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8th Workshop

FOOD AND DRUG SAFETY AND QUALITY

September 26th 2024, Vinča Institute of Nuclear Sciences - National Institute of the Republic of Serbia, University of Belgrade, Belgrade, Serbia

PROCEEDINGS

FOOD AND DRUG SAFETY AND QUALITY

8th WORKSHOP: FOOD AND DRUG SAFETY AND QUALITY

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BELGRADE, SERBIA 2024

Research paper **P A2**

DESIGN AND EVALUATION OF LIPOSOMAL FORMULATION OF CAROB (*Ceratonia siliqua* L.) PULP EXTRACT

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ABSTRACT
The carob (*Cerationa siliqua* L.) pulp flour is used primarily in the food industry. As a valuable source of bioactive compounds (polyphenols), it is a good candidate for pharmaceutical formulation research and development. In this study, carob pulp extract-loaded liposomal particles were developed using the proliposome procedure and characterized *via* encapsulation efficiency (EE), vesicle size, polydispersity index (PDI), zeta potential, mobility, and viscosity. The obtained results suggest that the EE is high (80.6±3.3%). The particle size and PDI results indicated the existence of the multilamellar and uniform liposomal system. Lower values of zeta potential and mobility (-17.8±1.0 mV and -1.40±0.08 μmcm/Vs, respectively) of developed liposomes suggest that future experiments should be focused on the carob pulp liposomal nutraceutical formulation improvement, particularly its stability.

INTRODUCTION
Carob (*Ceratonia siliqua* L.) is an evergreen tree belonging to the Leguminosae family, widely cultivated in the Mediterranean region. Carob pulp is the seedless part of the carob pod and has been recognized as a valuable source of many phytochemicals such as polyphenols (tannins, phenolic acids, flavonoids, and their derivatives), alongside amino acids, minerals, vitamins, and insoluble fibers [1]. This plethora of bioactive compounds makes it a promising product and the focus of many studies related to its use in human nutrition [2].

Despite the promising health effects of dietary polyphenols in preclinical studies, the clinical use of polyphenol-based functional foods is still very limited due to their low bio-accessibility and/or bioavailability. The encapsulation process protects polyphenols from decomposition throughout the processing and storage stages, prevents them from degradation in the gastrointestinal

environment, and controls their release in the target tissue/organs [3]. Thus, the aim of this study was to design liposomal nutraceutical formulation and to evaluate the physicochemical characteristics of carob pulp flour extract-loaded liposomes.

EXPERIMENTAL

Carob extract was prepared using 1 g of the plant material and 10 mL of 40% ethanol in microwave-assisted extraction (power of microwaves was 800 W) for 25 min [4]. Ultrapure water from a Simplicity UV® water purification system (Merck Millipore, Germany) and soy L-α-phosphatidylcholine (Avanti Polar Lipids, USA) were employed for the preparation of carob extract-loaded liposomes.

C. siliqua extract-loaded liposomal particles were obtained in the previously published proliposome method [5]. The extract (20 mL) was mixed with phosphatidylcholine (2 g), and heated to 60°C for 45 min. After cooling, ultrapure water was added in small portions to a total volume of 40 mL, and the dispersion was stirred for 2 h at 800 rpm. The sample was stored at 4°C until further analysis.

The encapsulation efficiency (EE), vesicle size, polydispersity index (PDI), zeta potential, mobility, and viscosity of the obtained extract-loaded liposomes were investigated using the spectrophotometric method (UV Spectrophotometer UV-1800, Shimadzu, Japan), dynamic light scattering (Zetasizer Nano Series, Malvern Instruments, UK), and rotation viscometer (Rotavisc *lo-vi*, IKA, Germany), respectively. For the dynamic light scattering, the liposomes were diluted 200 times, and the measurements were performed in triplicates at 25°C.

RESULTS AND DISCUSSION

In the present study, *C. siliqua* extract-loaded liposomes were prepared and characterized via EE, particle size, PDI, zeta potential, mobility, and viscosity. The EE of carob extract was 80.6±3.3%. Regarding the literature data, the EE of biologically active components from plants within liposomes can be in a very wide range (25-80%) [6-9]. However, the EE of polyphenols from rosehip extract in liposomes was higher and amounted to ~90% [5]. The vesicle size and PDI of the prepared liposomes amounted to 2055.0±120.0 nm and 0.137±0.021, respectively, indicating the existence of the multilamellar and uniform liposomal system [5,9]. The zeta potential and mobility were -17.8±1.0 mV and -1.40±0.08 μmcm/Vs, respectively. A negative value of zeta potential gives evidence of the phospholipid molecules' organization, whereas a medium value suggests that the liposomal suspension is moderately electrostatically stabilized [9]. Namely, the desired value of the zeta potential should be ~30 mV, making particle aggregation slower and longer, and therefore the liposomal stability was prolonged [5,9]. The mobility of the liposomal vesicle is a function of size, surface charge, and membrane composition [10]. Flavonoids from plant extract can be adsorbed at the liposome bilayer's surface, lowering liposome mobility [11]. The determined

viscosity of carob extract-loaded liposomes at room temperature was 6.76±0.12 mPa·s. The viscosity of the liposomal dispersions has a crucial role in their long-term storage, and it is also one of the essential factors of stability and release of encapsulated bioactive compounds [9,12]. According to the literature data, the viscosity values of the liposomes varied in a very wide range [12]. Since the viscosity of the liposomal system depends on used type and concentration of phospholipids and bioactive compounds, as well as temperature conditions and the employed viscosimeter tool, the comparison with the literature data is not possible.

CONCLUSION

In this study, the physicochemical characteristics of *C. siliqua* (carob) pulp flour extract-loaded liposomes are evaluated. The vesicle size and polydispersity index results indicated the existence of the multilamellar and uniform liposomal system. The encapsulation efficiency was high; however, lower values of zeta potential and mobility of developed liposomes suggest that future experiments should focus on improving carob pulp liposomal nutraceutical formulation, particularly its stability.

Acknowledgement

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Research paper **P A3**

RADICAL SCAVENGING POTENTIAL OF DIFFERENT TYPES OF LIPOSOME PARTICLES WITH ENCAPSULATED CAROB EXTRACT

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ABSTRACT

Carob pulp extract contains bioactive phenolic compounds, making it a suitable functional food ingredient. This study aimed to investigate the radical scavenging potential of pure carob extract and different types of carob extract-loaded liposomes (multilamellar, UV-irradiated, and sonicated unilamellar vesicles). Their antioxidant capacity was measured by employing DPPH and ABTS assays. The encapsulation of carob extract within liposomes as well as UV irradiation and sonication did not cause a decrease in the anti-DPPH activity, however, the ABTS radical scavenging potential of pure extract was significantly lower compared to encapsulated counterparts. UV irradiation and sonication significantly decreased the anti-ABTS effect of the liposome with the extract. Carob pulp extract and its liposomal formulations displayed significant *in vitro* antioxidant potential that can be of interest for further development as a functional food ingredient or in the prevention/treatment of various diseases.

INTRODUCTION

The carob tree (*Ceratonia siliqua* L.), an evergreen perennial tree from the Fabaceae (Leguminosae) family has been utilized by humans since ancient times. Its leaves, bark, and seeds have traditionally been used in medicine to treat various diseases, including diabetes and hypertension [1]. The pulp flour, obtained by drying, roasting, and grinding the pods after stripping them of their seeds, is used primarily in the food industry, in the preparation of sweet juices, chocolates, and biscuits, and as a cocoa substitute [1,2]. Currently, carob is being investigated as a source of new natural antioxidants, specifically those found in seed coats and fruit pulp. Its antioxidant activity is mainly attributed to the presence of phenolic compounds.

The aim of this study was to investigate the radical scavenging potential of carob pulp flour extract obtained by a microwave-assisted extraction technique

[2] and three types of carob pulp flour extract-loaded liposomes (multilamellar, UV-irradiated, and small sonicated unilamellar vesicles). Their antioxidant activity was evaluated using two *in vitro* assays.

EXPERIMENTAL

C. siliqua extract was obtained employing microwave-assisted extraction and 40% (v/v) ethanol at a solid-to-solvent ratio of 1:10 g/mL, while the extraction time was 25 min [2]. Carob extract-loaded liposomes (as multilamellar vesicles) were prepared using the proliposome technique [3]. The volume of 20 mL of carob extract was mixed with 2 g of phospholipids and stirred at 60°C for 45 min. Further, at 25°C, 40 mL of ultrapure water was added, and the emulsion was mixed for 2 h at 800 rpm. With the aim to investigate the influence of UV irradiation on the antioxidant potential of prepared liposomes, 10 mL of the liposomal suspension was exposed to UV-C irradiation (253.7 nm) in uncovered Petri dishes for 30 min in a laminar flow cabinet (AC2-4G8, ESCo, Singapore). In addition, 15 mL of liposomes was sonicated employing an ultrasound probe (Sonopuls, Bandelin, Germany) for 15 min (on 40 s-off 10 s) using the ultrasound probe at 45% amplitude and 25°C (a flask with the liposomes was continuously cooled using ice coating during sonication and the temperature was measured and controlled) [4].

The antioxidant capacity of all obtained carob extract-loaded liposomes and pure extract was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging potential [5,6]. In the DPPH assay, liposomal samples or pure extract (20 μL) were mixed with 2 mL of ethanol DPPH• radical solution (an absorbance of ~0.800 at 517 nm, UV-VIS Spectrophotometer UV-1900i, Shimadzu, Japan). After 20 min of incubation at 25°C, the absorbance was measured, and the anti-DPPH potential was expressed in the percentages (% of free DPPH radical neutralization). In the ABTS assay, ABTS•+ solution (2 mL, an absorbance of ~0.700 at 734 nm) was mixed with previously diluted liposomal samples or pure extract (1:10, 20 μL). After 6 min of incubation at 25°C, the absorbance was measured, and the results were expressed as μmol Trolox equivalent (TE)/mL of sample (pure extract or liposomes).

RESULTS AND DISCUSSION

The high phenolic content of carob pulp has been deeply associated with several nutritional and functional benefits [2,7]. In this study, carob extract and extract-loaded liposome antioxidant activity was evaluated using *in vitro* assays.

Table 1. The radical scavenging potential of liposome particles with encapsulated carob extract (multilamellar, UV-irradiated, and sonicated unilamellar vesicles) and pure carob extract measured in the DPPH and ABTS assays.

Sample	Neutralization of DPPH radicals (%)	Concentration (μmol TE*/mL)
Multilamellar vesicles	69.6±0.8[a]	7.05±0.04[a]
UV-irradiated	70.3±0.7[a]	6.51±0.08[b]
Sonicated unilamellar vesicles	69.4±0.6[a]	6.17±0.26[c]
Pure extract	69.1±1.0[a]	5.49±0.12[d]

*TE, Trolox equivalent; different letters in each column (a-d) indicated the differences that were considered statistically significant (analysis of variance followed by Duncan's *post hoc* test, $p<0.05$, n=3).

As can be seen from Table 1, the potential for neutralization of DPPH radicals varied in a narrow range, from 67.9 to 70.3%. Namely, the encapsulation of carob extract within liposomes did not cause a decrease in its antioxidant potential measured in the DPPH assay. Additionally, UV irradiation and sonication did not affect the anti-DPPH activity of the liposomes. However, the trend was different in the results of the ABTS test (Table 1). The ABTS radical scavenging potential of pure extract (5.49±0.12 μmol TE/mL) was significantly lower in comparison to encapsulated counterparts (6.17-7.05 μmol TE/mL). UV irradiation and particularly sonication significantly decreased the anti-ABTS capacity of the extract-liposome system. The obtained differences between the results from the two antioxidant assays are not surprising due to differences in the reactivity of employed free radicals, kinetics of the reactions, targeted compounds, and experimental conditions.

CONCLUSION

Our results showed that carob pulp extract, as well as its different liposomal nutraceutical formulations, show a significant *in vitro* antioxidant potential that may be of interest for the further development of functional food, in order to improve the prevention and treatment of various diseases whose pathogenesis is based on oxidative stress.

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This work was supported by the Ministry of Science, Technological Development and Innovation, Republic of Serbia (No. 451-03-66/2024-03/200019 and by the Provincial Secretariat for Higher Education and Scientific Research of Vojvodina (No. 142-451-3531/2023-01). The authors express gratitude to Z. Šereš, D. Šoronja-Simović, and J. Zahorec for extract preparation.

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Research paper **P A4**

MULTILAMELLAR LIPOSOMES AS A CARRIER FOR *Cotinus coggygria* Scop. EXTRACT

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ABSTRACT

In the present paper, *Cotinus coggygria* ethanol extract-loaded liposomes were formulated to provide better stability and bioavailability of smoke tree biologically active compounds, as well as their prolonged release. The encapsulation efficiency (EE), size, polydispersity index (PDI), zeta potential, and mobility of the prepared liposomal particles with extract were investigated. The EE of extract in liposomes was 96.05±0.06%. The particle size and PDI values were 2888.7±219.9 nm and 0.273±0.089, respectively. The zeta potential was -27.7±0.5 mV, while the mobility was -2.172 μmcm/Vs. Future experiments can be developed with the aim to investigate the biological potential of the obtained liposomes with *C. coggygria* extract.

INTRODUCTION

Cotinus coggygria, also known as the "smoke tree", is one of the two species constituting a small genus of the family Anacardiaceae. The plant, as an important source of essential oils and extracts, shows a wide range of health-promoting effects [1]. Different parts of this plant possess pharmacological properties, such as antiseptic, anti-inflammatory, antimicrobial, hepatoprotective, and antihemorrhagic agents [2]. In addition, ethanol infusions from the wooden parts of the plant were employed to treat gastric ulcers and diarrhea. *C. coggygria* extracts were also used to treat cancer, for eye ailments, as a cholagogue, and as an antipyretic [3].

Liposomes are one of the most widely used carrier systems to protect and deliver bioactive compounds, as they can be manufactured from natural components using simple procedures [4,5]. In addition, liposomal vesicles are of great interest due to their biocompatibility and ability to transport and improve

the bioavailability of both hydrophilic and hydrophobic molecules [5]. Thus, in the present paper, *C. coggygria* ethanol extract-loaded liposomes were formulated to provide better stability and bioavailability of smoke tree biologically active compounds, and their prolonged release.

EXPERIMENTAL

Ethanol (Fisher Science, United Kingdom) and highly comminuted plant material of the wooden part of *C. coggygria* (collected in Belgrade, Serbia) were used for the extraction. Soy L-α-phosphatidylcholine (Avanti Polar Lipids, USA) was employed for the liposomal preparation. *C. coggygria* extract was prepared using 5 g of the wooden part of the plant and 200 mL of 80% ethanol in the ultrasound bath (Sonorex Super RK, Bandelin, Germany) for 30 min. Extract-loaded nanoliposomes were obtained in the previously published proliposome procedure [6]. Ethanol extract (20 mL) was mixed with phosphatidylcholine (2 g), and heated to 60°C for 30 min. After cooling, ultra-pure water was added in small portions to a total volume of 20 mL, and the dispersion was stirred for 2 h at 800 rpm. The encapsulation efficiency (EE), liposome size, polydispersity index (PDI), zeta potential, and mobility of the prepared liposomal particles with extract were investigated using the spectrophotometric method (UV Spectrophotometer UV-1800, Shimadzu, Japan) and photon correlation spectroscopy (PCS, Zetasizer Nano Series, Malvern Instruments, UK), respectively. For the photon correlation spectroscopy, the sample was diluted 500 times, and the measurements were performed in triplicates at room temperature.

RESULTS AND DISCUSSION

The data of the PCS measurements are presented graphically in Figure 1. The EE of *C. coggygria* extract in liposomes amounted to 96.05±0.06%. The vesicle size and PDI values were 2888.7±219.9 nm and 0.273±0.089, respectively. The zeta potential was -27.7±0.5 mV, while the mobility was -2.172 μmcm/Vs. Due to the presented results, it can be concluded that the liposomal population was uniform and multilamellar, whereas a high value of the zeta potential can provide a satisfied stabilization of the system.

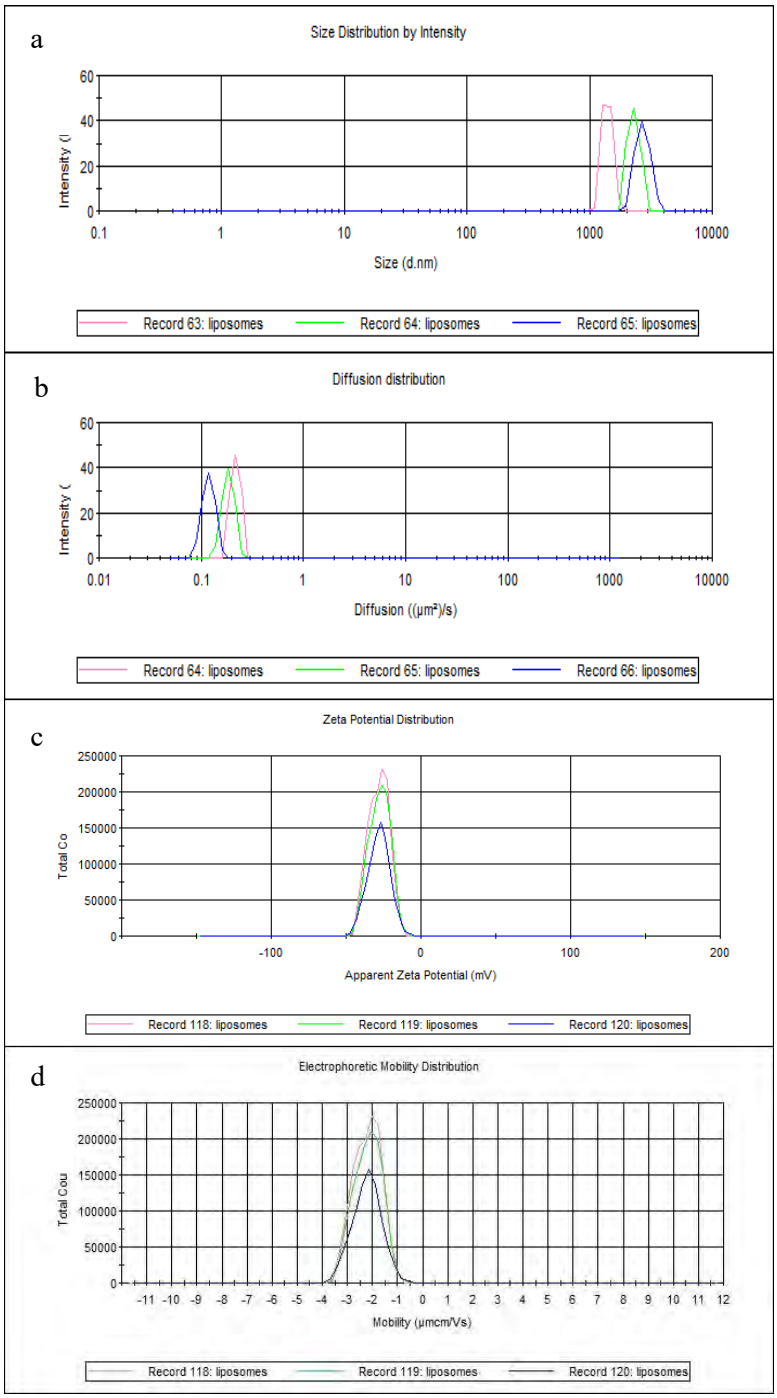

Figure 1. Graphical presentation of *Cotinus coggygria* ethanol extract-loaded liposome (a) size distribution by volume, (b) size distribution by intensity, (c) zeta potential distribution, and (d) mobility distribution.

CONCLUSION

C. coggygria extract-loaded multilamellar liposomes were formulated with the aim of providing better stability and bioavailability of smoke tree biologically active compounds, as well as their prolonged release. The liposomal population was uniform, and a high value of the zeta potential can provide a satisfied stabilization of the system. Further research can be focused on the biological properties of the liposomes.

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