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The Stability of Wild Thyme Extract-Loaded Phospholipid-Cholesterol Liposomal Particles

Stabilnost lipozomalnih čestica sa inkapsuliranim ekstraktom majčine dušice

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Abstract

Wild thyme contains biologically active compounds, particularly polyphenols (flavonoids and phenolic acids) that exert antitumor, antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, analgesic, and spasmolytic activities. Nevertheless, the mentioned bioactive compounds have low stability, solubility, and bioavailability, thus their application is limited. Liposomal particles have been widely used for the encapsulation of bioactive components, due to their high structural integrity, stability during storage, and controlled release capability. In the present study, phospholipid-cholesterol liposomal particles, as the carrier for wild thyme extract, were developed. The stability of extract-loaded liposomes was monitored for 21 days by measuring vesicle size, polydispersity index (PDI), and zeta potential. The particle size and PDI of extract-loaded liposomes did not change drastically during 21 days of storage and amounted to ~450 nm and ~0.155, respectively. The zeta potential varied in the liposomes and started to decrease after 7 days of storage (from -21.0 mV to 20.3 mV, without a statistically significant difference), while the zeta potential value after 21 days was statistically significantly lower in comparison to the 1st day (19.3 mV). The beneficial effects of polyphenols on human health, as well as showed storage stability of the prepared liposomes highlight the use of the wild thyme extract-loaded phospholipid-cholesterol liposomal particles for potential application in food, pharmaceutical, and cosmetic industries.
Keywords: *liposomal particles; phospholipids; stability; wild thyme*

Izvod

Majčina dušica sadrži biološki aktivna jedinjenja, posebno polifenole (flavonoide i fenolne kiseline) koji imaju antitumorsko, antimikrobno, antiinflamatorno, antioksidativno, imunomodulatorno, analgetično i spazmolitično dejstvo. Ipak, pomenuta bioaktivna jedinjenja imaju nisku stabilnost, rastvorljivost i bioraspoloživost, te je njihova primena ograničena. Lipozomalne čestice se široko koriste za inkapsulaciju bioaktivnih komponenti, zbog njihovog visokog strukturnog integriteta, stabilnosti tokom čuvanja i sposobnosti kontrolisanog oslobađanja aktivnih komponenti. U ovoj

studiji su razvijene lipozomalne čestice sa fosfolipidima i holesterolom, kao nosači za ekstrakt majčine dušice. Stabilnost lipozoma sa inkapsuliranim ekstraktom je praćena 21 dan merenjem veličine čestica, indeksa polidisperzije (PDI) i zeta potencijala. Veličina čestica i PDI lipozoma sa inkapsuliranim ekstraktom nisu se drastično promenili tokom 21 dana čuvanja i vrednosti su iznosile ~450 nm i ~0,150. Zeta potencijal je varirao u lipozomima i počeo je da opada nakon 7 dana čuvanja (od -21,0 mV do 20,3 mV, bez statistički značajne razlike), dok je vrednost zeta potencijala nakon 21 dana bila statistički značajno niža u odnosu na 1. dan (19,3 mV). Blagotvorni efekti polifenola na zdravlje ljudi, kao i pokazana stabilnost pri čuvanju pripremljenih lipozoma, ističu upotrebu lipozomalnih čestica sa inkapsuliranim ekstraktom majčine dušice za potencijalnu primenu u prehrambenoj, farmaceutskoj i kozmetičkoj industriji.
Ključne reči: *lipozomalne čestice; fosfolipidi; stabilnost; majčina dušica*

Introduction

Wild thyme contains biologically active compounds, polyphenols (flavonoids and phenolic acids), essential oil, monoterpenes, polysaccharides, and proteins that exert antitumor, antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, analgesic, and spasmolytic activities [1,2]. Nevertheless, the mentioned bioactive compounds have low stability, solubility, and bioavailability, thus their application is limited [3].

With the aim to overcome the mentioned disadvantages, various encapsulation techniques were established [3-5]. Liposomal particles have been widely used as the carrier for bioactive components, due to their high structural integrity, storage stability, and controlled release of target compounds [4,6]. Liposomal spheres are usually constituted by phospholipids or other kinds of lipids and can be used for the encapsulation of hydrophilic, lipophilic, and amphiphilic molecules [7,8]. Furthermore, liposomal preparation is simple and readily functionalized for active targeted delivery. Liposomes represent an ideal drug delivery carrier due to their superior biocompatibility since their bilayer is an analog of a biological membrane [6]. Depending on the formulation process, liposomal vesicles can have a quite small diameter, thus the water-dispersible preparations have a practically clear appearance [7]. Additionally, the addition of sterols, particularly cholesterol, during liposomal formulation can result in liposomes with satisfied physicochemical characteristics.

In the present study, phospholipid-cholesterol liposomal particles, as the carrier for wild thyme extract, were developed. The stability of extract-loaded liposomes was monitored for 21 days by measuring vesicle size, polydispersity index (PDI), and zeta potential.

Materials and Methods

Reagents and plant material

The following reagents were used: ethanol (Fisher Scientific, UK), Phospholipon 90 G, as unsaturated diacyl-phosphatidylcholine (Lipoid GmbH, Germany), and cholesterol (Sigma-Aldrich, Germany). Distilled water was purified through a Simplicity UV® water purification system (Merck Millipore, Merck KGaA, Germany).

Wild thyme herba was from the Institute for Medicinal Plants Research ”Dr Josif Pančić”, Pančevo, Serbia.

Preparation of the extract

Wild thyme extract was prepared using dried herba (1 g), 50% ethanol as the extraction medium (30 mL), and heat-assisted extraction at 80°C using the incubator shaker KS 4000i control (IKA, Germany) for 30 min [4]. The extraction was performed in Erlenmeyer flasks covered by aluminum foil to avoid light exposure and evaporation of the solvent. After the extraction, the sample was filtered through a cellulose filter (fine pore, 0.45 µm) and stored at 4°C until further experiments.

Preparation of liposomal particles with extract

Liposomal particles with wild thyme extract were obtained using the proliposome method and a mixture of phospholipids (Phospholipon) [9]. Phospholipids and 10 mol % of cholesterol (a total amount of 1 g) and ethanol wild thyme extract (4 mL) were stirred at 50°C to homogenize a mixture and evaporate ethanol. After cooling to 25°C, ultrapure water (20 mL) was added and the formulation was stirred at 800 rpm for 2 h. Subsequently, the liposomes were exposed to the ultrasound waves in an ultrasound bath (Sonorex, Bandelin, Germany) for 15 min, with the aim to reduce liposomal particle size.

Stability study

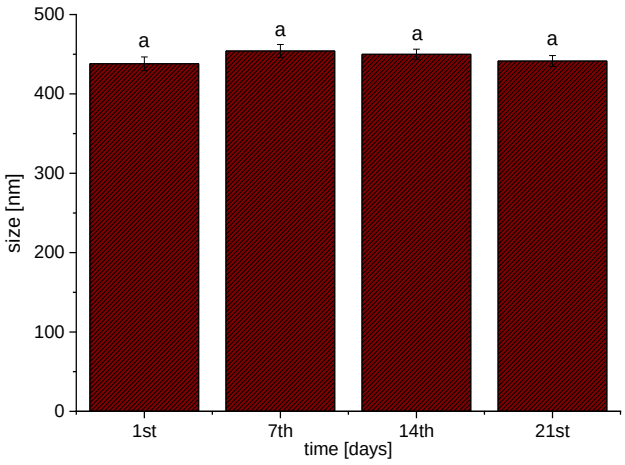
The measurements of vesicle size, PDI, zeta potential, conductivity, and mobility of liposomal particles with phospholipids, cholesterol, and wild thyme extract were performed using photon correlation spectroscopy in Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., UK). The measurement was repeated on the 1st, 7th, 14th, and 21st days after the preparation of extract-loaded liposomes to monitor their stability at 4°C. Each sample was diluted 500 times and measured in triplicate at room temperature.

Statistical analysis

The statistical analysis was done by using analysis of variance (one-way ANOVA) and Duncan's *post hoc* test in STATISTICA 7.0. The differences were considered statistically significant at $p<0.05$.

Results and Discussion

Phospholipid-cholesterol liposomal particles with encapsulated wild thyme extract were developed and vesicle size, PDI, and zeta potential were measured during the 21-days storage study. The results are shown in the graphs of Figure 1.
Since the vesicle size of liposomes represents a relevant parameter for their stability, biodistribution, and release of the encapsulated components [10], the monitoring of this variable was done during 21 days of storage at 4ºC (Figure 1A).



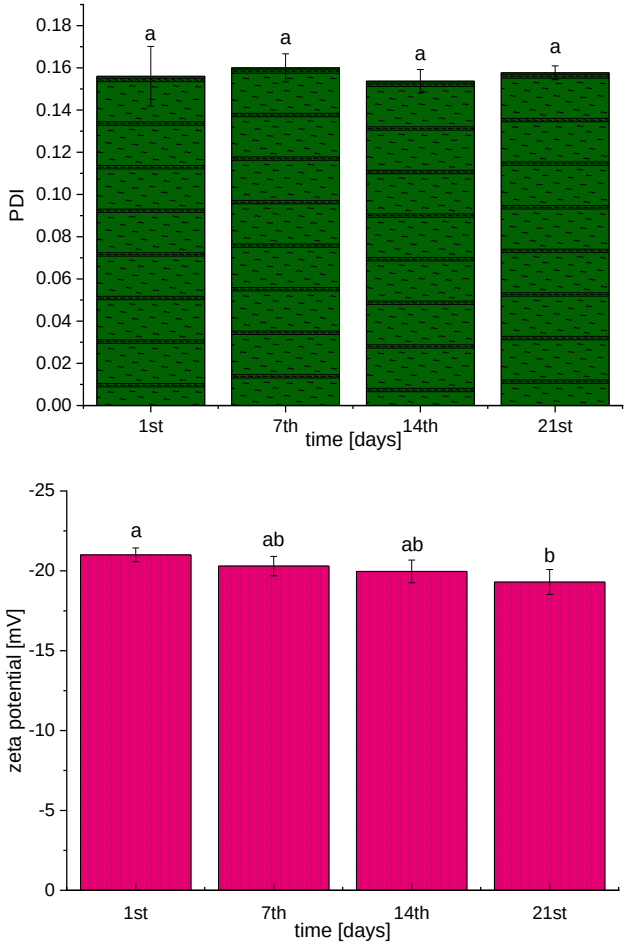


Figure 1. *(A) Vesicle size, (B) polydispersity index, and (C) zeta potential of wild thyme extract-loaded phospholipid-cholesterol liposomes, monitored during 21 days of storage at 4°C; the letters above bars showed statistically significant differences ($p<0.05$; $n=3$; analysis of variance, Duncan's post-hoc test).*

As can be seen from Figure 1A, the vesicle size of liposomal particles with wild thyme extract was 438.1±8.5 nm on the 1st day. The obtained values of particle size are in agreement with the literature data, where phospholipid liposomal particles with ergosterol and plant extract had a diameter of ~400 nm [11]. Namely, the type of lipids, the procedure for the liposomal preparation, the presence or absence of sterols, and the physicochemical characteristics of the entrapped compounds significantly influenced the vesicle size of the liposomes [4,9]. The measurements were repeated on the 7th, 14th, and 21st days and it can be concluded that there were no drastic changes in the liposome sizes (454.1±8.2 nm, 450.0±6.4 nm, and 441.4±6.9 nm, respectively). Due to a relatively high value of zeta potential (described below, Figure 1C), the absence of a significant change in vesicle size was expected.

Furthermore, PDI, as a measure of the vesicle size distribution in the liposomal suspension, was monitored as well, and the results are presented in Figure 1B. PDI value was 0.156±0.002 which indicates the existence of a uniform system. According to the literature data [12], higher liposomes (multilamellar vesicles, such as phospholipid-cholesterol liposomal particles with encapsulated wild thyme extract) possessed lower PDI values compared to smaller liposomes (small unilamellar vesicles, size ≤ 100 nm). The procedure used for liposomal formulation affects the uniformity of the liposomal population as well [4,9]. For example, Isailović et al. study [9] showed that liposomes produced using the proliposome technique (as in our case) had a PDI value of ~0.2, while the PDI of

the liposomal particles prepared using the thin film procedure was significantly higher, ~0.4. As can be seen in Figure 1B, PDI did not change during the 21-day storage study, as in the case of liposomal particle size (0.160±0.006, 0.154±0.002, and 0.158±0.003).
The zeta potential, as a measure of the stability of the liposomal system, was also measured during 21 days of storage at 4ºC (Figure 1C). The zeta potential was -21.0±0.4 mV indicating the presence of a stable system. Although phosphatidylcholines are neutral lipids in the water surrounding, the reorientation of the groups of the lipid heads results in the presence of a surface charge, which depends on the phase state and lipids/sterols types [12]. According to the literature [4], the negative value of zeta potential is related to the exposure of the phosphate group lying in an outer plane concerning the choline groups. The measured values of the zeta potential are in agreement with the Isailović et al. study [9], where the liposomal particles with polyphenol antioxidant formed using the proliposome method had a zeta potential of ~-25 mV. The zeta potential of wild thyme extract-loaded liposomes varied in the liposomal suspension and started to decrease after 7 and 14 days of storage (20.3±0.6 mV and 20.0±0.7 mV). However, there was no statistically significant difference between mentioned measured values. The zeta potential was statistically significantly lower after 21 days in comparison to the 1st day and amounted to 19.3±0.8 mV. Nevertheless, the mentioned zeta potential value was not statistically significantly lower compared to the values measured on the 7th and 14th days. Therefore, the results of zeta potential prove that liposomal particles with extract were stable during 21 days of storage at 4ºC. The stability was also confirmed by the absence of vesicle size and PDI value changes, i.e. there was no fusion or fission of the liposomal particles.

Conclusion

In the present paper, wild thyme extract-loaded phospholipid-cholesterol liposomal particles were prepared using the proliposome method, and their storage stability was monitored through the measuring of particle size, PDI, and zeta potential. The obtained liposomal particles were physically stable during 21 days of storage, i.e. there was no occurrence of agglomeration (changes in vesicle size) or significant changes in the size distribution and zeta potential of the liposomal suspension. The presented results qualify wild thyme extract-loaded phospholipid-cholesterol liposomal particles for application in food, functional foods, pharmaceutical or cosmetic products. Nevertheless, future experiments should deal with the biological potential of the developed liposomes and their widespread use in humans or industry.

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