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Antioxidant Potential of Ergosterol–Phospholipid Liposomes with *Thymus serpyllum* Extract †

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Abstract: The antioxidant activity of ergosterol–phospholipid liposomes with chemically characterized, encapsulated wild thyme (*Thymus serpyllum*) extract was examined. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity and ferric ion-reducing potential of the extract-loaded liposomes with different proportions of ergosterol were investigated. The neutralization of DPPH radicals for the samples with 10 and 20 mol% of ergosterol was 56.3 ± 2.2% and 53.1 ± 3.5%, respectively. The elimination of ABTS radicals was significantly higher and amounted to 95.3 ± 2.6% (10 mol% of ergosterol) and 98.2 ± 1.7% (20 mol% of ergosterol). The Fe^{3+}-reducing potential of the liposomes was 0.14 ± 0.01 and 0.15 ± 0.03 mmol $FeSO_4$/L. Therefore, there was no significant difference between the antioxidant capacity of the liposomes with various amounts of ergosterol. The shown antioxidant potential highlights the employment of prepared liposomes with ergosterol and *T. serpyllum* extract active compounds in functional foods, pharmaceutics, or cosmetics.

Keywords: encapsulation; *Thymus serpyllum*; liposomes; antioxidant potential; polyphenols

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1. Introduction

Thymus serpyllum extracts express antioxidative, antispasmodic, anti-inflammatory, antihypertensive, cytotoxic, antiseptic, antibacterial, and antifungal activities due to the presence of polyphenols [1]. Polyphenol compounds from *T. serpyllum* extract are sensitive to temperature, light, oxidation, and pH value changes and possess lower bioavailability and bitter taste [2]. Thus, despite their great bioactive potential, their application in different industries is quite limited. Micro- or nano-vesicles known as liposomes are created when lipid components, including phospholipids, are distributed across an aqueous medium. The inner water phase is separated from the outer phase by one or more bilayers that are generated by and have a structure similar to the cell membrane [3,4]. Liposomes can enhance the stability of encapsulated sensitive compounds and the bioavailability of poorly hydrosoluble components. Additionally, liposomal particles have outstanding biodegradability and a strong affinity for cells [5,6]. Furthermore, the bilayer membrane of liposomes and its permeability can be modified by adding sterols, such as ergosterol, which can change the end product's properties and pharmacological behavior [7]. Namely,

ergosterol contributes to the performance of liposomal vesicles by increasing membrane packing density, as well as physical stability [8]. According to the literature data, ergosterol can reduce membrane flexibility less than cholesterol due to a higher degree of unsaturation [9]. In comparison to cholesterol, which forms a tightly packed arrangement with phospholipids and β-sitosterol, which markedly reduces the effectiveness of lipid ordering, the liposomal membranes containing ergosterol are more fragile and fluid [10]. Yoda's study [11] showed that the addition of ergosterol increased the homogeneity of the liposome membrane with charged lipids; the membrane with cholesterol is more sensitive to a charged state, whereas liposomes with ergosterol showed lower responses to charged lipids. Additionally, ergosterol-containing nutraceuticals can promote immune function, liver health, and overall well-being [12]. Fungus sterols can lower the concentration of cholesterol and triacylglycerides in plasma and therefore lower the risk of cardiovascular disorders [13].

Thus, in the present study, the antioxidant activity of ergosterol–phospholipid liposomes with encapsulated *T. serpyllum* ethanol extract was investigated. Namely, DPPH and ABTS radical scavenging activity and ferric ion-reducing potential of the prepared extract-loaded liposomes with different proportions of ergosterol (10 and 20 mol%) were determined.

2. Materials and Methods

2.1. Chemicals

The aerial and dried part of *T. serpyllum* was grinded in the Institute for Medicinal Plants Research "Dr Josif Pančić", Pančevo, Serbia. Ultrapure water was prepared in a Simplicity UV® water purification system (Merck Millipore, Darmstadt, Germany). Ethanol (Fisher Science, Loughborough, UK), ABTS, DPPH, 2,4,6-tri-(2-pyridyl)-5-triazine, iron (III) chloride, iron (II) sulphate, and ammonium acetate (Sigma–Aldrich, Hamburg, Germany), Phospholipon (Lipoid, Ludwigshafen am Rhein, Germany), and potassium persulfate (Centrohem, Stara Pazova, Serbia) were also used.

2.2. Extract Preparation

The liquid wild thyme extract was obtained at 80 °C in the incubator shaker KS 4000i control (IKA, Staufen, Germany) for 15 min at 200 rpm; a particle size of *T. serpyllum* plant material of 0.3 mm, solid-to-solvent ratio of 1:30 g/mL, and 50% (*w*/*w*) ethanol were employed in the extract formulation. After the extraction process, the sample was filtered using fine filter paper, and the extract was used for further liposome preparation.

2.3. Liposome Preparation

Extract-loaded liposomes with ergosterol were prepared using the proliposome method [14]. A mixture of phospholipids and ergosterol (10 or 20 mol%) in the amount of 1 g and ethanol extract in a volume of 4 mL was stirred at 50 °C for 20 min. After cooling, ultrapure water (20 mL) was added in small portions, and the emulsion was stirred at 800 rpm for 1 h at the ambient temperature. The data on the stability of developed liposomes with ergosterol and wild thyme extract were previously published [13], and the study showed that the sizes of liposomes did not change during 21 days of storage, whereas a slight increase in polydispersity index appeared after 14 days. The zeta potential and mobility varied in all developed liposomes, but the trend depended on the lipid/sterol composition.

2.4. Measurement of the Encapsulation Efficiency

Encapsulation efficiency was calculated by the concentration of total polyphenols in the supernatant as shown in Equation (1):

$$\text{Encapsulation efficiency} = (C_i - C_{sup})/C_i \times 100 \quad (1)$$

where C_i is the initial concentration of total polyphenols used for the preparation of liposomes, and C_{sup} is the content of total polyphenols determined in the supernatant.

Free wild thyme extract was removed from liposome dispersions by centrifugation at 17,500 rpm and 4 °C for 45 min in a Thermo Scientific Sorval WX Ultra series ultracentrifuge (ThermoScientific, Waltham, MA, USA). The concentration of total polyphenols was determined spectrophotometrically at 280 nm (UV-1800, Shimadzu, Kyoto, Japan).

2.5. Determination of the Liposome Antioxidant Potential

The antioxidant potential of liposomes was measured via hydrogen-donating or radical scavenging ability using the stable DPPH radicals [15]. Liposomes (20 µL) were mixed with 2 mL of ethanol DPPH$^{\bullet}$ radical solution. The absorbance of DPPH• radicals in ethanol was ~0.800 at 517 nm. The absorbance of ethanol DPPH$^{\bullet}$ radical solution mixed with extract-loaded liposomes or pure extract (diluted to achieve the same concentration as that of the liposomes) was recorded after 20 min of incubation in the dark at room temperature, and the percentage of inhibition was calculated using the following equation:

$$\%\ \text{inhibition} = (A_{0DPPH} - A_x) \times 100/A_{0DPPH} \quad (2)$$

where A_{0DPPH} was the absorbance of the control, and A_x was the absorbance of the DPPH$^{\bullet}$ solution and liposomes.

The ABTS assay is based on the reduction in ABTS$^{\bullet+}$ free radicals by antioxidant compounds from the sample [16]. The ABTS radicals were produced in the reaction of 7 mM ABTS in distilled water and 2.45 mM potassium persulfate in the dark at 25 °C for 18 h. The ABTS$^{\bullet+}$ working solution was diluted using ethanol (an absorbance of ~0.700 at 734 nm). ABTS$^{\bullet+}$ solution (2 mL) was mixed with liposomes (20 µL). After 6 min of incubation in the dark at room temperature, the absorbance was measured, and the radical scavenging activity of the samples was calculated using the following equation:

$$\%\ \text{inhibition} = (A_{0ABTS} - A_x) \times 100/A_{0ABTS} \quad (3)$$

where A_{0ABTS} was the absorbance of the control, and A_x was the absorbance of ABTS$^{\bullet+}$ solution and liposomes.

The ferric-reducing antioxidant potential (FRAP) of prepared liposomes was determined as well [17]. The FRAP reagent was prepared using 2.5 mL of a 10 mmol/L 2,4,6-tri-(2-pyridyl)-5-triazine solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L $FeCl_3$, and 25 mL of 0.3 mol/L acetate buffer (pH 3.6) at 37 °C. The liposome (40 µL) was mixed with distilled water (200 µL) and FRAP reagent (1.8 mL). The absorbance was measured at 593 nm after 10 min of incubation at 37 °C. Trolox was used as a positive control (1.7 mmol $FeSO_4$/L at the concentration of 0.5 mmol/L). The results were expressed as the concentration of antioxidant compounds having a ferric-reducing ability equivalent to that of 0.5 mmol/L of $FeSO_4$.

2.6. Statistical Analysis

The statistical analysis was performed by using analysis of variance followed by Duncan's post-hoc test within the statistical software STATISTICA 7.0. Differences were considered statistically significant at $p < 0.05$, and measurements were done in triplicate.

3. Results and Discussion

The antioxidant potential of wild thyme extract-loaded liposomes with ergosterol was determined using three antioxidant assays, namely the DPPH, ABTS, and FRAP methods.

As can be seen in Table 1, DPPH radical scavenging capacity was 56.3 ± 2.2 (for liposomes with 10 mol% of ergosterol) and 53.1 ± 3.5% (for liposomes with 20 mol% of ergosterol). The values of the ABTS radical neutralization were 95.3 ± 4.6% for the liposomes with 10 mol% of ergosterol and 98.2 ± 1.7% for the liposomes with 20 mol% of ergosterol. The ferric ion-reducing potential of the liposomes with 10 and 20 mol% of ergosterol amounted to 0.14 ± 0.01 and 0.15 ± 0.03 mmol $FeSO_4$/L, respectively.

Table 1. Antioxidant activity of ergosterol–phospholipid liposomes with encapsulated *Thymus serpyllum* extract.

Tests	Samples		
	Liposomes with 10 mol% of Ergosterol	**Liposomes with 20 mol% of Ergosterol**	**Extract**
DPPH [1] assay (% of neutralization)	56.3 ± 2.2 [a]	53.1 ± 3.5 [a]	47.7 ± 0.6 [b]
ABTS assay (% of neutralization)	95.3 ± 4.6 [a]	98.2 ± 1.7 [a]	74.0 ± 1.3 [b]
FRAP assay (mmol $FeSO_4$/L)	0.14 ± 0.01 [a]	0.15 ± 0.03 [a]	0.17 ± 0.04 [a]

[1] DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); FRAP, ferric ion-reducing potential; values with the same letter in each row showed no statistically significant difference ($p > 0.05$; n = 3; analysis of variance, Duncan's post-hoc test).

Regarding the results from the antioxidant tests, it can be concluded that antioxidant components contained in *T. serpyllum* extract of a given concentration provide an effective antioxidant effect. However, the obtained extract-loaded liposomes more effectively neutralized ABTS radicals in comparison to DPPH radicals. The discrepancy between the three antioxidant tests is not surprising. This is because oxidation probes, kinetics of the reactions, targeted compounds, and experimental conditions (pH values, wavelengths for the measurements, incubation periods, etc.) are different depending on the employed test [18]. Additionally, there was no significant difference in antioxidant capacity for samples containing various amounts of ergosterol. Considering that the carriers of antioxidant activity are wild thyme polyphenols [1], this can explain the absence of statistically significant differences among wild thyme extract-loaded liposomes with 10 and 20 mol% of fungal sterol, i.e., ergosterol; all of these contained the same amount of extract and exhibited a similar encapsulation efficiency (91.2–92.8%). Sterols, including ergosterol, represent key compounds in liposome preparation due to their ability to enhance the membrane stability, improve the entrapped efficiency, and decrease the leakage of encapsulated bioactives [10,19]. According to the literature data, incorporation of 10 and 20 mol% ergosterol in the liposomal bilayer led to a significant increase in anisotropy due to the conjugated double bond system in the ergosterol nucleus, interactions with the acyl chains, and enhancement of van der Waals forces [20]. Song et al.'s study [10] reported that ergosterol can change the permeability of the liposomal membrane, i.e., increase its rigidity, but to a lesser extent compared to sterols.

Using LC/DAD/MS analysis, in the previous study, polyphenol compounds identified in the encapsulated extract include chlorogenic, caffeic, rosmarinic, and salvianolic acids, salvianolic acid K isomer, 6,8-Di-C-glucosylapigenin, 6-hydroxyluteolin 7-O-glucoside, luteolin 7-O-glucuronide, and apigenin glucuronide, while the total polyphenol content amounted to up to 29.8 mg gallic acid equivalent/L of the extract [21]. The mentioned phenolic acids and flavonoid compounds (as subgroups of polyphenols) are recognized as potent antioxidant agents [22]. The obtained results for the extract antioxidant potential are correlated with the polyphenol concentration in the pure extract. This is because polyphenols represent the main plant-based antioxidant compounds. The correlation between the antioxidant activity of extract formulations and polyphenol yield was shown in numerous studies [23,24]. The free radical neutralization potential of the pure extract amounted to 47.7% in the DPPH assay and 74.0% in the ABTS assay. It can be seen that the encapsulation of the extract in liposomal particles improved the antioxidant capacity of the system. Namely, according to the literature data, green tea extract encapsulated in liposomes showed a higher antioxidant potential compared to the non-encapsulated extract [25]. In all samples (extract-loaded liposomes and pure extract), ABTS radical scavenging capacity was higher compared to the antioxidant effect shown in the DPPH assay. The reason for the observed difference in radical neutralization capacity may lie in the fact that the antioxidant potential of wild thyme extract depends not only on the polyphenol amount but also on the presence of non-phenolic compounds, as well as on the interactions between those compounds [26].

4. Conclusions

This work aimed to determine the antioxidant potential of phospholipid liposomes with encapsulated *T. serpyllum* extract and various amounts of ergosterol. The prepared extract-loaded liposomes more effectively scavenged ABTS radicals compared to DPPH radicals, while there was no significant difference in the antioxidant activity of samples with different ergosterol proportions. The demonstrated antioxidant potential of developed liposomes and the biological activities of the active compounds contained in *T. serpyllum* extract and ergosterol can highlight the employment of prepared liposomes in functional foods, pharmaceutics, or cosmetics. Nevertheless, future experiments will be focused on the other biological activities of developed wild thyme extract-loaded liposomes with ergosterol using in vitro cell models.

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Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	ferric-reducing antioxidant potential

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