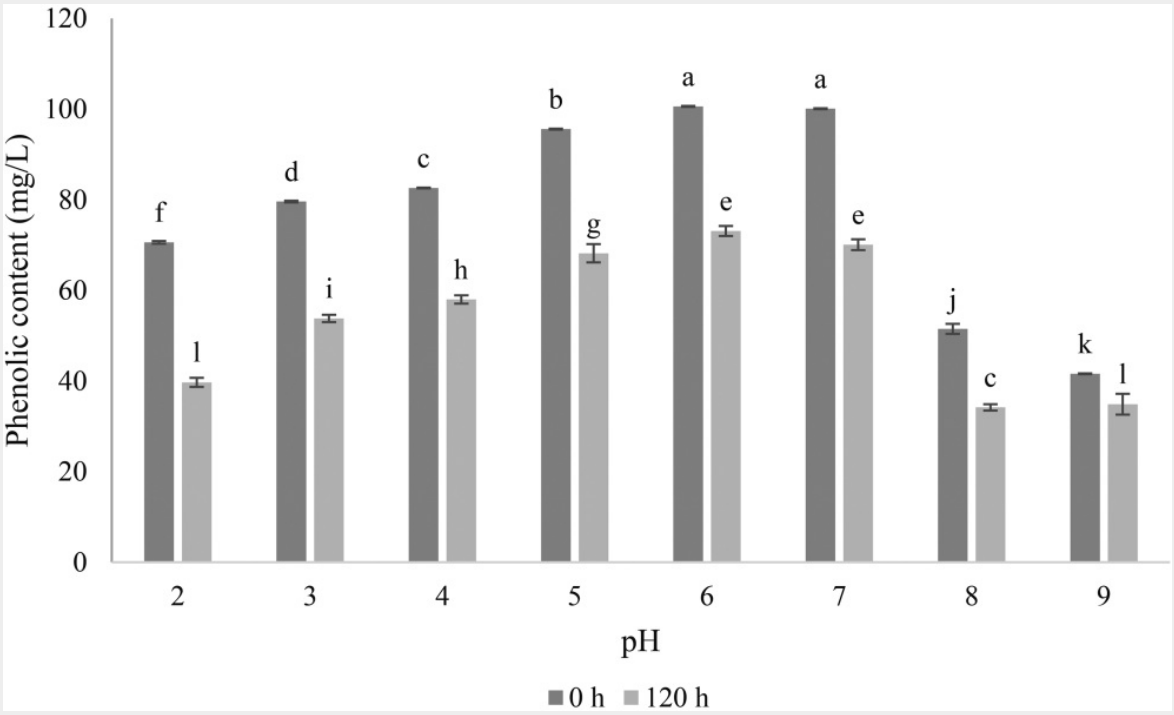


Figure 2. Effect of heating (at 25, 40 or 80 °C, for 30 minutes), on the visual appearance and UV visible absorption spectra of the walnut green husk extract (0.6% v/v) in ethanol and 10 mM phosphate buffer solution (4:1 v/v, pH 7) across the 200–450 nm wavelength range.

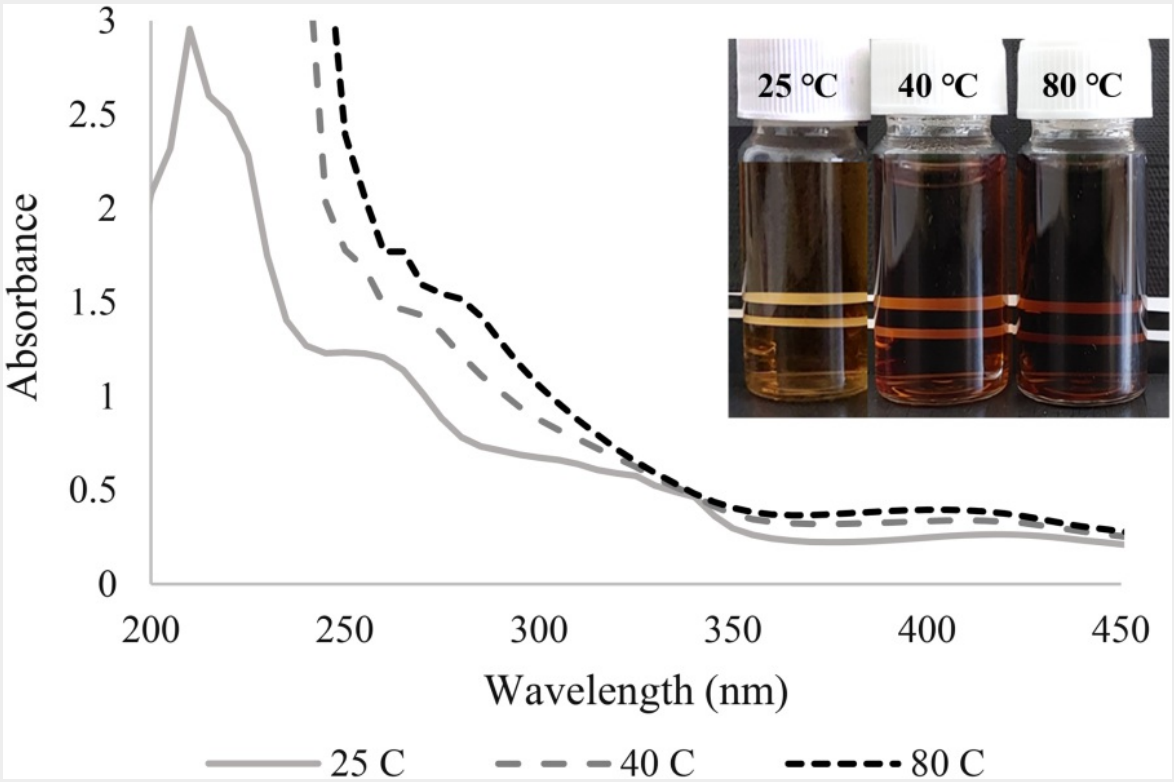


Table 2. The total phenolic content (TPC) of walnut green husk extract (0.6% v/v) in ethanol and 10 mM phosphate buffer solution (4:1 v/v, pH 7) as a function of storage time after heating at 25, 40 or 80 °C for 30 minutes

Preliminary heating (°C)	Storage time (h)	Phenolic content (mg/L)
25	0	100.1[a] ± 0.1
	8	99.7[a] ± 0.1
	48	87.1[b] ± 0.1
	120	70.1[d] ± 2.1

40	0	78.1^{c} ± 3.5
	120	61.2^{e} ± 2.3
80	0	46.2^{f} ± 1.0
	120	39.2^{g} ± 1.2
Note: Different superscript letters show significant differences at $P < 0.05$.		

When the storage time was extended to 120 hours, the color of the solution changed from light yellow to light brown. The absorbance spectra after 0 and 8 hours were almost identical, but the absorption spectrum after 48 hours was slightly different. The greatest differences were found in the 260–300 nm and 340–400 nm ranges, with higher peak heights. The phenolic contents over time (Table 2) significantly decreased after 8 hours, and a 30% decrease in total phenolic content was observed after 120 hours. Hence, the visual color changes might be related to the decrease in phenolics content.

By increasing the temperature from 25 to 40 and 80 °C, the color intensity and turbidity increased. The decrease in transparency was clearly visible in the sample heated at 80 °C (Figure 2). Most of the phenolic compounds have a pronounced absorbance in the range of 200–300 nm. A significant difference between the spectra of the heated and control sample (25 °C) was also observed in this range (Figure 2). In the food industry, heat treatment is widely applied with various purposes such as pasteurization, cooking, eliminating spoilage and pathogenic agents and blanching of enzymes. Phenolics are heat-sensitive compounds and are easily oxidized under high temperature.[31,32] The measurement of the total phenolic content showed a decrease of about 22% and 54% for samples treated at 40 °C and 80 °C, respectively. Storage of heated samples (30 minutes heating then cooled to room temperature) also caused a considerable reduction in phenolic content.

The effect of pH on the stability was also investigated (Figure 1). The color of the dispersed extracts was lighter at acidic pH and darker under alkaline conditions. The absorption spectra decreased slightly at acidic pH and increased in height at alkaline conditions. In the range of 260 to 330 nm at pH 9, the absorption spectrum had a bulge instead of a depression, which may indicate changes in chemical structure. To investigate the reversibility of the spectral changes, first, the extract was brought from pH 7 to 9 and after 2 hours it was returned to a pH of 7. After several hours of storage, precipitation of some compounds was observed. Examination of the content of phenolic compounds also showed a decreasing trend as the pH shifted away from the 6–7 range. This decrease was more pronounced at alkaline pH than at acidic conditions. This finding aligns with previous studies, which reported a degradation of 20.2% in taxifolin after a 30 minutes exposure to 1 M HCl and 16.3% after a 15 minutes exposure to 1 mM NaOH. Taxifolin, a flavonoid compound, undergoes degradation at alkaline pH through auto-oxidation, wherein the B ring is more susceptible to oxidation compared to the A ring. This degradation process involves the formation of radicals and peroxides, which attack the B ring, leading to water loss and the formation of a ketone group. Subsequently, the aromatic system of the B ring is regenerated.[33] At pH 9, a more than 50% reduction in phenolic content was observed (Figure 1). The effect of storage time during incubation at different pH conditions on the reduction of phenolics was significant. These changes can have a direct impact on the measured total phenolics. As a result, the significant decrease observed at alkaline pH may be attributed to the degradation of the dissociated state of the phenolic compounds and alterations in their chemical structure.[34] In this regard, Friedman et al.[34] stated that the shift in spectra as well as the

sensitivity of phenolics to pH, temperature and time is strongly related to their chemical structure. The number of hydroxyl groups, double bonds, number of rings in the phenolic structure, spatial structure, and the position of the hydroxyl groups on the benzene ring influence the sensitivity and reversibility of the changes.

It is worth noting that the nature of these chemical changes and transformations is not yet fully understood, even for purified phenolics.[34] In the case of WGHE, due to the presence of various natural phenolics (such as catechin, quercetin, taxifolin, and their isomers) as well as other compounds, it is difficult to interpret the exact cause of the observed changes. In summary, based on the results obtained, it can be stated that the most important problem in the field of direct use of WGHE was its poor stability. As most food products may be exposed to the above-mentioned stress conditions, it is necessary to protect the phenolic compounds by an encapsulating system.

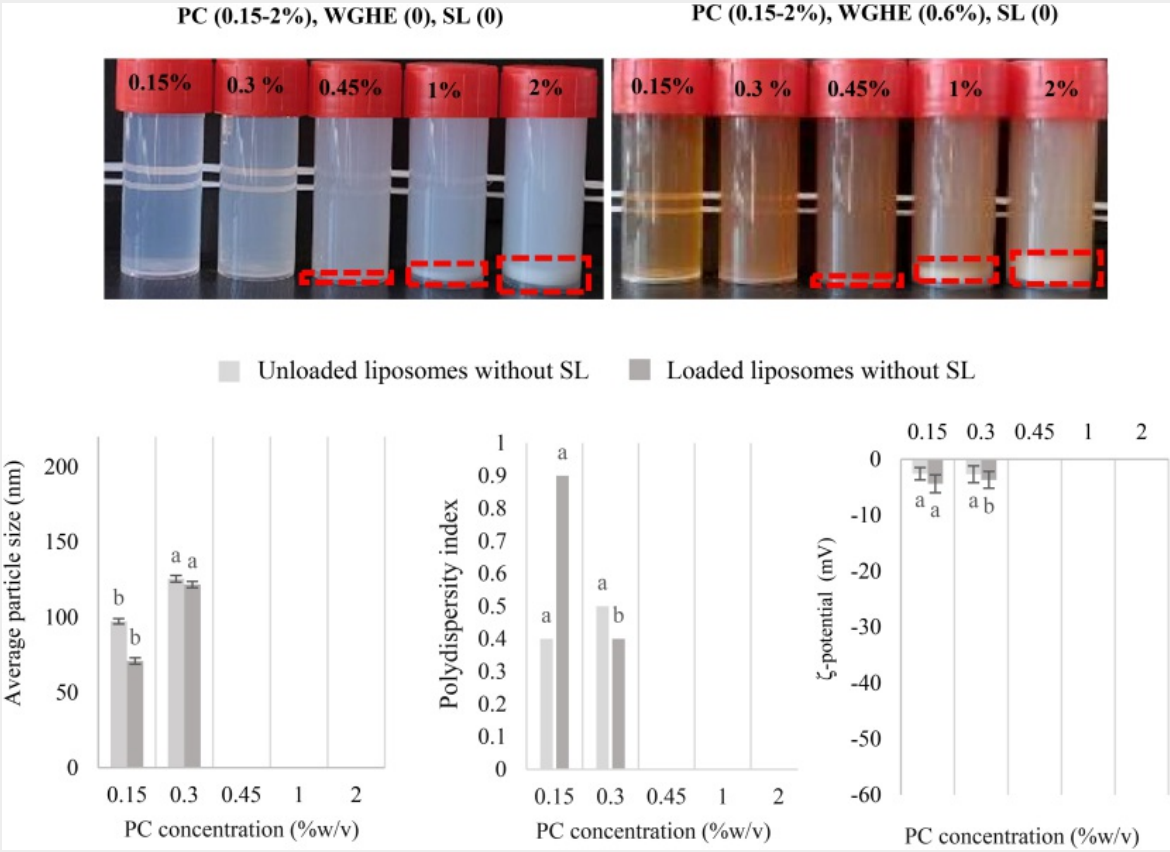
Formulation and Characterization of Liposomal System

Many factors, such as PC and SL concentration, loaded WGHE, and pH, can affect the liposomes formation process and their stability. Therefore, these conditions need to be optimized to achieve an optimal formulation of liposomes. Initially, concentrations of 2% w/v PC, 0.2% w/v SL, and 0.6% v/v WGHE were selected based on experimental testing. Then, the effect of changes in their concentrations was evaluated assuming that the other factors remained constant. Since phospholipids are susceptible to hydrolysis under strongly acidic conditions, whereas alkaline pH conditions are rarely used in the food and pharmaceutical industry, pH 7 was selected as a starting point to formulate the liposomal systems.[35]

Effect of Phosphatidylcholine Concentration

The concentration of PC is the most important factor in the formation of liposomes. The lowest and highest concentration of PC was selected based on the minimum concentration necessary to form liposomes and the maximum concentration necessary to achieve the highest loading of the extract, respectively. The visual appearance, z-average particle size, PDI, and ζ-potential of unloaded liposomes, and loaded liposomes without SL are shown in Figure 3.

Figure 3. Effect of phosphatidylcholine concentration (0–2% w/v) on the visual appearance, average particle size, polydispersity index, and ζ-potential of liposomes that either contained 0.0 (i.e., unloaded) or 0.6% (v/v) ~~WGHE~~walnut green husk extract (i.e., loaded liposomes). Different letters for each parameter show significant differences at $p < .05$.



Loaded and unloaded liposomes were formed for all selected concentrations of PC. However, concentrations of 0.45, 1, and 2% (w/v) PC showed an increased turbidity with a pronounced sediment, indicating the aggregation and formation of larger vesicles. In general, a smaller size, lower PDI (<0.3), and higher absolute value of the ζ-potential induce a more uniform system with greater repulsion between the liposomes and thus a longer stability.[36] Stable liposomes were formed at PC concentrations of 0.15 and 0.3% (w/v) in the absence of anionic surfactant. The z-average size in unloaded liposomes was 97.3 nm for 0.15% (w/v) and 125.6 nm for 0.3% (w/v) PC, whereas the ζ-potential was –2.6 mV and –2.7 mV, respectively. The loaded liposomes were smaller (70.9 nm and 121.7 nm) and exhibited a slightly more negative ζ-potential of about –3.7 mV and –4.4 mV. A similar trend was observed by Marín et al. (2018) for pomegranate (Punica granatum) peel and albedo encapsulated in soy phosphatidylcholine liposomes.[37]

Since the encapsulation efficiency was less than 40% at PC concentrations of 0.15 and 0.3% (w/v), whereas pronounced aggregation and sedimentation of the liposomes occurred at higher PC content, the use of a stabilizer to

increase the stability of the liposomes was necessary. One of the suggested ways in this field is to increase the electrostatic repulsion between the liposomes by adding a charge inducer. SL with a concentration of 0.2% (w/v) was used to induce a negative charge on the surface of the liposomes as it is a food-grade additive.

Effect of Sodium Laurate Concentration

As shown in Figure 3, at a concentration of at least 0.45% (w/v) of (zwitterionic) PC, liposomes aggregated and sedimented. Therefore, it was essential to improve the stability of the PC-liposomes. Different methods either for inducing steric or electrostatic repulsion, such as adding cholesterol, nonionic surfactants (tweens, spans or poloxamers), charge-inducing agents (cationic or anionic surfactants and polycationic compounds such as stearylamine, dicetylphosphate, and chitosan), or polymers (carbomers) have been reported in previous studies to prevent fusion of liposomes and to increase their stability.[38–40] In this respect, the use of charge-inducing agents is the simplest solution. The effect of addition of SL at different concentrations of 0.05, 0.1, and 0.2% (w/v) was investigated. At 0.05 and 0.1% (w/v) SL, relatively stable dispersions were achieved with a translucent appearance. However, an improved stability was achieved at 0.2% (w/v): the appearance was transparent, indicating the small size and stability of the liposomal formulation. Table 3 clearly indicates that the z-average size decreased with increasing SL concentration. Moreover, the PDI decreased from 0.9 to 0.4 with increasing SL concentration, and its change was statistically significant, which indicates a more uniform size distribution (Table 3, Figure 4).

Figure 4. Effect of sodium laurate concentration (0.05–0.2% w/v) on the visual appearance of liposomes that contained 2% (w/v) ~~phosphatidylcholinePC~~phosphatidylcholine and 0.6% (v/v) ~~WGHE~~walnut green husk extract.

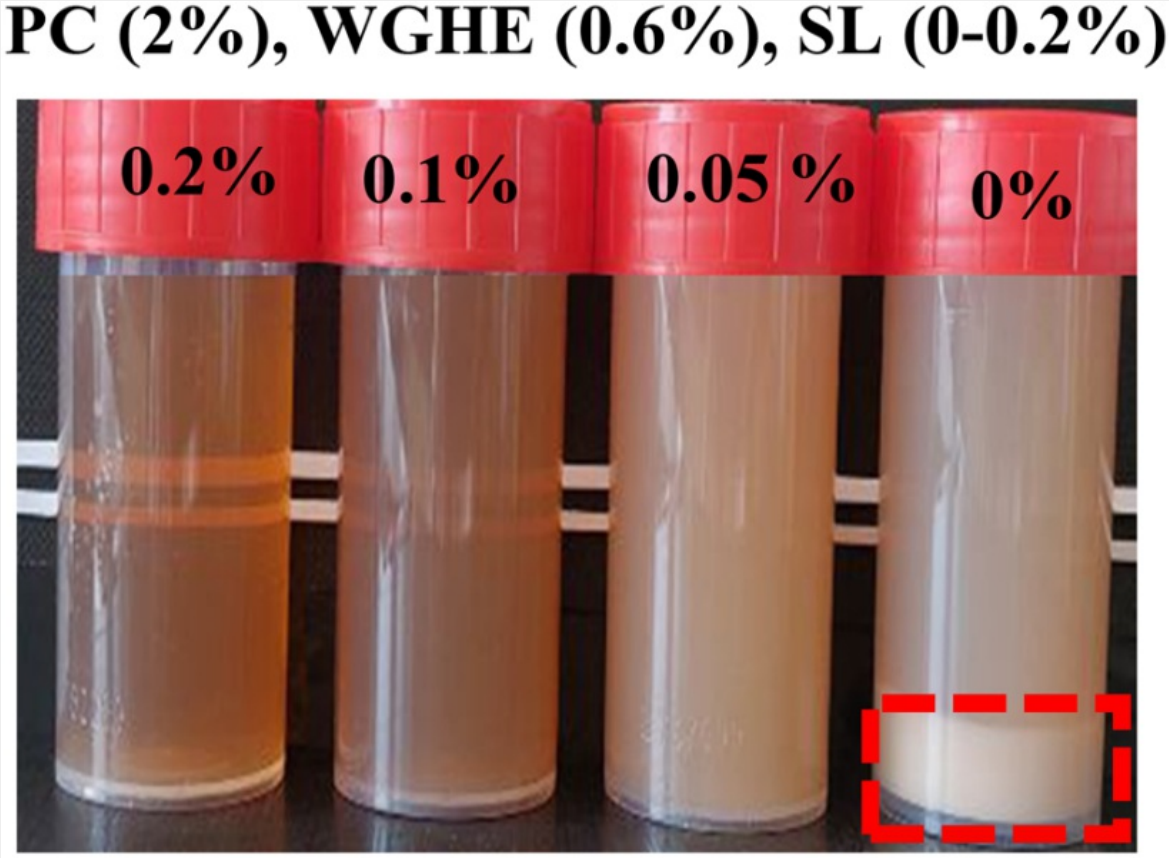


Table 3. Effect of sodium laurate concentration on average size, polydispersity index, and ζ-potential of liposomes in dispersions containing 0.15 to 2% (w/v) **of** ~~PC~~phosphatidylcholine

PC (%)	Sodium laurate (%)	Average size (nm)	Polydispersity index (–)	ζ-potential (mV)
0.15	0.2	–	–	–
0.3		–	–	–
0.45		216.2[a] ± 9.2	0.3[c] ± 0.0	–55.2[a] ± 0.9

1		90.9^{d} ± 0.5	0.3^{c} ± 0.0	–29.7^{c} ± 0.3
2		97.5^{c} ± 0.9	0.5^{b} ± 0.0	–39.8^{b} ± 0.9
	0.1	108.8^{b} ± 1.1	0.5^{b} ± 0.0	–20.9^{d} ± 1.2
	0.05	220.7^{a} ± 6.9	0.9^{a} ± 0.1	–40.2^{b} ± 0.6
Note: Different superscript letters show significant differences at $p < .05$.				

Completely stable liposomes were formed at PC concentrations of 0.45, 1, and 2% (w/v), in combination with 0.2% (w/v) SL. This effect follows logically from the strongly increased (negative) surface charge density, as can be clearly deduced from the roughly tenfold increase in ζ-potential. The increased surface charge density induced by sodium laurate enhances the electrostatic repulsion and hence minimizes the collision between liposomes. Moreover, the added sodium laurate, apart from inducing electrostatic repulsion, might also intercalate within the fatty acid chains of the phospholipid membrane and hence contribute to a more closely packed bilayer.

Despite our expectation, at concentrations of 0.15 and 0.3% (w/v) of PC, liposomes were formed in the first hours, but after that, SL and PC became separated, especially at the lowest PC concentration, that is 0.15% (w/v). The z-average diameter of the liposomes decreased with increasing PC concentration from 0.45% (w/v) and was in the range of 90.5 to 97.5 nm. The absolute value of the ζ-potential of the liposomes was at least 30 mV at a PC content of 0.45–2%, which is typically considered to give rise to stable dispersions. The instability of the liposomes at low concentrations of PC (i.e., 0.15 and 0.30%) can be attributed to the limited solubility or dispersibility of SL in water (as illustrated by Figure S1), which led to the formation of a precipitate. However, as the PC content increased, the SL molecules became more likely to solubilize within the liposomes.

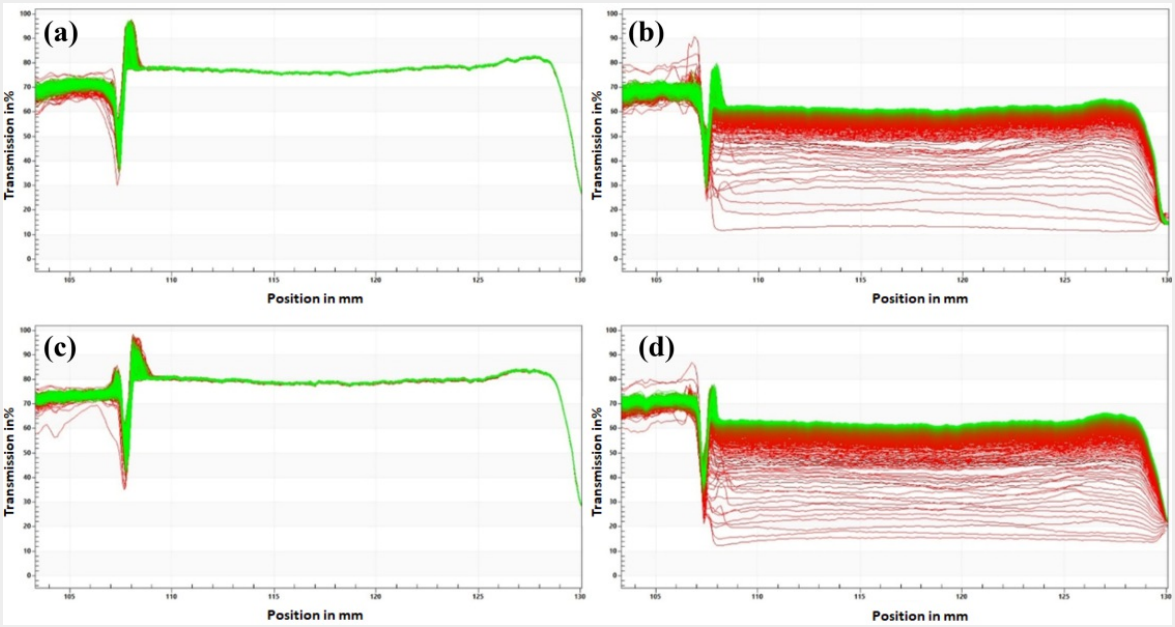
At pH 7.0, the anionic SL became incorporated into the liposomes, thereby increasing their negative charge density.[35] This effect is evident in our results from the considerably larger absolute value of the ζ-potential in the presence of SL. Additionally, the ~~polydispersity index (~~PDI~~)~~ reflects the width of the particle size distribution of the vesicles. Table 3 clearly indicates lower PDI values upon increasing the SL content (at a fixed PC concentration of 2%), indicating a higher homogeneity.

Physical Stability Analyzed by Centrifugal Photosedimentometry

The stability of the liposomes was further tested using centrifugal photosedimentometry. This technique is used to evaluate the physical instability of encapsulation systems (i.e., creaming or sedimentation) under intensified conditions, that is in a centrifugal field.[30] An overview of the different transmission profiles is provided in Figure 5. The profiles show the light transmission as a function of the radial position from the centrifugation axis, that is from the top (at a radial distance of about 108 mm) to the bottom of the sample (at a radial distance of about 129 mm) upon centrifugation in polycarbonate tubes. In the case of sedimentation, the transmission increases as a function of centrifugation time at the top and in the middle of the tube. Hereby, the time evolution is indicated by colors changing from red (at the beginning of the experiment) to green (at the end of the experiment). In Figure 5a and 5c, all profiles nearly overlap, indicating hardly any changes in transmission (e.g., due to sedimentation or creaming) during the whole centrifugation procedure. However, in Figure 5b and 5d, the profiles clearly change with time, which indicates rapid movement and sedimentation of liposomes to the bottom of the tube. Cheng et al.[11] also reported that liposomes

prepared with phospholipids are unstable and require the use of an additional compound to modify the structure of the wall materials. It is clear from Figure 5a and 5c that the sample with the optimal formula was stable at the maximum rotational speed of the apparatus during the complete cycle of approximately 12 hours, irrespective of the presence of the extract, which means that sedimentation or creaming did not occur. In addition, similar SL-free formulated samples (containing 2% (w/v) PC in the absence or presence of 0.6% (v/v) WGHE) were investigated as well, which showed pronounced instability (Figure 5b and 5d).

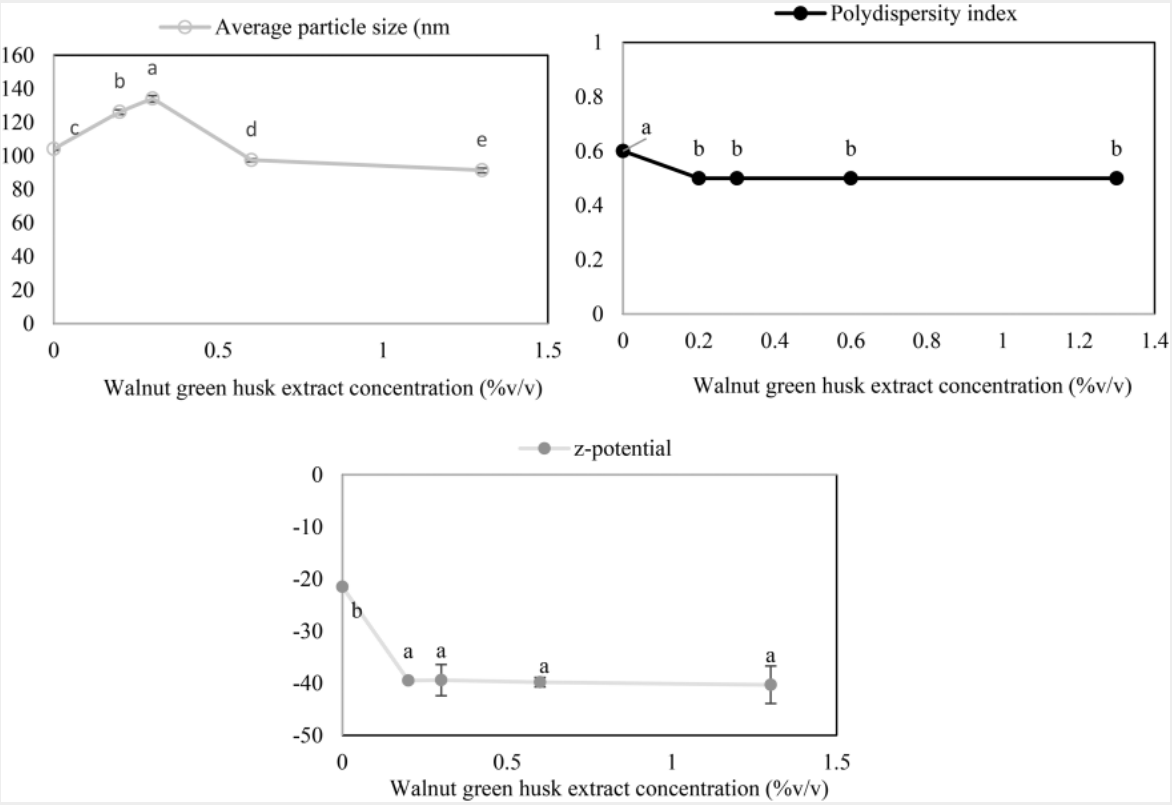
Figure 5. Transmission profiles of loaded liposomes with 0.6% (v/v) ~~WGHE~~walnut green husk extract (a, b) and unloaded liposomes (c, d) in the absence (b, d) or presence of 0.2% (w/v) sodium laurate (a, c). These investigations were carried out using a fixed concentration of 2% (w/v) ~~PC~~phosphatidylcholine.



Effect of Extract Concentration

The effect of the concentration of walnut green husk phenolic-rich extract was investigated within the 0.2 to 1.3% (v/v) range as another key factor. As the concentration of WGHE increased, the color intensity of the liposomal dispersion increased appreciably. The size and ζ-potential were also studied, and the results showed some dependence on the WGHE concentration (Figure 6). The z-average size of the liposomes decreased after loading the extract from 134.2 ± 1.9, over 97.5 ± 0.9, to 91.4 ± 1.4 nm for liposomes loaded with 0.3, 0.6, and 1.3% (v/v) of extract, respectively. The ζ-potential in liposomes containing the extract was more negative than that of the unloaded liposomes. This indicated that the presence of phenolics and their interaction with phospholipids increased the negative charge of the liposomes and hence their ζ-potential and electrophoretic mobility.[41]

Figure 6. Effect of walnut green husk extract concentration (0–1.3% v/v) on average particle size, polydispersity index, and ζ-potential of liposomes in a dispersion containing 2.0% (w/v) ~~PC~~phosphatidylcholine and 0.2% (w/v) ~~SL~~sodium laurate. Different letters for each parameter show significant differences at $p < .05$.



Encapsulation Efficiency

Based on the obtained results in 'Physical stability analyzed by centrifugal photosedimentometry' section, concentrations of 2.0 and 0.2% (w/v) of PC and SL were selected for the formation of liposomes. The encapsulation efficiency at increasing concentrations of WGHE was 70.0 ± 1.0, 79.7 ± 0.7, and 78.5 ± 0.5% at 0.3, 0.6, and 1.3% (v/v) WGHE, respectively. For completeness, it can be added that the encapsulation efficiency of a blank experiment (i.e., liposomes without any loaded WGHE) was verified to be zero, indicating that the liposomal components did not contain phenolic compounds. By increasing the amount of extract, the encapsulation efficiency of the liposomes increased at first. There was, however, no significant difference between the EE at 0.6 and 1.3% WGHE. This indicated a very effective incorporation of WGHE at around 1.3% inside the phospholipid bilayers. Cheng et al.[11] reported a decrease in encapsulation efficiency resulting from the saturation of the hydrophobic domains of PC with the extract. On the other hand, the encapsulation efficiency of the liposomal dispersion containing 1% PC and 0.6% WGHE was significantly lower (70.1 ± 1.2%) than in the presence of 2% PC. In general, the encapsulation efficiency varies depending on the technique used to prepare the liposomes, the composition of the coating materials, and the

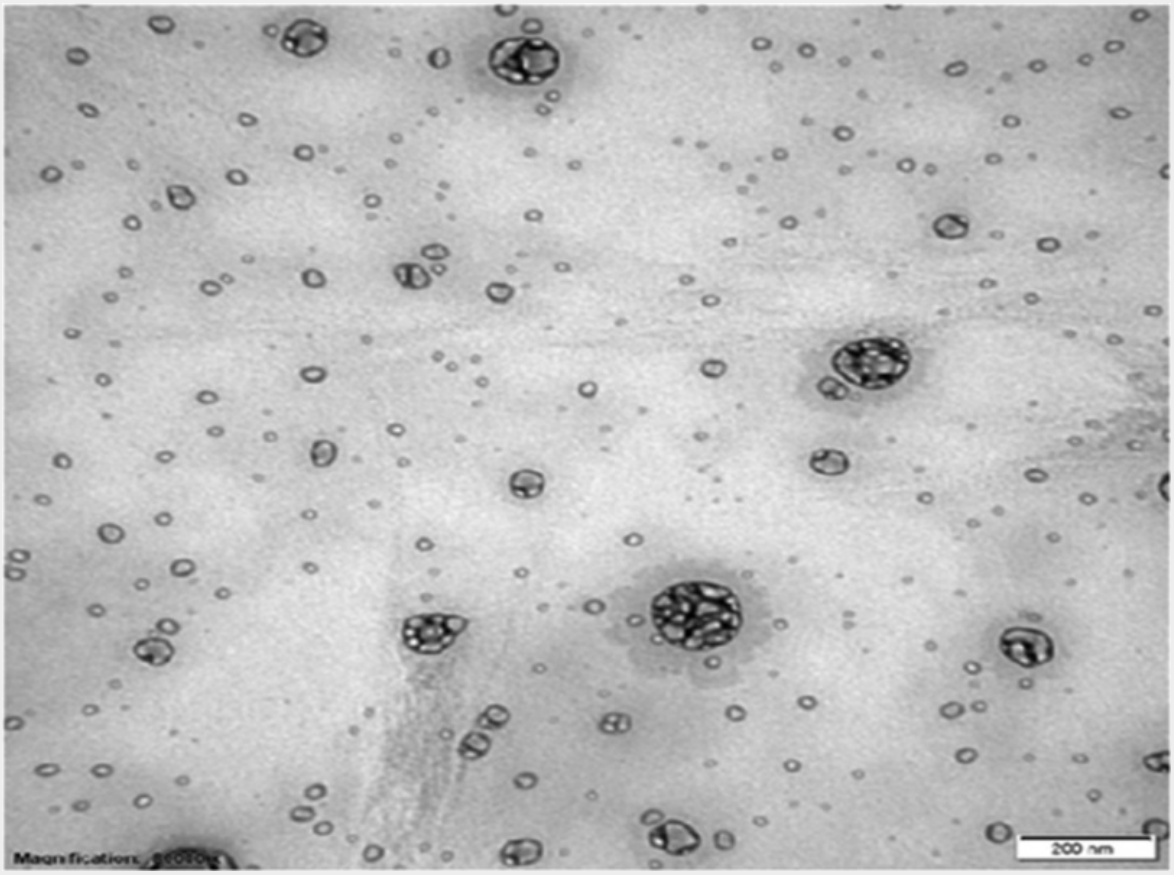
nature of the extract used for encapsulation.[12,36] Machado et al.[12] studied reverse phase evaporation to encapsulate phenolic extracts of Spirulina and obtained encapsulation efficiency values of 97.4 and 88.3% for the PC of rice and soybean, respectively.

In summary, also considering economic aspects, the concentration of 0.6% (v/v) WGHE was chosen to achieve a high encapsulation efficiency with minimal extract consumption.

Transmission Electron Microscopy

Considering all previous outcomes, the formulation containing 0.6% (v/v) WGHE, 2% (w/v) PC and 0.2% (w/v) SL was selected as the optimum for further analyses. A microstructural visualization was performed at first to study the liposomes morphology. TEM revealed that the majority of the liposomes were unilamellar spherical vesicles smaller than 100 nm in size, although some aggregation was observed (Figure 7). The latter might, however, (at least partly) also result from the inherent drying step that is needed during sample preparation. In fact, liposomes can encapsulate both hydrophilic (in the enclosed aqueous phase) and lipophilic substances (in their membranes).[7,10] According to the reported partition coefficients of the main components of WGHE, they are expected to be mostly encapsulated into the membranes.

Figure 7. Transmission electron microscopy of loaded liposomes with the optimum formulation (i.e., 2% (w/v) phosphatidylcholine, 0.6% (v/v) walnut green husk extract, and 0.2% (w/v) sodium laurate) after 100 times dilution with distilled water (at 80,000 × magnification).

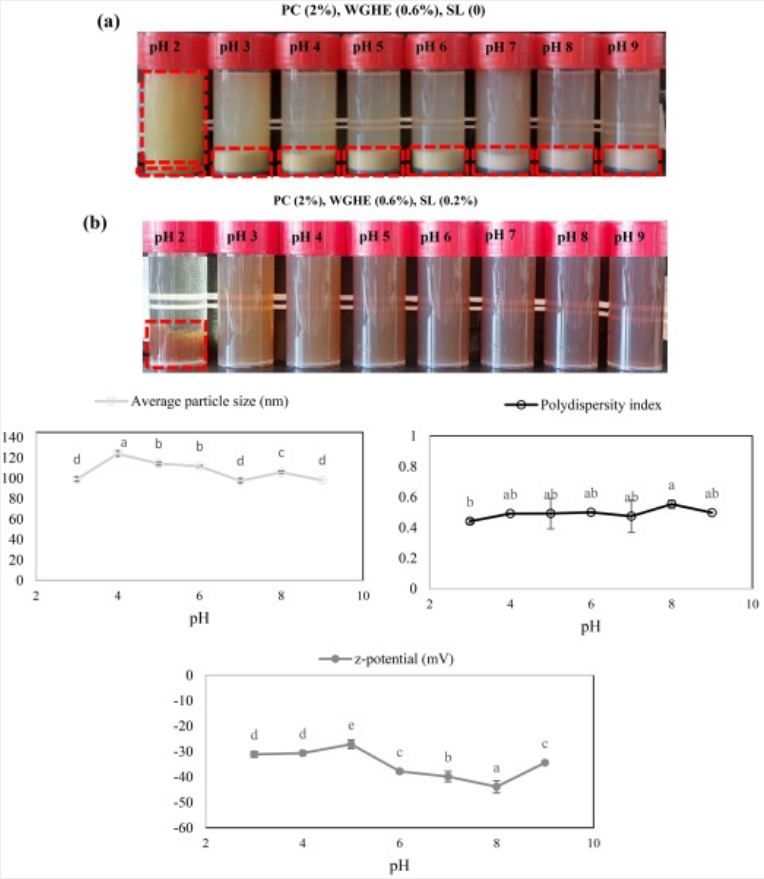


Stability of Liposomes

Effect of pH

WGHE-loaded liposomes in the absence of SL were incubated at different acidic and alkaline conditions (pH 2–9) for 24 hours, and then their visual appearance, average particle size, and ζ-potential were determined. As can be seen in Figure 8a, sedimentation occurred at all investigated pH conditions. As the pH was reduced, the turbidity in the supernatant increased which can be assigned to increased protonation of the phosphate group, thus converting zwitterionic PC (at neutral pH conditions) to its cationic equivalent. The increased positive surface charge stabilized the formed aggregates by electrostatic repulsion.

Figure 8. Effect of acidic and alkaline conditions (pH 2–9) on the visual appearance, average particle size, polydispersity index, and ζ-potential of loaded liposomes (with 2% (w/v) ~~PC~~phosphatidylcholine, and 0.6% (v/v) ~~WGHE~~ walnut green husk extract) formulated (a) without, and (b) with 0.2% (w/v) sodium laureate. Different letters for each parameter show significant differences at $p < .05$.



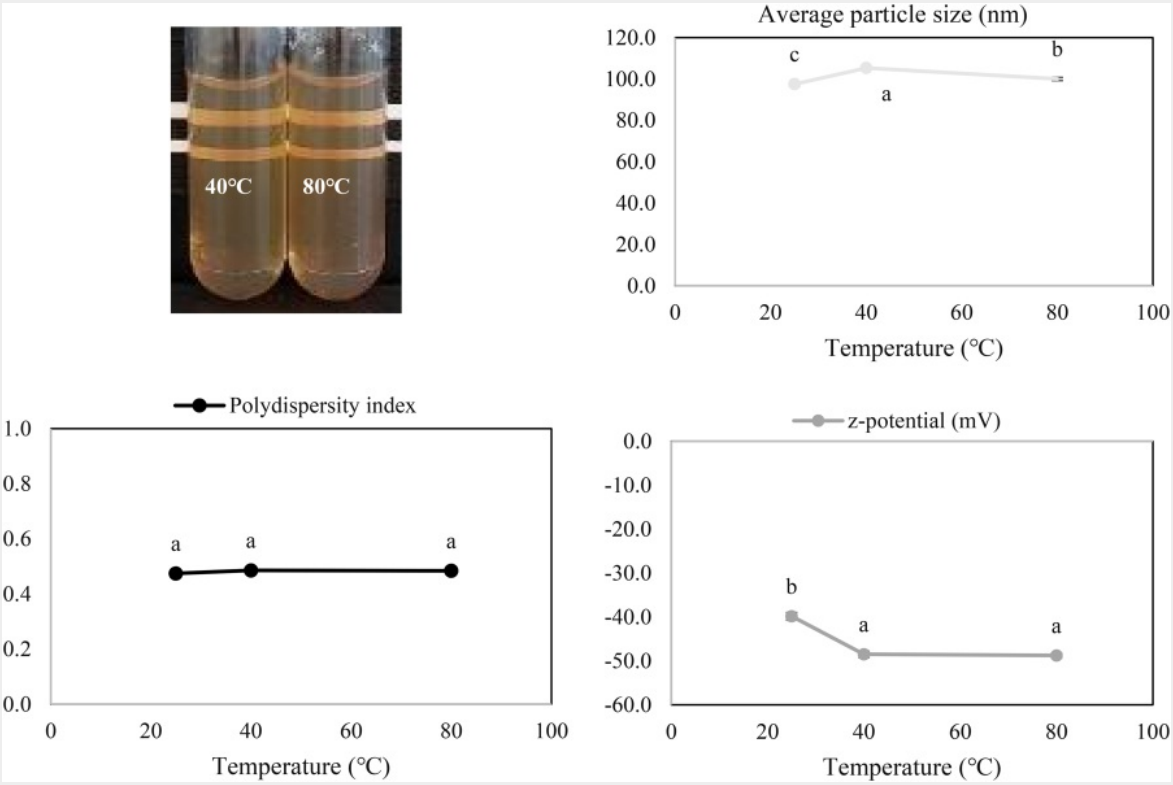
In the liposomal formulation with 2% (w/v) PC and 0.6% (v/v) WGHE in the presence of 0.2% SL, the liposomes were completely stable at all pH conditions, except pH 2. At pH 2, the liposomes precipitated. This happened most probably because of the decreased surface charge effect whereby the anionic charge of the vesicles at low pH became neutralized by protonation of the SL carboxylic groups as well as the PC phosphate groups. Additionally, it has been reported in a previous study that due to the presence of ester bonds, PC is very sensitive to strongly acidic pH conditions, whereby chemical degradation may happen. Consequently, the acid-induced hydrolysis of phosphatidylcholine may have caused the precipitation and leakage of the phenolic extract. Pu et al. also observed that extreme pH conditions, such as pH 2, can disrupt the lipid bilayer structure of liposomes, leading to the leakage of encapsulated contents and compromising their integrity.[35] Based on the measurement of extract release and the information illustrated in Figure 8b, the majority of the extracts was released from the liposomes at pH 2. The pH also influenced the mean size and ζ-potential, and the results are shown in Figure [Figure 8b] 8. The average size of the

particles at all investigated pH conditions was smaller than 130 nm, and the lowest and highest size were related to pH 7 and 4, respectively. SL induced a negative charge on the surface of the liposomes, and the resulting electrostatic repulsion increased as the pH increased. This effect may be explained from the fact that SL becomes less negatively charged as the pH is decreased from 9 to 2, as this pH reduction results in pH conditions steadily approaching the fatty acid pKa (which is the pH where 50% dissociation takes place). For completeness, it can be mentioned that an aqueous dispersion of SL and the extract in the absence of PC had a ζ-potential of about –62.3 mV (data not shown), whereby significant light scattering, and precipitation occurred. The increase in the absolute value of the ζ-potential from –25 mV at pH 3 to –46 mV at pH 9 indicated more electrostatic repulsion. The PDI in most of the examined samples was around 0.5, independent from the pH, which indicated a relatively uniform size distribution.

Effect of Temperature

The average particle size, PDI, and ζ-potential of the liposomes as a function of the incubation temperature (during 30 minutes) are shown in Figure 9. After 30 minutes heat treatment at either 40 or 80 °C, the liposomes remained structurally stable. The heated liposomes had a mean diameter of 100–105 nm, with a PDI of about 0.5, and a zeta-potential of –49 mV. These particle size and ζ-potential values were higher as compared to the untreated sample (i.e., kept at 25 °C). The increase in ζ-potential was probably due to the better incorporation of SL within the PC bilayers during heating. Overall, these results indicated that the formulated liposomal system had good stability against heat. However, since the encapsulation efficiency of the WGHE was only about 80%, there is still a possibility of degradation of the fraction of the WGHE that was not encapsulated.

Figure 9. Effect of heating temperatures (25–80 °C for 30 minutes) on the visual appearance, average particle size, polydispersity index, and ζ-potential of liposomes with optimum formulation (2% (w/v) phosphatidylcholine, 0.6% (v/v) walnut green husk extract, 0.2% (w/v) sodium laurate). Different letters for each parameter show significant differences at $p < .05$.

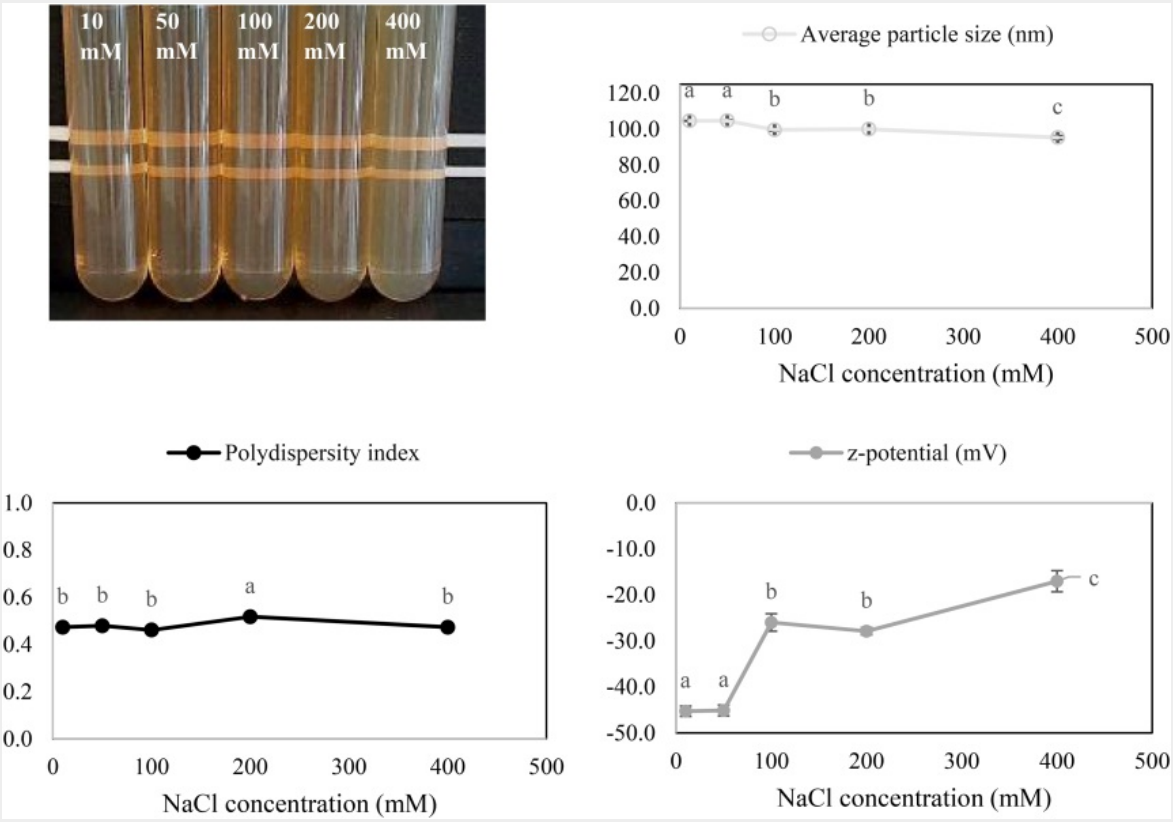


Effect of Ionic Strength

The ionic strength of the surrounding medium may have a strong influence on the stability of the liposomes.[11] The addition of sodium chloride at different concentrations from 10 to 400 mM after 3 days of incubation did not affect the stability and visual appearance of the liposomal dispersions. The ζ-potential, on the other hand, became less negative under the influence of the increased salinity, which is fully in line with the electric double-layer theory (as described by the Grahame equation). However, the increased shielding of the electrostatic repulsion at higher ionic strength did not give rise to an increased size of the particles: in fact, the z-average size decreased slightly, which may be due to the elevated outside osmotic pressure at the highest salt levels,[42] whereas the PDI of the samples remained constant (Figure 10). A decrease in size and ζ-potential with increasing ionic strength has also been reported in previous studies. Cheng et al.[11] also studied the effect of an increasing salinity from 10 to 400 mM: at the highest salt concentration studied, the turbidity of liposomes incorporated with curcumin increased significantly and sedimentation occurred. This instability and liposomal aggregation occurred due to a decrease in electrostatic repulsion on the surface. To overcome

this problem, Cheng et al.[11] modified the structure of the PC-coated liposomes using rhamnolipids. These modified liposomes were more stable, and sedimentation did not occur. However, in terms of the appearance of the samples, they became more opaque. On the other hand, the particle size also decreased slightly at the highest sodium chloride concentration used.

Figure 10. Effect of ionic strength (10–400 mM) on the visual appearance, average particle size, polydispersity index, and ζ-potential of liposomes with optimum formulation (2% (w/v) phosphatidylcholine, 0.6% (v/v) walnut green husk extract, 0.2% (w/v) sodium laurate). Different letters for each parameter show significant differences at $p < .05$.



Conclusions

This study aimed to determine the optimal concentration of constituents in a liposomal system for encapsulating a phenolic-rich extract from green walnut husks, ensuring a high stability. Phenolics often face limitations such as instability, degradation, low bioactivity, and poor solubility. Unlike previous studies,[5,7] which focused on phenolic encapsulation, we investigated the challenges of direct phenolic use from green walnut husk. Without encapsulation, the extract exhibited significant limitations, including a decreased phenolic content, unwanted visual changes during storage, and susceptibility to alkaline pH and heat. Unlike studies using pure phenolics or specific extracts,[17–19] we employed a

phenolic mixture. Natural phosphatidylcholine was chosen over synthetic alternatives for its superior properties.[8] Our innovative approach involved the addition of sodium laurate, which played a crucial role in imparting surface charges to enhance the electrostatic stabilization of the liposomes, effectively preventing aggregation and precipitation. Encapsulation into small liposomes was achieved through a combination of ethanol injection and sonication in the presence of phosphatidylcholine and sodium laurate. The selected optimal concentrations for phosphatidylcholine, sodium laurate, and extract were 2% (w/v), 0.2% (w/v), and 0.6% (v/v), respectively. The resulting liposomes were nano-sized spherical unilamellar vesicles with an approximate diameter of 100 nm, a ~~zeta~~ζ-potential of –30 to –40 mV, and a polydispersity index of 0.4. These vesicles demonstrated an excellent stability under various stress conditions. Our findings hold significant value for the encapsulation of other natural phenolic mixtures in liposomal dispersions.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

All data generated or analyzed during this study are included in this article.

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