

THE CHARACTERISATION OF SILYMARIN AND SILIBININ LOADED LIPOSOMES

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Abstract

The aim of the present study was the characterization of silymarin and silibinin loaded liposomes *via* determination of encapsulation efficiency, particle size, polydispersity index (PDI), zeta potential, mobility, and conductivity, as well as storage stability during 28 days at 4°C and stability after UV irradiation. Encapsulation efficiency of silymarin and silibinin were 92.05±1.41% and 87.86±2.06%, respectively. Particle size and PDI of the liposomes with silymarin were changed from 3541.3±62.5 nm to 2677.0±44.2 nm and from 0.346±0.044 to 0.228±0.036, respectively, during the 28-days stability study; particle size and PDI of the liposomes with silibinin were changed from 2074.7±19.4 nm to 2704.0±35.0 nm and from 0.328±0.030 to 0.456±0.026, respectively. Zeta potential of the silymarin-liposomes and silibinin-liposomes was changed from -27.0±0.7 mV to -26.4±0.4 mV and from -29.4±0.6 mV to -29.0±0.4 mV, respectively. Mobility and conductivity of the liposomes with silymarin were changed from -2.120±0.057 μmcm/Vs to -2.067±0.028 μmcm/Vs and from 0.017±0.005 mS/cm to 0.009±0.004 mS/cm, respectively. Mobility and conductivity of the liposomal particles with silibinin were changed from -2.307±0.053 μmcm/Vs to -2.110±0.033 μmcm/Vs and from 0.018±0.003 mS/cm to 0.060±0.001 mS/cm, respectively. UV irradiation did not affect particle size and PDI of all liposomes, but it caused a decrease in zeta potential: -23.9±0.8 mV for silymarin and -24.5±0.7 mV for silibinin, in mobility: -1.874±0.064 μmcm/Vs for silymarin and -1.920±0.057 μmcm/Vs for silibinin, and in conductivity: 0.014±0.001 mS/cm for silymarin and 0.007±0.003 mS/cm for silibinin. Overall, the obtained results qualify liposomes to be used as silymarin and silibinin carriers for application in functional foods and pharmaceutical products.

Keywords: silymarin, silibinin, liposomes, characterization.

Introduction

Silymarin represents the group of bioactive polyphenol compounds from milk thistle (*Silybum marianum*) and contains silibinin, isosilybin, silydianin, and silychristand. Silibinin is the most prevalent component (Zhang et al., 2022). According to previous studies, these polyphenolic compounds exhibit various biological activities that may promote human health and wellbeing, such as antioxidant, antimicrobial, anti-inflammatory (Zhang et al., 2022), antiviral (Liu et al., 2019), immunomodulatory (Zhao & Li, 2015), and anticancer properties (Ahmad et al., 2018). Nevertheless, silymarin and silibinin are quite sensitive to temperature, light, and oxidation, as well as have poor water solubility and low bioavailability, thus, their application in food, pharmaceutical, and cosmetic formulation is limited (Zhang et al., 2022).

Silibinin, the flavonolignan, is the major active constituent of silymarin, a standardized extract of the milk thistle seeds mentioned above (Sara et al., 2021; Zhang et al., 2022). Silibinin was traditionally used as a chemopreventive and therapeutic agent in human lung cancer (Sara et al., 2021). Song et al. (2022) suggest that silibinin has hepatoprotective activity through the protection of liver cells against toxins. According to the literature, silibinin can also inhibit amyloid beta aggregation by affecting the human islet amyloid polypeptide (García-Viñuales et al., 2022). However, its application is limited due to poor water solubility, limited intestinal resorption, and consequently low bioavailability (Mohammadi et al., 2022).

With the aim to overcome the disadvantages of bioactive components, numerous encapsulation techniques have been established (Jovanović et al., 2018; Kalušević et al., 2022; Zhang et al., 2022). The emphasis is on the protection of the target substances, the increase of oral or transdermal bioavailability, as well as the controlled release of the active molecules (Jovanović et al., 2018). Furthermore, the utilization of various carriers to improve the water-dispersibility, chemical stability, and bioavailability of silymarin and silibinin, and consequently increase its implementation within functional foods, supplements, pharmaceuticals, and cosmetics is examined (Zhang et al., 2022). Liposomes are non-toxic, biodegradable, and biocompatible lipid micro- or nano-carriers with one or more phospholipids bilayers. Liposomes, as lipophilic and hydrophilic drugs delivery systems, can provide controlled delivery of bioactive components, as well as their protection from degradation caused by light, oxygen, UV irradiation, different pH values, and enzymes (Jovanović et al., 2019; Zhang et al., 2022). The main advantage of the mentioned encapsulation technology is the stability that liposomes provide in various food, pharmaceutical, and cosmetic products with a high amount of water (Isailović et al., 2013). In addition, lipids from the liposomes do not provoke a reaction with taste receptors, therefore liposomal bilayer is an appropriate carrier for covering the unpleasant taste of numerous polyphenols (Jovanović et al., 2019).

Liposomes can be produced using the common thin film hydration method or proliposome method (Isailović et al., 2013; Jovanović et al., 2019). The common thin film hydration procedure is considered unsuitable for producing liposomes on an industrial scale, whereas the proliposome technique may be suitable for liposome production on a large scale (Isailović et al., 2013). Therefore, in the present research, silymarin and silibinin loaded liposomes were prepared using proliposomes procedures and analyzed in terms of encapsulation efficiency, particle size, polydispersity index (PDI), zeta potential, mobility, and conductivity, as well as storage stability during 28 days at 4°C and stability after UV irradiation.

Materials and Methods

Materials

Phospholipon 90G (phosphatidylcholine from soybean) was supplied by Natterman Phospholipids (Germany). The following reagent and standards were used: ethanol (Fisher Scientific, UK), silymarin, and silibinin (Sigma-Aldrich, Germany).

Liposomes preparation

Liposomes with silymarin and silibinin were prepared using the proliposome method according to Isailović et al. (2013). In specific, a mixture of 1 g of phospholipids, 0.02 g of silymarin or silibinin, and 8 mL of ethanol was stirred and heated to 60°C for 10 min. After cooling to 25°C, 20 mL of distilled water was added in small portions. Subsequently, the mixture was stirred for 1 h at 800 rpm.

Determination of extraction efficiency

Free silymarin or silibinin were removed from liposome dispersions by centrifugation at 17,500 rpm for 45 min at 40C in a Thermo Scientific Sorval WX Ultra series ultracentrifuge (Thermo Scientific, Waltham, MA, USA). The amount of silymarin or silibinin in the supernatant was determined spectrophotometrically at 280 nm. Entrapment efficiency (EE%) was calculated as the content of silymarin or silibinin encapsulated in liposomal particles divided by the content of silymarin or silibinin used for the preparation of the liposomes bilayer: $EE (\%) = (m_i - m_s) / m_i \cdot 100$, where m_i is the initial amount of silymarin or silibinin used for the liposomal preparation and m_s is the amount of silymarin or silibinin determined in the supernatant.

Analysis of particle size, polydispersity index, zeta potential, mobility, and conductivity

The particle size, polydispersity index, zeta potential, mobility, and conductivity of the silymarin or silibinin loaded liposomes were determined by photon correlation spectroscopy in Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., UK). Each sample was measured three times at room temperature.

Storage and UV-irradiation stability of the liposomes

The particle size, polydispersity index, zeta potential, mobility, and conductivity of the silymarin or silibinin loaded liposomes were monitored for 28 days of storage at 40C (on 1st, 7th, 14th, 21st, and 28th day) and immediately after UV irradiation. UV irradiation was performed in a laminar flow cabinet (AC2-4G8, ESCo, Singapore). Namely, the liposomal sample (2 mL) was exposed to UV-C irradiation (253.7 nm) for 20 min at 250C in uncovered Petri dishes (Petrović et al., 2017; Yao et al., 2021). Subsequently, all measurements for physicochemical characterization were performed.

Results and discussion

Extraction efficiency in silymarin and silibinin loaded liposomes

In order to determine the silymarin and silibinin efficiency of encapsulation into liposomes, the concentration of silymarin or silibinin in the supernatant was quantified spectrophotometrically at 280 nm; the liposomes were separated from free silymarin and silibinin by centrifugation. The results are presented in Table 1. The encapsulation efficiency of silymarin and silibinin were $92.05 \pm 1.41\%$ and $87.86 \pm 2.06\%$, respectively. The obtained results are similar to the liposomes with resveratrol prepared using the proliposome method ($97.36 \pm 2.00\%$, Isailović et al., 2013), and in addition, significantly higher in comparison to the liposomes with gentisic acid ($\sim 54\%$, Jovanović et al., 2019). In the present study, the liposomes contain only phospholipids (without the addition of sterols), which makes their bilayer more rigid (Jovanović et al., 2018), consequently preventing leakage of silymarin and silibinin, and providing higher encapsulation efficiency. The liposomal membrane containing sterols (cholesterol, ergosterol, lanosterol, β -sitosterol, etc.) possesses higher permeability (Jovanović et al., 2018), and thus, encapsulation efficiency was lower, as in the case of previously mentioned gentisic acid (Jovanović et al., 2019).

Particle size, polydispersity index, zeta potential, mobility, and conductivity

Since the average size of liposomal particles represents an essential and relevant parameter for liposome stability, biodistribution, as well as for the release of encapsulated compounds (Mozafari et al., 2008) the measurement of the mentioned variable was performed. The results are presented in Figure 1A. Additionally, PDI, as a measure of the particle size distribution, was determined as well, and the results are presented as the values above the bars in Figure 1A.

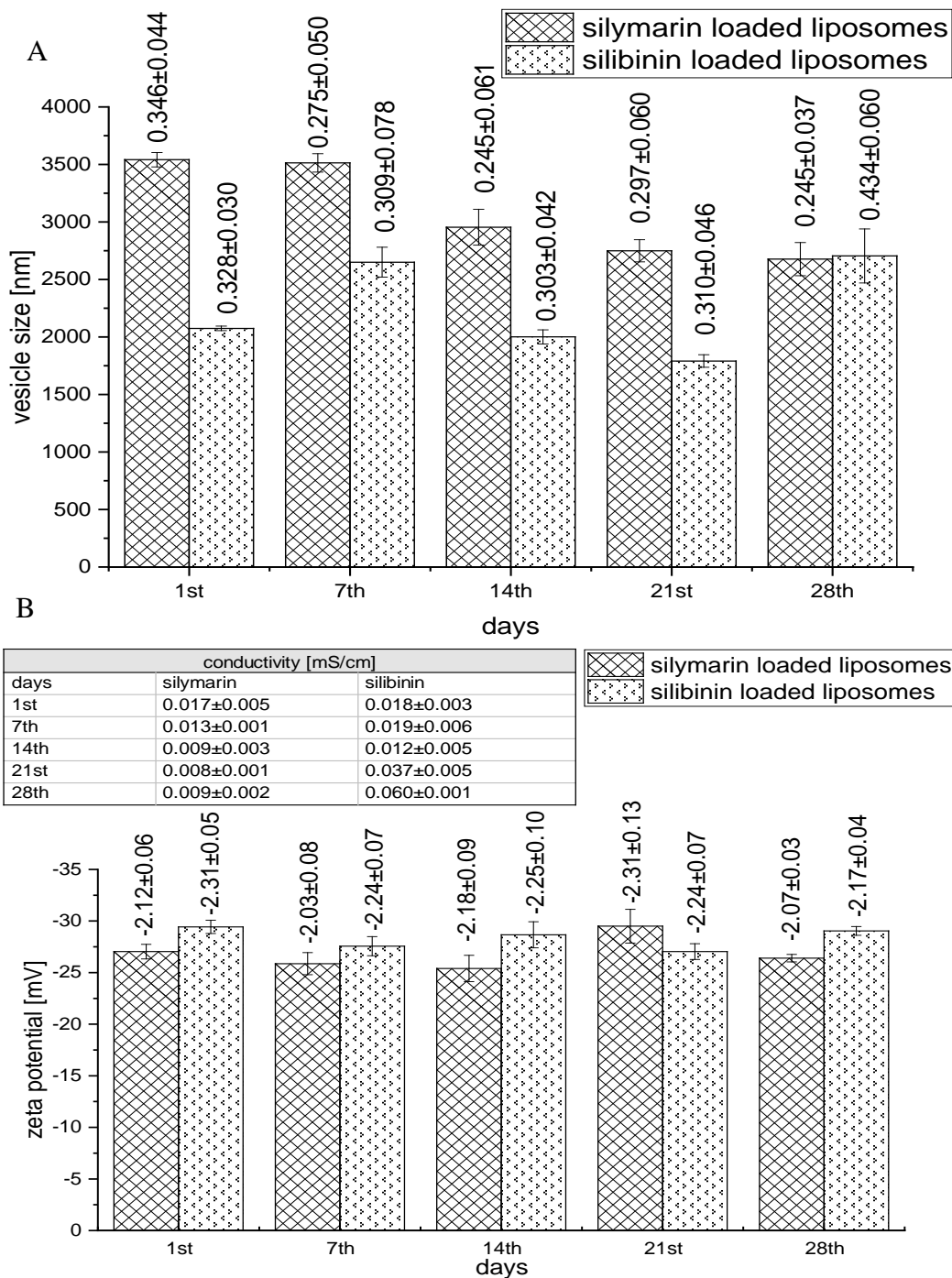


Figure 1. Particle size - bars and polydispersity index - numbers above bars (A) and zeta potential - bars, mobility - numbers above bars [$\mu\text{mcm/Vs}$], and conductivity - table (B) of silymarin and silibinin loaded liposomes, measured immediately after the liposomal preparation and for 28 days of storage at 40C.

The average size of the liposomal particles with silymarin was 3541.3 ± 62.5 nm, while particle size of the liposomes with silibinin was 2074.7 ± 19.4 nm (Figure 1A). The obtained values for liposome size are in agreement with the literature data, where pure phospholipids liposomes

(without the sterols) had diameter of 2974 ± 140 nm (Jovanović et al., 2018). It can be noticed that silymarin-liposomes had higher diameter in comparison to silibinin sample. The explanation can be in the fact that some of the compounds from silymarin are probably incorporated within the liposomal bilayer, which causes the formation of inter-lipid space and membrane expansion, and consequently the increase of liposomes size (Jovanović et al., 2018). Namely, particle size of the liposomes is significantly affected by the lipid composition, liposomal preparation technique, and the nature of the encapsulated substances (Isailović et al., 2013; Jovanović et al., 2018; Jovanović et al., 2019).

PDI for silymarin and silibinin loaded liposomes was similar, 0.346 ± 0.044 and 0.328 ± 0.030 , respectively (the values above the bars in Figure 1A). However, a single phospholipid, such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, provides better uniformity (PDI of ~ 0.1) compared to the mixture of phospholipids (such as Phospholipon 90G, commercial phospholipid mixture used in the present research). Namely, a single phospholipid eliminates the imperfect packing that can occur in the case of various hydrophobic fatty acyl chain lengths, head groups, and degrees of saturation present in the mixture (Jovanović et al., 2018). Nevertheless, the obtained values for PDI are acceptable from the point of view of further application of the liposomes. Additionally, according to Jovanović et al. (2018), higher liposomes (multilamellar vesicles, such as silymarin and silibinin loaded liposomes) possessed lower PDIs in comparison to smaller liposomes (small unilamellar vesicles). The applied technique for liposomal preparation also influenced uniformity of the system (Isailović et al., 2013; Jovanović et al., 2019). PDI values for gentisic acid loaded liposomal samples prepared using thin film method were higher than in the case of silymarin and silibinin liposomes where proliposome methods were used (0.4 and 0.5, Jovanović et al., 2019). Isailović et al. (2013) have reported that resveratrol loaded liposomes produced by proliposome method had PDI of ~ 0.2 , while PDI of the same sample prepared in the thin film method was ~ 0.4 .

According to the literature data, in water surrounding, phosphatidylcholines are neutral lipids. However, the reorientation groups belong to the lipid heads cause the presence of a surface charge, which depend on the phase state and types of the lipids (Jovanović et al., 2018). Thus, the zeta potential (as a measure of system stability) of the obtained silymarin and silibinin loaded liposomes was examined and the results are presented in Figure 1B. The results of the liposomes mobility are presented as the values above the bars, while conductivity values are presented in the tables, within Figure 1B.

The zeta potential of the silymarin-liposomes was -27.0 ± 0.7 mV, whereas the zeta potential was -29.4 ± 0.6 mV for silibinin-liposomes (Figure 1B). The negative values of zeta potential are related to the exposure of the phosphate group lying in an outer plane concerning the choline groups (Jovanović et al., 2019). The obtained results of zeta potential are in agreement with the literature data, where the liposomes with resveratrol prepared using proliposome technique had zeta potential of ~ -25 mV (Isailović et al., 2013). The zeta potential of silibinin-liposomes was slightly higher in comparison to silymarin-liposomes, due to the changes in the space between head group of phospholipids within the bilayer membrane (Jovanović et al., 2019).

According to the literature, conductivity represents an indicator of total dissolved compounds and a predictor of the antioxidant capacity of a sample as well (Suliman et al., 2015). Further, the number of ions per unit volume and their drift velocity affect a liquid's electrical conductivity. An ion's drift velocity changes depending on the strength of the electric field, the ion's mass, the temperature of the solution, as well as on other variables. Thus, the electrical conductivity of various liquids may thus be anticipated to have a wide range of values (Rhoades et al., 1976).

The conductivity of the liposomes with silymarin and silibinin immediately after the preparation was 0.017 ± 0.005 and 0.018 ± 0.003 mS/cm, respectively, while mobility was -2.12 ± 0.06 and -2.31 ± 0.05 (Figure 1B). According to Fathi-Azarbay et al. (2009), higher capture volume corresponds to a decrease in conductivity. Lidgate et al. (1993) have also reported that greater lipid concentrations=higher capture volume=the effective removal of ions from the liposome dispersions=the reduction in conductivity. Indeed the liposomes containing silymarin and silibinin possessed a high concentration of lipids (50 mg/mL), showed higher encapsulation efficiency and had lower conductivity (Figure 1B). The mobility of liposomes is a function of vesicle size, zeta potential, and bilayer membrane composition (Duffy et al., 2001). Therefore, the obtained differences among various liposomal populations were expected. Namely, liposomes with lower zeta potential correspondingly possess lower mobility, which was the case with silymarin and silibinin loaded liposomes. Furthermore, some bilayer membranes are rigid, whereas others are high-permeable, flexible, and deformable, which depend on the composition of the bilayer, as well as the encapsulated compounds. The liposomes that have higher membrane fluidity also show better mobility. Since any changes in the liposome's mobility were attributed to the mechanical rigidity, or the ability of the liposomes to deform (Pysher and Hayes, 2004), it can be concluded that liposomes with silibinin (slightly higher mobility) were softer and more fluid (consequently lower extraction efficiency, Table 1) than liposomes with silymarin which exhibited lower mobility and probably higher rigidity (consequently higher extraction efficiency, Table 1). In addition, when flavonoids (among which are silymarin and silibinin) are adsorbed at the surface of the liposomes, it can reduce liposome mobility (Yang et al., 2015).

Storage stability of silymarin and silibinin loaded liposomes

The size of the liposomes, PDI, zeta potential, mobility, and conductivity of silymarin and silibinin loaded liposomes were measured on the 1st, 7th, 14th, 21st, and 28th days after preparation. As can be seen from Figure 1A, the particle size of the liposomes with silymarin was changed from 3541.3 ± 62.5 nm (1st day) to 2677.0 ± 44.2 nm (28th day) during the 28-day stability study, while PDI varied from 0.346 ± 0.044 to 0.228 ± 0.036 . The decrease in the diameter of silymarin-liposomes was detected on the 14th day of storage in refrigeration, to 2954.0 ± 55.9 nm and it continued to decrease continuously up to the 28th day. On the other hand, particle size and PDI of the liposomes with silibinin were changed from 2074.7 ± 19.4 nm (1st day) to 2704.0 ± 35.0 nm (28th day) and from 0.328 ± 0.030 to 0.456 ± 0.026 , respectively. The results presented in Figure 1A show that liposomes were physically stable during 28 days of storage, i.e. there was no occurrence of agglomeration or significant changes in uniformity of the liposomal system.

The zeta potential of the silymarin-liposomes did not change; it amounted -27.0 ± 0.7 mV (1st day) and -26.4 ± 0.4 mV (28th day). The zeta potential of silibinin-liposomes did not vary as well and was -29.4 ± 0.6 mV (1st day) and -29.0 ± 0.4 mV (28th day) (Figure 1B). The results of zeta potential presented in Figure 1B prove that liposomes were stable during 28 days of storage at 40C, i.e. there were no changes in values of zeta potential. Mobility and conductivity of the liposomes with silymarin were changed from -2.120 ± 0.057 $\mu\text{mcm/Vs}$ (1st day) to -2.067 ± 0.028 $\mu\text{mcm/Vs}$ (28th day) and from 0.017 ± 0.005 mS/cm (1st day) to 0.009 ± 0.004 mS/cm (28th day), respectively (Figure 1B). Mobility and conductivity of the liposomal particles with silibinin were changed from -2.307 ± 0.053 $\mu\text{mcm/Vs}$ (1st day) to -2.110 ± 0.033 $\mu\text{mcm/Vs}$ (28th day) and from 0.018 ± 0.003 mS/cm (1st day) to 0.060 ± 0.001 mS/cm (28th day), respectively (Figure 1B). The conductivity of silymarin-liposomes and silibinin-liposomes differs only between some measurements during the 28-day stability study, but values of conductivity are pretty low, thus

some differences are minor (Figures 1B). Additionally, conductivity was continuously decreased in silymarin-liposomes during the 28-day stability study, whereas in silibinin-liposomes, conductivity increased from the 1st to the 28th day. It can be explained by the fact that silibinin-liposomes are more fluid in comparison to silymarin-liposomes, therefore leakage of encapsulated compounds (i.e. silibinin) into the surrounding water medium can occur increasing conductivity (Lidgate et al., 1993). The interactions between liposomes, which depend among others on the concentration of liposomes, affect modifications in liposomal particles, as well as changes in mobility. Thus, liposome fusion or fission can cause a redistribution of phospholipids between liposomes and variations in mobility (Duffy et al., 2001).

UV irradiation stability of silymarin and silibinin loaded liposomes

UV irradiation stability of silymarin and silibinin loaded liposomes was examined by measuring vesicle size, PDI, zeta potential, mobility, and conductivity of the liposomes after 20 min of UV irradiation. The results are presented in Table 1.

Table 1. Particle size, polydispersity index (PDI), zeta potential, mobility, and conductivity of UV-irradiated silymarin and silibinin loaded liposomes, measured immediately after the 20-min UV irradiation.

variable	sample	
	silymarin-liposomes UV	silibinin-liposomes UV
particle size [nm]	3552.7±45.3	2234.0±50.2
PDI	0.356±0.032	0.317±0.024
zeta potential [mV]	-23.9±0.8	-24.5±0.7
mobility [$\mu\text{mcm/Vs}$]	-1.87±0.06	-1.92±0.06
conductivity [mS/cm]	0.014±0.001	0.007±0.003

As can be seen from Table 1, UV irradiation did not have an influence on vesicle size and PDI of all liposomes, but it caused a decrease in zeta potential: -23.9 ± 0.8 mV for silymarin-liposomes and -24.5 ± 0.7 mV for silibinin-liposomes, in mobility: -1.874 ± 0.064 $\mu\text{mcm/Vs}$ for silymarin- and -1.920 ± 0.057 $\mu\text{mcm/Vs}$ for silibinin-liposomes, and in conductivity: 0.014 ± 0.001 mS/cm for silymarin and 0.007 ± 0.003 mS/cm for silibinin.

Conclusion

In the present research study, silymarin and silibinin loaded liposomes were developed using proliposomes procedures and characterized via encapsulation efficiency, vesicle size, PDI, zeta potential, mobility, and conductivity, as well as storage stability and stability after UV irradiation. The encapsulation efficiency of silymarin and silibinin loaded liposomes were $92.05\pm 1.41\%$ and $87.86\pm 2.06\%$, respectively. Silymarin-liposomes had a higher diameter in comparison to silibinin-liposomes, while PDIs and conductivity were similar. The zeta potential and mobility (absolute value) of silibinin-liposomes were slightly higher in comparison to silymarin-liposomes. The obtained liposomes were physically stable during 28 days of storage, i.e. there was no occurrence of agglomeration and significant changes in uniformity and zeta potential of the liposomal system. UV irradiation did not cause changes in vesicle size and PDI of liposomes, but it caused a decrease in zeta potential, mobility, and conductivity. The obtained results qualify silymarin and silibinin loaded liposomes for application in functional foods and

pharmaceutical products, however, future experiments should deal with the biological activities of the developed liposomes.

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