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LIPOSOMES WITH PLANT EXTRACTS IN THE TREATMENT OF CARDIOVASCULAR DISEASES

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Abstract

Worlwide, cardiovascular diseases (CVDs) is the leading cause of death, taking an estimated 17.8 million lives each year. CVDs include coronary heart disease, rheumatic heart disease, cerebrovascular disease and other conditions. In order to help the treatment of CVDs, this work aims to develop a food supplement based on liposomes. The market for food supplements is growing rapidly but many active ingredients have low bioavailability, especially when taken in high doses. Therefore, this paper is focused on obtaining and characterisation liposomes containing a combination of two plant extracts of Sambucus ebulus and Lycium barbarum. The preparation of liposomes was done by the technique of hydration of the lipid film. Reduction of particle size and degree of uniformity was ensured by the sonication process of the liposomal solutions. The formulations were characterised in terms of entrappment efficiency, particle size, polydispersity index, stability, and the evaluation of antioxidant activity was assessed by DPPH method.

Key words*: lipsomes, cardiovascular, Sambucus ebulus, Lycium barbarum, plant extracts.*

INTRODUCTION

Worldwide, cardiovascular diseases (CVDs) are a multifactorial condition responsible for approximately 17.5 million deaths each year. An estimated 17.9 million people died from CVDs in 2016, accounting for 31% of all deaths globally. Of these deaths, 85% occurred as a result of heart attack and stroke.The World Health Organization estimates that by 2030, this number of patients will increase to approximately 24 million (McMurray et al., 2012; W.H.O Report, 2019).

In Europe, CVDs rank first as a cause of mortality, with a higher prevalence in women (56 %) compared to men (44 %). Each year, approximately 4.40 million deaths in the member states of the World Health Organization - European Region, and approximately 2.0 million deaths in the European Union states were caused by CVDs (W.H.O Report, 2019).

According to the European Society of Cardiology, there is an increasing slope in the risk of death from CVDs in Europe, starting in western countries and reaching to the eastern countries. Therefore, Romania is included in the category of high-risk countries, immediately followed by Ukraine and Bulgaria. Thus, annually, approximately 60% of Romanians die due to a form of CVDs. Also, in terms of the mortality rate of the population due to CVDs, at the end of 2017, Romania ranked first in Europe and third in the world (approximately 109 deaths per 100,000 inhabitants, compared to the average offered by the European Union - about 44 per 100,000 inhabitants) (W.H.O Report, 2019).

Following these statistics, currently, the medical-pharmaceutical specialists places a special emphasis on the prophylaxis of CVDs, on early diagnosis, the correct treatment of these categories of patients, as well as on the long-term follow-up of patients by applying primary, secondary preventive measures and some cardiovascular recovery programs.

The market for dietary supplements for prevention of CVDs is expanding quickly, but many of the active ingredients - vitamins, minerals, and other substances - have low bioavailability, particularly when taken in high dosages.

Herbal therapeutic products are used worldwide, according to the WHO, with an estimated 80% of the world's population preferring natural treatments as the first alternative in preventing and combating various diseases. The advantages of products based on

natural compounds over conventional drugs are: a lower risk of side effects, wide availability, low cost, etc. (Sobhani et al., 2017). However, the use of natural extracts/natural compounds in biomedical applications is limited, due to the fact that they present various drawbacks, such as: toxicity, instability in very acidic pH environments, solubility and absorption problems; these inconveniences can lead to a low level of the concentration of the active principle(s) in the plasma, resulting in an ineffective therapeutic effect. To overcome these drawbacks, various innovative delivery systems (NDDS) are proposed: liposomes, micro/nano emulsions, microspheres, nanoparticles, etc. (Lacombe et al, 2017).

Particulate delivery technologies, such as liposomes, have gained attention because they can restructure a drug's *in vivo* behavior to lessen toxicity and solubilize water-insoluble active substances into nanoscale structures (Guo & Huang, 2014; Lee, 2020). Because liposomes are biocompatible enough to be allowed for parenteral administration, they have undergone the most testing of any nanoparticles (Allen & Cullis, 2013; Lee, 2020). Water-insoluble substances can be dissolved into the lipid domain of the liposomal membrane by liposomes, which are vesicles surrounded by phospholipid bilayers (Guo & Huang, 2014; Allen & Cullis, 2013; Has & Sunthar, 2019).

The structural and compositional similarity of liposomes with bio-membranes, in addition to their solubilizing ability and biocompatibility, has promoted their application for non-invasive oral delivery of poorly-permeable substances. The comparatively large size of liposomes has presented a number of challenges, including instability in the gastrointestinal tract and low permeability across the intestinal epithelia (He et al., 2019).There are numerous studies on the use of liposomes for the transport and release of natural phytoconstituents/extracts. Liposomes have been used to encapsulate plant extracts, such as: ginkgo biloba (*Ginkgo biloba*), grape seed (*Vitis vinifera*), milk thistle (*Silymarin marianum*), green tea (*Camelia sinensis*), ginseng (*Panax ginseng*), etc., or phytoconstituents, such as: vincristine, curcumin, quercitin, resveratrol (Alexander et al., 2016)

Formulation of natural extracts/natural compounds into liposomes is one of the strategies to improve bioavailability and thus potentiate the biological activity of natural extracts/compounds.

Moreover, there are numerous clinical studies for the evaluation of liposomes with natural compounds/plant extracts in various biomedical applications, such as:

a) evaluation of the therapeutic activity of liposomes with Ginkgo biloba (Agheron phytosome) - the study was conducted on patients affected by chronic venous disorders of the lower limbs (Montes et al., 2015)

b) evaluation of the effect of liposomes with Ginko biloba in the treatment of migraines (Montes et al., 2015)

c) evaluation of the release of silybin from liposomes to a target organ - study carried out on patients who previously underwent surgery to remove gallstones (Kidd & Head, 2005)

d) evaluation of the efficiency of liposomes with silybin - study carried out on patients with chronic hepatitis (Kidd and Head, 2005)

e) the effect of liposomes with green tea extract on obesity (Matsuda et al., 2016; Lin et al., 2020)

f) the effect of lipsomes with curcumin on diabetes symptoms (Antiga et al., 2015)

Therefore, this paper is focused on obtaining and characterisation liposomes containing a combination of two plant extracts of *Sambucus ebulus* and *Lycium barbarum*. The preparation of liposomes was done by the technique of hydration of the lipid film. Reduction of particle size and degree of uniformity was ensured by the sonication process of the liposomal solutions.

The formulation was characterised in terms of entrappment efficiency determined by UV-Vis spectrophotometry, particle size and polydispersity index assessed by the technique of dynamic light diffusion, and antioxidant activity evaluated by DPPH method. Also, all liposomes were characterised in terms of stability for three months.

MATERIALS AND METHODS

Materials

Phosphatidylcholine (from egg yolk), Folin-Ciocalteu reagent, 2,2-diphenyl-1-

picrylhydrazyl (DPPH), sodium carbonate, sodium cholate, Triton X-100, were purchased from Sigma-Aldrich Co (Germany).

Plant material and Lycium barbarum and Sambucus ebulus extract preparation

The mature leaves of *Lycium barbarum* (LB) and *Sambucus ebulus* (SE) were collected in Dambovita County, Romania (45°18'15.4" N; 25°23'28.4" E). The botanical team at the National Institute of Chemical-Pharmaceutical R&D (ICCF) in Bucharest, Romania, made the taxonomic identification. Based on their greater quantities of polyphenolic compounds that have a strong protection capacity against reactive oxygen species, LB and SE leaves were chosen as the vegetal material for the preparation of the extracts (Koehn & Carter, 2005; Vlachojannis et al, 2010; Zhou et al, 2017). LB and SE extracts were obtained by a technique described by Pavaloiu et al. (Pavaloiu et al, 2019; Pavaloiu et al, 2021). Therefore, an ethanolic extract of LB leaves with a polyphenol content of 18.30 ± 0.010 milligrams of gallic acid equivalents (mg GAE)/g of dry material and respectively an ethanolic extract from SE leaves with 25.50 ± 0.010 mg GAE/g dry material were combined and entrapped into liposomes.

Preparation of liposomes loaded with LB and SE extracts

The thin-film hydration method was used to produce liposomes that were loaded with SE and LB, fixed-dose (1:1 w/w) before being sonicated and extruded. In a brief, 10 mL of methanol was used to dissolve phosphatidylcholine, sodium cholate, and LB and SE extracts with various ratio (8:2:2.5; 9:1:2.5; 10:0:2.5 w/w).

The solutions were then maintained at room temperature for one night to facilitate the swelling of the phosphatidylcholine. The lipid solutions were added to a round-bottom flask and evaporated for two hours at 37°C under vacuum using a Laboranta 4000 Rotary evaporator from Heidolph Instruments GmbH & Co. KG. The resulting thin lipid layer was hydrated with distilled water at 37°C after the entire solvent had been removed. The resultant dispersions were stabilized for 2 hours at room temperature. Liposomes were sonicated, then extruded, to shrink their size. During 20 minutes, the sonication procedure was carried out in an ice-filled sonication bath (Sonorex Digital 10P, Bandelin Electronic GmbH & Co), with power delivery controlled by a 20% percentage amplitude. By utilizing 0.4 m and then 0.2 m pore size filters with 6 cycle extrusions for each, the extrusion was done sequentially. Centrifugation at 10,000 rpm and 5°C for 30 minutes was used to separate the loaded liposomes from the free extracts. The supernatant was then removed, and the sediment containing the loaded liposomes with LB and SE was re-dispersed in water. As controls, empty liposomes were prepared. Triplicates of each sample were prepared, and they were all kept at 4°C.

Table 1. Preparation of liposomes - parameters

Sample	PC: Sodium colat (w/w)	Plant Extracts (mg)	Parameters
P1	80:20	25	$t_{evaporation}$ = 37°C
P2	90:10	25	
P3	100:0	25	$t_{hydration}$ = 37°C
L1	80:20	-	
L2	90:10	-	Stirring rate = 200 rpm
L3	100:0	-	

Characterisation of liposomes loaded with LB and SE extracts

Particle size, polydispersity index (PDI), and entrappment efficiency (EE) of liposomes containing LB and SE extracts were assessed. The first step to evaluate the EE was the separation of liposomes loaded with SE and LB extracts from the free extracts combination by centrifugation (10,000 rpm at 5°C, 30 min), followed by redispersion of sediment in water. After two centrifugation cycles, the pellets were mixed with 0.5 ml Triton X-100 (0.5%) and vortexed to break up the lipid membrane. The final suspensions were diluted ten times with methanol. The Folin-Ciocalteu method, which has been described in multiple studies (Miere et al., 2019; Miere et al., 2021; Pavaloiu et al., 2019) was used to measure the EE. Samples without the Folin-Ciocalteu reagent were used as a control. Using a particle size analyzer and the Dynamic Light Scattering (DLS) method, the mean diameter and PDI of liposomes were determined (Beckman Coulter N4 PCS Submicron, Coulter Company, France). The stability of liposomes containing the combination of SE and LB extracts was

evaluated by EE assessment at different storage periods of time. The samples were kept in amber-coloured glass vials and stored at a temperature of 4°C for three months.

Radical Scavenging Activity Assessment

Using the Sanchez-Moreno et al. method, antioxidant activity was evaluated for the liposomes containing LB and SE extracts (Sanchez-Moreno et al., 1998). 2950 mL of the DPPH methanolic solution (0.025 g/L) were combined with 50 mL of each sample at various concentrations. For 30 minutes, the mixtures were maintained at 25°C in the dark. Using a UV/VIS spectrophotometer (Helios, Thermo Electron Corporation), the absorbance was determined at 517 nm. Five repetitions of the experiment for the antioxidant activity were run.

The following equation was used to calculate the percentage of DPPH inhibition:

$$\%inhibition = \frac{A_0 - A_{sample}}{A_0} \times 100$$

where A_0 is the absorbance of the control (without sample) and A_{sample} is the absorbance in the presence of the sample.

Statistical analysis

At least three replicate samples were used in each experiment, and the results were given as mean ±standard deviation (SD). At $p < 0.05$, differences were considered significant.

RESULTS AND DISCUSSIONS

Liposomes have attracted much attention as one of the most promising vehicles for the delivery of bioactive substances, owing to their remarkable properties: biocompatible, biodegradable, non-immunogenic, non-toxic, and the ability to incorporate hydrophilic and hydrophobic compounds. These unique properties therefore enable liposomes to enhance solubility, improve bioavailability, modulate pharmacokinetics, increase cellular uptake, and provide greater stability of bioactive compounds from plant extracts. Several papers showed that liposomes can enhance therapeutic activity for quercetin (Priprem et al., 2008), silymarin (El-Samaligy et al., 2006), curcumin (Sinjari et al., 2019), resveratrol (Joraholmen et al., 2015), rutin (Bonechi et al., 2018), chamomile (Das et al., 2019), etc.

Characterization of liposomes loaded with combination of SE and LB

The film hydration process, followed by sonication and extrusion, was used to produce the liposomes loaded with SE and LB extracts. The characteristics of liposomes influence their behavior *in vitro* and *in vivo*. Therefore, their characterization is an essential step in order to use them in the biomedical field. The size, PDI, EE and three-month stability of liposomes loaded with SE and LB extracts were determined. As a control, empty liposome were used. Table 2 lists the features of the liposomes.

Table 2. Features of liposomes loaded with combination of SE and LB extracts

Sample Code	Particle size (nm)	PDI
P1	196.1 ± 0.421	0.177 ± 0.001
P2	187.6 ± 0.560	0.114 ± 0.001
P3	167.6 ± 0.360	0.124 ± 0.001
L1	100.01± 0.141	0.300 ± 0.003
L2	96.76± 0.151	0.280 ± 0.003
L3	86.1± 0.211	0.298 ± 0.003

The empty liposomes had a smaller value (86.1± 0.211 - 100.01± 0.141 nm), while the encapsulation of combination of SE and LB extracts increased the particle size (167.6 ± 0.360 - 196.1 ± 0.421 nm).All liposomal formulations had particle sizes that were less than 200 nm.

A surfactant called sodium cholate may destabilize the phospholipids in an empty liposome, which would increase the size of the vesicle because of its intercalation in the phospholipid bilayer's structure (Gupta et al., 2012). The incorporation of compounds from plant extracts in the vesicle structure led to the increase of particle size following the extracts loading process. Differently polarized molecules are present in both the SE and LB extracts. Their distribution within liposomes is distinct: non-polar molecules are situated in the bilayer, and polar ones in the watery inner core, both of which lead to an increase in particle size (Castangia et al., 2015). Furthermore, the

addition of non-polar substances may result in a fluidization of the liposome membrane by disrupting the phospholipid bilayer by a deep insertion of polyphenols (Castangia et al., 2015). The EE of the combination of SE and LB extracts in liposomes was higher than 75% (reported in Figure 1). These results demonstrated that the preparation technique was effective and that there was minimal extract loss during preparation. Similar outcomes for the entrapment of *Polygonum aviculare* (EE = 83%), *Glycyrrhiza glabra* (EE = 84%), and *Artemisia arborescens* (EE = 74%) (Castangia et al., 2015; Sinico et al, 2005; Soon et al, 2015). Moreover, sodium cholate used as an "edge activator" in liposomes can improve the flexibility of the lipid layer, which leads to the incorporation of a greater amount of polyphenols (Gupta et al., 2012).

The PDI values show the homogeneity of the systems. In general, PDI values less than 0.1 indicate a homogeneous population, while values greater than 0.3 show high heterogeneity (Danaei et al., 2018). All liposomes showed a narrow size distribution (PDI < 0.3), demonstrating good homogeneity and low aggregation tendency.

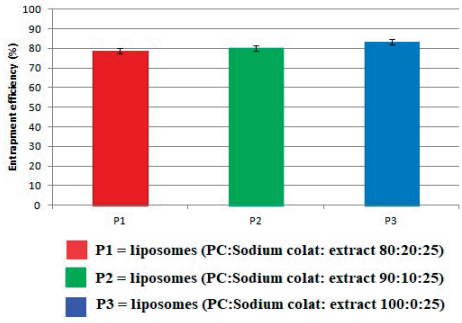

Figure 1. Values of EE of the combination of SE and LB extracts in liposomes

Antioxidant activity of liposomes loaded with SE and LB extracts

Serious health problems like cancer, cardiovascular disorders, cataracts, and diabetes are brought on by the oxidation of biomolecules (Berger, 2005). Utilizing antioxidant chemicals is one potential defense against oxidation. Due to the negative consequences of synthetic antioxidants, such as the potential for liver damage and carcinogenesis, more people are choosing to use natural antioxidants instead of synthetic ones. To guard diverse food products from oxidation, herbal extracts must be used as natural antioxidants (Komes et al., 2011). Polyphenolic chemicals, which can be utilized to stop oxidation and lengthen the shelf-life of delicate food material, are one of the most significant families of natural antioxidants (Rispail et al., 2005).

Numerous therapeutic plants contain antioxidants, primarily polyphenols, which are known as free radical scavengers and are recognised to reduce the degenerative consequences caused by oxidative stress (Forman et al., 2014; Halliwell, 2008; Vinson et al., 2001). As a by-product of aerobic respiration's mitochondrial electron transport or as a result of oxido-reductase biocatalysts and metal-catalyzed oxidation, ROS (Reactive Oxygen Species) are frequently created in a variety of reactions in living organisms (Hancock et al, 2001). Even at low doses, polyphenols can protect cells by inhibiting oxidative stress, which halts the oxidative stress-dependent molecular damage via a number of pathways (Galano et al., 2016). Due to the presence of several particular groups, specifically phenylhydroxyl groups, the major mechanisms of phenolic compounds as antioxidant agents have been documented in the literature (Galano et al., 2016; Pereira et al., 2009). Due to the way these groups behave as hydrogen donors, they have the ability to combine with reactive nitrogen species and ROS to produce a phenoxy radical (Pereira et al., 2009).

In this paper, the antioxidant activity of ethanolic extracts of LB and SE leaves was determined compared to the combination of SE and LB extracts. Also, as a control, empty liposome were used. The highest value of the antioxidant activity was presented by sample P3. Due to the lack of sodium colate in the structure of P3 liposome, there was not a competition between the plant extracts mixture and the sodium colate, therefore the EE and the ability of protection against reactive oxygen species were higher than other samples. Figure 2 shows the values of the antioxidant activity of the lipid vesicles loaded with extracts determined by the DPPH method. As controls

were used free liposomes and the combination of SE and LB extracts.

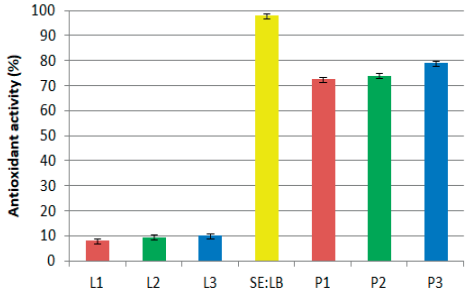

Figure 2. Antioxidant activity of liposomes loaded with SE and LB extracts (P1, P2, P3) compared to the mixture of SE and LB
L1,L2, L3 - liposomes without plant extract (PC:Sodium colat 80:20; 90:10; 100:0)
P1, P2, P3 - liposomes with plant extract (PC:Sodium colat:plant extract 80:20:25; 90:10:25;100:0:25)

Stability of liposomes loaded with combination of SE and LB

The stability of liposomes containing SE and LB extracts was investigated during a three-month period. The samples were kept in the dark at 4°C to prevent the oxidation and hydrolysis of lipids, processes caused by light. EE evaluation was used to determine the samples' stability across varied storage durations (up to three months). Liposomes containing SE and LB extracts were stable during three-month period, losing about the same amounts of plant compounds after one month (below 0.75%) and after three months (plant compunds loss below 4.50%) (Table 3). The liposomes loaded with SE and LB extracts were also visually stable because storage didn't result in any sedimentation. These findings could most likely be explained by higher Brownian motion and diffusion rates than those caused by gravitational-induced sedimentation (due to liposomes) (Gharib et al., 2017). Antioxidants with high sensitivity are polyphenols. In light of this, a number of variables, including oxygen content, an alkaline pH, and even their concentration, can weaken their antioxidant abilities (Zou et al., 2017). To prevent oxidation and degradation, increase the stability of the molecules during storage, and maintain their antioxidant action, these compounds can be encapsulated in a carrier system (Dehkharghanian et al., 2009). Lipid-based carrier systems have the most applications in the food and nutraceutical industries of all the entrapment methods now in use. These carrier systems are utilized to transfer materials to the desired place inside or outside the body while encapsulating and protecting compounds with varied solubilities.

The stability of the plant extracts against unfavorable environmental factors is increased when it is enclosed in a liposome. This effect might be brought on by high concentration of polyphenols and controlled oxygen levels, which considerably improve polyphenol stability (Bummer, 2004; Dehkharghanian et al., 2009). Because liposomes have membranes, the reaction between oxygen and polyphenols can only take place to a certain extent. Additionally, encapsulated polyphenols in liposomes degrade more slowly than free polyphenols because of their sluggish release (Bummer, 2004). The most widely used lipid-based entrapment technology, liposomes have a number of benefits, such as being made of natural materials, having the capacity to entrap bioactive materials with various solubilities, and preventing the oxidation of ingredients by free radicals, metal ions, and enzymes (Bummer, 2004).

Tabel 3. Entrapment efficiency of liposomes loaded with SE and LB for three-month period

Code	EE (%)	EE (%) 1 month	EE (%) 2 months	EE (%) 3 months
P1	78.60 ± 1.230	77.98 ± 1.030	76.50 ± 1.050	75.80 ± 1.130
P2	80.23 ± 1.761	79.89 ± 1.361	78.21 ± 1.091	77.23 ± 1.341
P3	83.43 ± 1.061	82.69 ± 1.071	81.33 ± 1.081	79.23 ± 1.231

CONCLUSIONS

Three formulations of liposomes loaded with a combination in a fixed-dose of *Sambucus ebulus* and *Lycium barbarum* with various ratio of PC:sodium colat:mixture of plant extracts (8:2:2.5; 9:1:2.5; 10:0:2.5 w/w) were obtained by the film hydration method combined with sonication and extrusion and characterized in terms of entrapment efficiency, size, polydispersity, and stability. Also, for all liposomal formulations it was investigated the antioxidant activity using the DPPH method.

Loaded liposomes presented small sizes (less than 200 nm), high entrapment efficiency (more than 75%), and a low polydispersity index. Also, all samples presented good stability over three months at 4 °C and a significant antioxidant activity. Our findings imply that liposome encapsulation might be a great method for delivering some antioxidant compounds, such as polyphenols, but more research is required.

Our results suggest that liposome encapsulation may be considered a great strategy for the delivery of plant extracts/polyphenols, but further research is needed to evaluate the cytotoxicity, the efficiency and the release profile of polyphenols from liposome formulations.

In conclusion, an innovative and alternative technology that can enhance health is liposomal technology.

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