Release of Polyphenols from Liposomes Loaded with *Echinacea purpurea*

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The aim of this study was to develop a delivery system for polyphenols from an extract of Echinacea purpurea leaves, based on liposomes. Liposomes loaded with Echinacea purpurea were prepared and characterized in terms of entrapment efficiency, size, polydispersity index, stability and release behavior. Results showed good entrapment efficiency, small sizes, low polydispersity index and good stability over 90 days at 4°C. Also, the liposomal formulations presented reduced burst release and slow release of polyphenols compared with free extract. Therefore, liposomes offer a great potential in the development of drug delivery systems for polyphenols.

Keywords: polyphenols, Echinacea purpurea, liposomes, drug delivery systems

Most of plant extracts are a rich source of polyphenols. Polyphenols have beneficial implications in health, in the treatment and prevention of digestion issues, diabetes, neurodegenerative diseases, cardiovascular diseases, cancer, weight management difficulties, etc. Also, they are used in food industry as additives (natural antioxidants, natural coloring agents, nutritional additives, preservatives) and in cosmetic industry, especially in anti-aging products. However, polyphenols often present drawbacks, such us: degradation in gastrointestinal media, a low biodisponibility due to poor water solubility, astringent and bitter taste [1, 2]. To improve these limitations, various delivery systems have been developed, like: liposomes, micro-/nano-emulsions, microspheres, micro-/nanoparticles, etc [3-5], and successfully applied to encapsulate various polyphenols. Liposomes are colloidal nanoparticles made of amphiphilic phospholipids with unique features: biodegradability, biocompatibility, non-toxicity, non-immunogenicity and possibility to encapsulate both hydrophobic and hydrophilic compounds. The advantages of encapsulation of polyphenols in liposomes are [6, 7]: better absorption, decrease of toxicity and side effects, decrease of frequency and dose of administration; consequently the patient compliance and the therapeutic effectiveness are improved.

The main objective of this paper was to develop a delivery system based on liposomes for polyphenols; an extract of *Echinacea purpurea* leaves was selected as source of polyphenols. *E. purpurea* contains caffeic acid derivatives (mainly choric and caftaric acids), alkamides, polysaccharides, melanins, lipopolysaccharides, lipoproteins and has immunostimulatory, anti-inflammatory, antioxidant, antibacterial, antiviral, and larvicidal activity [8]. To fulfill the aim of this paper, liposomes loaded with E. purpurea extract were prepared using thin-film hydration method followed by sonication and extrusion processes. The properties of loaded liposomes, like: entrapment efficiency, mean particle size, polydispersity index, stability were analyzed. Also, it was investigated the release behavior from liposomal formulations.

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Experimental part

Materials

Phosphatidylcholine from egg yolk (PC), Folin-Ciocalteu reagent, sodium carbonate, sodium cholate, Triton X-100, gallic acid (95%) were provided from Sigma-Aldrich Co (Germany). All other solvents were analytical grade and used without any other purification. Leaves of *E. purpurea* were harvested from Dambovita County, Romania, Europe and the identification was made by the botanical team of ICCF Bucharest, Romania; a voucher specimen was stored in ICCF Plant Material Storing Room, Romania.

Preparation of E. purpurea extract

E. purpurea was shade dried at room temperature and ground to a powder. Fifty grams (50 g) of raw plant material was extracted with 500 mL ethanol (50% v/v), at reflux temperature for one hour under continuous stirring. The resulted solution was filtered and concentrated at *residue* (60 °C, 72-75 mm Hg). The *spiss* residue (11.52 g) was further solved in ethanol (1:5 plant material/solvent ratio). The extract was stored in refrigerator at 4–8°C until analysis.

Preparation of lipid vesicles containing plant extract

Liposomes loaded with *E. purpurea* were prepared using thin-film hydration method. Phosphatidylcholine (80 mg), either alone or with sodium cholate (20 mg) were dissolved in 10 mL methanol. The ethanolic extract (50% v/v) of *E. purpurea* (corresponding to 25 mg) was added in the lipid solutions. Both solutions were evaporated for 2 h at vacuum at 35°C and after the complete solvent removal, the lipid film was hydrated for 1h with distilled water at 35°C. The dispersions were kept 2 h at room temperature for stabilization. The size of liposomes was reduced through sonication (20 min) followed by extrusion (5 cycle extrusions with 0.4 μm and then 0.2 μm pore size filters). Empty liposomes (coded L1 and L2) were prepared as control. All samples were stored in refrigerator (4–8°C) until analysis.

Entrapment efficiency

The amount of *E. purpurea* loaded in liposomes was separated from free extract by centrifugation at 10000 rpm

and 5°C for 30 min and washed with distilled water. An amount of 0.5 mL Triton X-100 (0.5% v/v) were added on loaded liposomes for breaking down the membranes; then the suspension was diluted with methanol and filtrated. The amount of *E. purpurea* loaded in liposomes was evaluated as total phenol content. Total phenol content was measured with Folin-Ciocalteu method [9], as described in a previous study [10], using gallic acid calibration curve (concentration range: 0.01-01 mg/mL, $y=0.01322x+0.0272$, $R^2=0.99574$). As blank were used samples without Folin-Ciocalteu reagent for liposomes and distilled water for extract. The entrapment efficiency (EE, %) was calculated using the equation (1), where M is the amount of polyphenols loaded in liposomes and M_t is the amount of polyphenols in extract.

$$EE(\%) = \frac{M}{M_t} \times 100 \quad (1)$$

Size and polydispersity index

Size and polydispersity index (PI) of liposomes were assessed by Dynamic Light Scattering (DLS) using a particle size analyzer (Beckman Coulter N4 PCS Submicron, Coulter Company). The determinations were performed at 25°C (detector position: angle 90°, solvent refractive index: 1.332; solvent viscosity: 0.871 cP) using 10 runs for each measurement on diluted samples (1:10).

In vitro release study

In vitro release studies were performed for liposomes and free extract (as control) using dialysis membrane method. An amount of 1.0 mL of each sample was placed in a dialysis bag (14000 molecular weight cut-off) and immersed into 50 mL distilled water, at 37°C and 100 rpm/min. An amount of 1.0 mL was taken at predetermined times (15, 30, 45, 60, 120, 180, 240, 300, 360, 600 min and 24 h) and an equal amount of fresh medium replaced. The cumulative release rate (CDR) was calculated using the equation, where Q_n was the phenol content at time n and Q_t was the phenol content initially entrapped in liposomes. The phenol content was determined by spectrophotometry using Folin-Ciocalteu assay.

$$CDR(\%) = \frac{Q_n}{Q_t} \times 100 \quad (2)$$

Statistical analysis

Values are represented as mean ± standard deviation (SD) for three replicate samples. Differences were considered significant at $p < 0.05$.

Results and discussions

Characterization of liposomes loaded with E. purpurea

The liposomes loaded with *E. purpurea* extract were characterizated in terms of size, polydispersity index, entrapment efficiency and stability over 90 days. Size and polydispersity index were shown in table 1. All samples were in nanometer size range, with values below 200 nm. It can be observed an increase of mean size at the incorporation of *E. purpurea* extract in liposomes with ~ 63.3 for EP_L2, and respectively ~ 113.7 for EP_L1. Similar results were reported in other papers, an increase of particle size was reported at the incorporation of *Polygonum aviculare* extract [11], grape-seed extract [12, 13] , *Glycyrrhiza glabra* root extract [14], etc. The significant difference between formulations (EP_L1 vs EP_L2) was caused by the addition of sodium cholate, an edge activator; this edge activator entered in competition with the extract and therefore only a smaller quantity of extract was accommodated in the liposomes, resulting in a size decrease. Also, all formulations presented polydispersity index values lower than 0.4 which indicate a less pronounced tendency to aggregation.

Entrapment efficiency and the effect of storage on the entrapment efficiency

The entrapment efficiency of *E. purpurea* and the stability of *E. purpurea* liposomes (evaluating entrapment efficiency at different storage time respectively 30, 60 and 90 days) were presented in figure 1. Samples presented high entrapment efficiency, 81.09 ± 0.23% for EP_L2 and 86.01 ± 0.57% for EP_L1, confirming that thin-film hydration provide an efficient encapsulation for *E. purpurea*. Similar results were reported in several papers, for example an EE = 83% for *Polygonum aviculare* liposomes [11], and respectively EE = 74% for *Artemisia arborescens* [14]. Also, the liposomes loaded with *E. purpurea* were stable at 4°C for at least 90 days with minimal plant material loss.

In vitro release study

In this experiment, the dialysis bag method was employed to study the release of *E. purpurea* loaded in liposomes comparatively with free extract; the resulted were shown in figure 2. The quantity of *E. purpurea* released was evaluated as cumulative release percent of polyphenols. All amount of pholyphenols from free extract were released in ten hours (99.35 ± 0.57%), while lipid formulations showed a slower release, reaching in one day 70.05 ± 4.05% and 60.85 ± 4.02% for EP_L1 and EP_L2, respectively. Release behaviour of sodium cholate formulations was higher than formulations without sodium cholate, a possible explanation being the deformable nature. Also, it can be observed a *burst release* in release profile from free extract (eg. 48.92 ± 2.03% was released in the first hour); the burst effect was attenuated by encapsulation of extract in lipid vesicles (34.29 ± 0.13% was released in first hour from EP_L1 and respectively 29.47 ± 0.33 % from EP_L2).

The mechanism of *in vitro* polyphenols release was investigated by applying several kinetics models (Zero-order, First order, Higuchi and Hixson-Crowell), the equations of the mathematical models are showed in tabel 2 . The models were compared using the correlation coefficient (R^2) and the highest value of R^2 showed the model that best fitted our data. It was noticed that *in vitro* release of polyphenols from free *E. purpurea* was better explained by First order model ($R^2 > 0.98$). Higuchi's model was found to be the best fitting model for release from liposomal formulations ($R^2 > 0.95$), indicating that polyphenols transported from lipid vesicles was driven mainly by a diffusion-controlled mechanism. Similar results

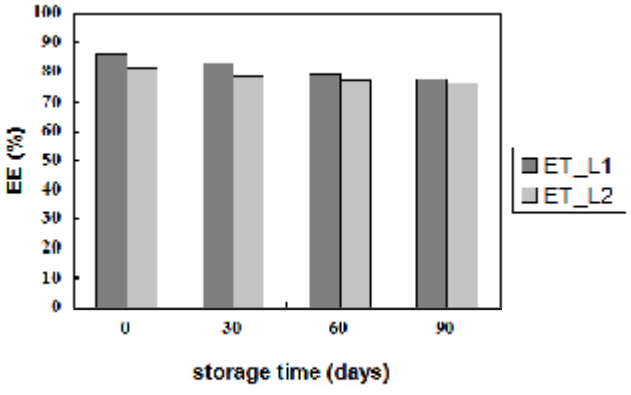

Fig. 1. The effect of storage on the entrapment efficiency of liposomes loaded with *E. Purpurea*

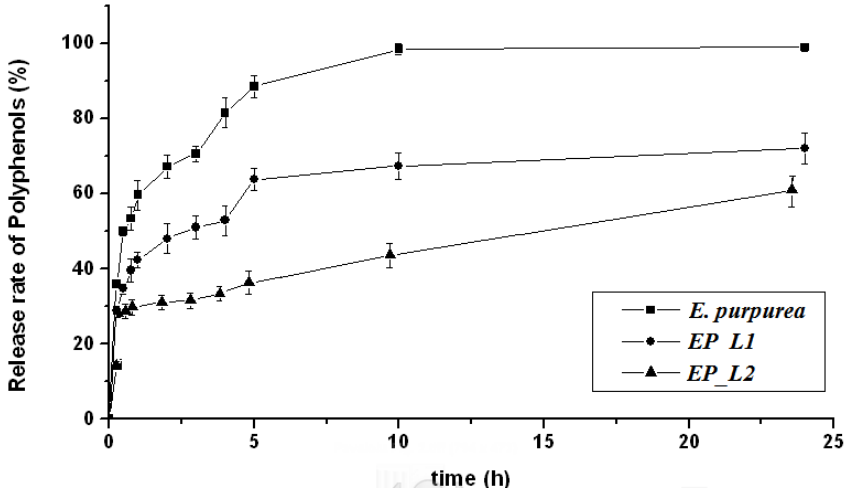

Fig. 2. Release of polyphenols from liposomes loaded with *E. purpurea* vs. free extract.

Sample code	PC (mg)	sodium cholate (mg)	Extract (mg)	size (nm)	polydispersity index
L1	80	-	-	85.40 ± 0.34	0.400 ± 0.01
L2	80	20	-	105.20 ± 2.04	0.379 ± 0.02
EP_L1	80	-	25	199.10 ± 0.32	0.455 ± 0.01
EP_L2	80	20	25	168.50 ± 0.11	0.009 ± 0.01

Table 1
COMPOSITION AND CHARACTERISTICS OF LIPID VESICLES

Model	Model equation	Sample code	R^2
Zero order	$Q_t = Q_0 + k_0 \cdot t$	*E. purpurea*	0.6122
		EP_L1	0.7338
		EP_L2	0.4153
Hixon	$Q_0^{1/3} - Q_t^{1/3} = k_{HC} \cdot t$	*E. purpurea*	0.7199
		EP_L1	0.8083
		EP_L2	0.6635
Higuchi	$Q = k_H \cdot t^{1/2}$	*E. purpurea*	0.8696
		EP_L1	0.9509
		EP_L2	0.9508
First order	$\ln(Q_t / Q_0) = -k_1 \cdot t$	*E. purpurea*	0.9800
		EP_L1	0.8371
		EP_L2	0.8044

Table 2
RELEASE KINETICS MODELING OF LIPOSOMES LOADED WITH *E. PURPUREA* AND FREE EXTRACT

were reported in other studies, for example release of pholyphenols from liposomes loaded with grape-seed extract was explained by a diffusion-controlled mechanism [15, 16].

Conclusions

Liposomes loaded with *E. purpurea* were prepared using thin-film hydration method and characterizated for entrapment efficiency, mean particle size, polydispersity index, stability and release. Evaluation of loaded lipid vesicles showed good entrapment efficiency (~80%), small sizes (lower than 200 nm), low polydispersity index and good stability over 90 days at 4°C. The liposomal formulations reduced burst release and provided slow release of polyphenols compared with free extract. Therefore, liposomes offer a great potential in the development of drug delivery systems for polyphenols.

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