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Original scientific paper

THE STABILITY OF LIPOSOMES WITH ERGOSTEROL AND *THYMUS SERPYLLUM* L. EXTRACT

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Abstract

Thymus serpyllum L. contains biologically active components, such as essential oil, polyphenols (flavonoids, phenolic acids, and anthocyanins), monoterpenes, polysaccharides, and proteins. Nevertheless, the use of the mentioned substances is rather limited, due to their low bioavailability, integrity, permeability, solubility, and stability. Therefore, their encapsulation in the liposomal particles can be advantageous. Apart from phospholipids, the addition of sterols during liposomal preparation can improve the physicochemical properties of the obtained particles. Thus, in the present study, ergosterol (10 and 20 mol %) and wild thyme extract loaded liposomes were developed. Vesicle size, polydispersity index (PDI), zeta potential, conductivity, and mobility of the obtained liposomes were determined using photon correlation spectroscopy. Additionally, the storage stability of the liposomes at 4ºC was investigated for 21 days. The liposomes with wild thyme extract were smaller than unloaded liposomes, while with the increase of ergosterol content vesicle size did not increase in all liposomes. The addition of extract caused the increase in the PDI, while it did not influence zeta potential. A phospholipid bilayer with 10 mol % of ergosterol possessed higher zeta potential (absolute value), conductivity, and mobility than a membrane containing 20 mol % of ergosterol. The vesicle sizes of all liposomes did not change drastically during 21 days of storage, whereas a slight increase of PDI appeared in extract loaded-liposomes after the 14th day. The zeta potential and mobility varied in all liposomes, and the trend depended on the composition of the membrane and the absence or the presence of the extract. The conductivity of the liposomes did not change during 21 days, except in the case of unloaded liposomes with 10 mol % ergosterol. The beneficial effects of polyphenols and ergosterol on human health, highlight the use of the liposomes with ergosterol and wild thyme L. extract for potential application in foods, pharmaceutics, and cosmetics.

Key words: *ergosterol, liposomes, polyphenols, stability, Thymus serpyllum.*

Introduction

Thymus serpyllum L. (wild thyme, Lamiaceae) contains biologically active compounds, such as essential oil, polyphenols (flavonoids, phenolic acids, and anthocyanins), monoterpenes, polysaccharides, sugars, and proteins (Čančarević et al., 2013; Jarić et al., 2014; Jovanović et al.,

2017). Polyphenols are widely used in different sectors of the food industry (natural antioxidants, nutritional, coloring, and antimicrobial agents). Additionally, polyphenol extracts are used as ingredients within pharmaceutical or cosmetic products (Munin and Edwards-Lévy, 2011). Nevertheless, the use of polyphenols is rather limited, due to their sensitivity, instability, and low bioavailability, permeability, and solubility (Fang and Bhandari, 2010). Therefore, the encapsulation of active compounds in liposomes represents an appropriate way to overcome all mentioned disadvantages (Jovanović et al., 2020). The hydrophilic, lipophilic, or amphiphilic substances encapsulated in liposomes are formulated as a solution, thus exhibiting higher bioavailability, and can achieve good physical stability during storage. Furthermore, the size of liposomes can have a quite small diameter, thus the water-dispersible formulations have a practically clear appearance (Ribeiro et al., 2010). Sterols, including cholesterol, lanosterol, stigmasterol, ergosterol, and β-sitosterol, are usually added during liposomal formulation, with the aim to modulate membrane permeability, promote stability of liposomal bilayer, and reduce leakage of the encapsulated active components (Miao et al., 2002; Silva et al., 2011). Ergosterol belongs to the group of fungus sterols, commonly used as cholesterol-lowering functional chemicals. The structure of ergosterol is similar to the sterols found in mammalian cells, with slight modifications in the side chain. Fungus sterols can lower plasma cholesterol and triacylglyceride concentration, and thus decrease the incidence of cardiovascular diseases (Zhao et al., 2015).

In the present study, phospholipid liposomes containing ergosterol (10 and 20 mol %), as the carrier for wild thyme polyphenol extract, were developed. The particle size, polydispersity index (PDI), zeta potential, conductivity, and mobility of the obtained liposomes were analyzed. Storage stability during 21 days was investigated as well.

Materials and Methods

Plant material and reagents

Wild thyme plant was from the Institute for Medicinal Plants Research "Dr Josif Pančić", Serbia. The following reagents were used: ethanol (Fisher Scientific, UK), Phospholipon 90 G (unsaturated diacyl-phosphatidylcholine) (Lipoid GmbH, Germany), ergosterol (Sigma-Aldrich, USA), and ultrapure water.

Polyphenol extraction

Wild thyme extract was obtained using 0.3 mm particle size of plant material, 50% ethanol, 30:1 solvent-to-solid ratio (module m/V) for 15 min of heat-assisted extraction (80°C), in the incubator shaker (KS 4000i control, IKA, Germany), according to previously published study (Jovanović et al., 2017).

Encapsulation in liposomes

Phospholipid liposomes containing ergosterol and wild thyme extract were prepared using the proliposome method (Isailović et al., 2013). Liposomes were obtained using a mixture of phospholipids (Phospholipon) and ergosterol (10 or 20 mol %). Phospholipids and ergosterol (a total of 1 g) and ethanol wild thyme extract (4 mL) were stirred at 50°C with the aim 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 1 h. Subsequently, the samples were sonicated using an ultrasound bath (SONOREX, Bandelin, Germany) for 15 min. Plain liposomes (as a control without wild thymeextract) were prepared as well.

Storage stability study

The measurements of vesicle size, PDI, zeta potential, conductivity, and mobility of liposomes were repeated on the 1st, 7th, 14th, and 21st days after the liposomal preparation with the aim to monitor their stability. During the stability study, the liposomal formulations were stored in the refrigerator at 4°C. The mentioned variables were determined using photon correlation spectroscopy in Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., UK). Each liposomal sample (25 μL) was diluted 500 times and measured three times at 25°C.

Statistical analysis

In the present study, the statistical analysis was performed by using analysis of variance (one-way ANOVA) followed by Duncan's *post hoc* test within the statistical software, STATISTICA 7.0. The differences were considered statistically significant at $p<0.05$.

Results and Discussion

The impact of different ergosterol content (10 and 20% of sterol) on vesicle size, PDI, zeta potential, conductivity, and mobility of liposomes was examined using photon correlation spectroscopy and the results are shown in Table 1 (the values measured after the liposomal preparation).

Table 1. Vesicle size, polydispersity index (PDI), zeta potential (ζ), conductivity (G), and mobility (μ) of phospholipid liposomes containing wild thyme extract and ergosterol (10 and 20 mol %, ergo 10% and ergo 20%, respectively) measured immediately after the preparation of liposomes.

sample	size [nm]	PDI	ζ [mV]	*G* [mS/cm]	*μ* [μmcm/Vs]
ergo 10%+extract	440.0±9.6[a*]	0.382±0.006[a]	-24.0±0.2[b]	0.012±0.001[a]	-1.57±0.04[b]
ergo 20%+extract	445.0±9.5[a]	0.389±0.006[a]	-23.0±0.2[a]	0.015±0.001[b]	-1.41±0.04[a]

**Values with different letters (a-b) in each column showed statistically significant differences ($p<0.05$; n=3; analysis of variance, Duncan's post-hoc test).*

As can be seen in Table 1, extract-loaded liposomes containing different amounts of ergosterol (10 and 20 mol %) did not show a significant difference in terms of particle size and PDI. According to the literature, sterols are usually part of the lipid composition of the liposomal bilayer, with the aim to modulate membrane fluidity, promote stability of the lipid bilayer, and prevent the leakage of the encapsulated components (Jovanović et al., 2018). Sterols are located in the lipid bilayer and the sterol carbohydrate tail connects with the hydrophobic fatty acyl chains, whereas the sterol hydroxyl group interacts with the hydrophilic head group of lipids, which leads to a more ordered liposomal membrane and the restriction of acid chains movement. Additionally, the presence of sterol resulted in increased packing, bilayer cohesion, and mechanical stiffness, and decreased membrane permeability and mobility of the carbohydrate chains (Farkas et al., 2004; Jovanović et al., 2018). Although literature data reported that a higher concentration of sterols caused the increase in the vesicle size of liposomes due to the interactions between phospholipids and sterols, and the formation of inter-lipid space (Jovanović et al., 2018), in the case of phospholipid liposomes with ergosterol and wild thyme extract, it did not occur (440.0±9.6 and 445.0±9.5 nm). Perhaps a further increase in the amount of ergosterol (over 20 mol %) would show a statistically significant difference in vesicle size. Nevertheless, the liposomal suspension with a higher concentration of ergosterol (data not presented) showed instability and ergosterol precipitation after centrifugation. As can be seen in Supplement 1, the plain liposomes (without extract) possessed statistically significantly higher size in comparison

to the extract-loaded liposomes. PDI values, as a measure of particle size distribution in the liposomal formulation, are also presented in Table 1. The obtained PDI values indicate the existence of a uniform system within both, extract-loaded liposomes with 10 and 20 mol % of ergosterol (0.382±0.006 and 0.389±0.006, respectively). However, liposomes without extract possessed lower PDI, i.e. lower heterogeneity (Supplement 1).

The zeta potential of the liposomes with extract and ergosterol (10 and 20 mol %) are shown in Table 1 as well. Extract-loaded liposomes containing 20 mol % of ergosterol had a statistically significantly higher absolute value of zeta potential (-24.0±0.2 mV) than liposomes with 10 mol % of ergosterol (-23.0±0.2 mV). According to the literature data, sterols caused the change in the phospholipid order, and the thickness of the liposomal membrane, and affected the zeta potential (Bhattacharya et al., 2000; Ricci et al., 2016). Therefore, the presence of sterol can provide the hydrophobic stabilization of the liposomal membrane (Bhattacharya et al., 2000). Additionally, there were no statistically significant differences in zeta potential between empty and extract loaded-liposomes (Supplement 1). As can be seen in Table 1, the conductivity of extract-loaded liposomes with 20 mol % of ergosterol was significantly lower (0.012±0.001 mS/cm) than the conductivity of the parallel with 10 mol % of ergosterol (0.015±0.001 mS/cm). According to the literature, a higher capture volume corresponds to a decrease in conductivity (Lidgate et al., 1993). Several studies have shown that indeed the liposomes containing a higher amount of sterols showed a higher encapsulation efficiency (Farkas et al., 2004; Jovanović et al., 2018). Namely, a lower amount of ions is removed as liposome capture volume decreased with decreasing lipid concentration, thus conductivity of the liposome dispersions increased as the lipid concentration decreased (Lidgate et al., 1993). Plain liposomes (without extract) have shown significantly lower conductivity compared to their extract-loaded parallels (Supplement 1). Liposomes with extract and 10 mol % of ergosterol exhibited mobility of -1.57±0.04 μmcm/Vs, whereas extract-loaded liposomes containing 20 mol % of ergosterol had a mobility of -1.41±0.04 μmcm/Vs (Table 1). Duffy et al. (2001) reported that the mobility of liposomal particles represents a function of vesicle size, total charge, and composition of the bilayer membrane. Therefore, the obtained differences between various liposomal populations were expected. Furthermore, some liposomal bilayers are fluid, flexible, and deformable, whereas others are rigid that depend on the composition of membrane and encapsulated compounds. According to Jovanović et al. (2018), the liposomal membrane with a higher content of sterol possessed higher fluidity and therefore better mobility. However, in our study, liposomes with a lower content of ergosterol (10 mol %) showed higher mobility. Nevertheless, the relatively minor change in mobility between different liposomal populations (with 10 and 20 mol % of ergosterol) does not account for the significant changes observed (~10%). It can be explained by smaller particles in both liposomal populations and their ability to deform (Pysher and Hayes, 2004). However, empty liposomes showed higher mobility in comparison to extract-loaded samples (Supplement 1).

In order to examine the stability of phospholipid liposomes with ergosterol and wild thyme extract, vesicle size, PDI, zeta potential, conductivity, and mobility were measured during 21 days; the results are presented in Figure 1.

The particle sizes of all liposomes did not change drastically during 21 days of storage, whereas a slight increase of PDI appeared in extract loaded-liposomes after the 14th day (data for the liposomes without extract not shown). The zeta potential and mobility varied in all liposomes, and the trend depended on the composition of the membrane and the absence or the presence of the extract (data for the liposomes without extract not shown). The conductivity of the liposomes did not change during 21 days, except in the case of unloaded liposomes with 10 mol % ergosterol (data for the liposomes without extract not shown).

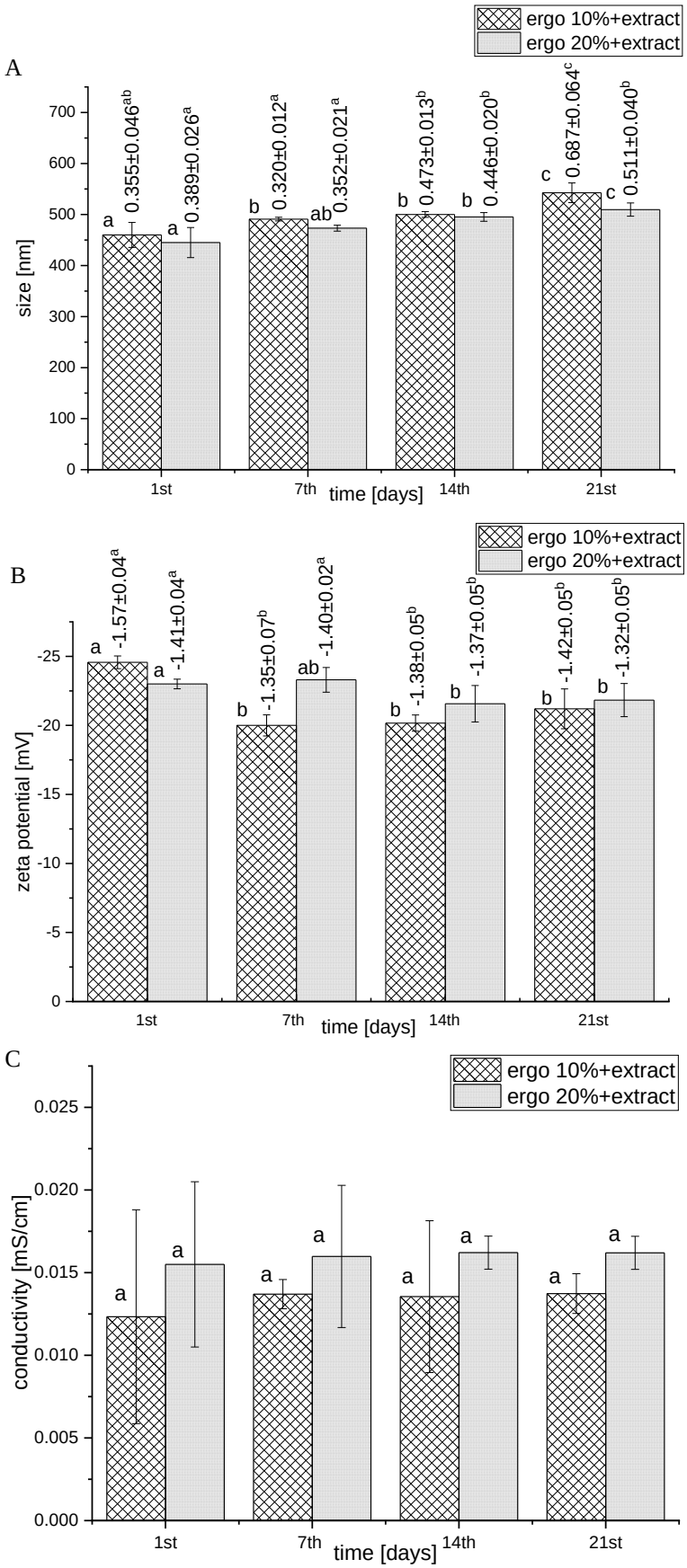

Figure 3. (A) Particle size - bars and polydispersity index - numbers above bars, (B) zeta potential - bars and mobility - numbers above bars (µmcm/Vs), and (C) conductivity of phospholipid liposomes containing ergosterol and Thymus serpyllum extract (10 and 20 mol % of ergosterol, ergo 10% and ergo 20%, respectively) for 21 days; values with different letters (a-c) showed statistically significant differences ($p<0.05$; n=3; analysis of variance, Duncan's post-hoc test).

Conclusions

In the present study, the stability of phospholipid liposomes containing ergosterol (10 and 20 mol %) and wild thyme polyphenol extract prepared using the proliposome technique, and sonication were investigated *via* determination of vesicle size, PDI, zeta potential, conductivity, and mobility. Both liposomes (with 10 and 20 mol % of ergosterol) had a similar diameter and PDI, whereas zeta potential, conductivity, and mobility were significantly different. After 21 days of storage, there was no drastic increase in the particle size, PDI, and conductivity, while the changes in zeta potential and mobility depended on the composition of the membrane. Therefore, the potential application of both liposomal populations in foods, functional foods, pharmaceutics, and cosmetics can be investigated in future experiments.

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Conflict of interest

The authors declare that they have no financial and commercial conflicts of interest.

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Supplement 1. Vesicle size, polydispersity index (PDI), zeta potential (ζ), conductivity (G), and mobility (μ) of phospholipid liposomes containing ergosterol (10 and 20 mol %, ergo 10% and ergo 20%, respectively) in the absence of Thymus serpyllum extract, measured immediately after the preparation of liposomes.

sample	size [nm]	PDI	ζ [mV]	*G* [mS/cm]	*μ* [μmcm/Vs]
ergo 10%	604.7 ± 7.2^{a}	0.289 ± 0.008^{b}	-24.3 ± 0.7^{a}	0.005 ± 0.000^{a}	-2.19 ± 0.10^{b}
ergo 20%	596.0 ± 15.6^{a}	0.235 ± 0.019^{a}	-23.9 ± 1.0^{a}	0.010 ± 0.001^{b}	-1.96 ± 0.06^{a}

**Values with different letters (a-b) in each row showed statistically significant differences ($p<0.05$; $n=3$; analysis of variance, Duncan's post-hoc test).*