FUNCTIONAL FOOD BASED ON A COMBINATION OF POLYPHENOLS AND POLYSACCHARIDES WITH EMERGENT PROPERTIES FOR HYPOCALORIC DIET

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Abstract

The aim of this work was to develop a functional food used as an adjuvant for hypocaloric diet, based on a combination of polyphenols and polysaccharides extracted from berries pomace and chia seeds, presenting emergent properties. These bioactive compounds were encapsulated into liposomes before being added in the composition of a new protein bar. The antioxidant activity of polyphenols-polysaccharides combinations in different ratios was determined by TEAC and DPPH assays. It was observed that the mixture of polyphenols extracted from berries and polysaccharides extracted from chia seeds in a weight ratio of 5:1 had the highest antioxidant activity. A study of α-amylase inhibition in the presence of polyphenols, polysaccharides and their combinations was also performed to investigate the synergism of these compounds. A liposomal formulation of the selected synergistic combination was obtained in powder form by lyophilization. A final product including the synergistic combination was prepared in the form of a hypoglycemic food bar with amplified antioxidant effects. The product is further recommended for potential application as an adjuvant for a low-calorie diet.

Keywords: *Functional food, polyphenols, polysaccharides, forest fruits pomace, chia seeds, antioxidant activity, protein bar*

1. INTRODUCTION

Food products and dietary supplements contain bioactive compounds that have beneficial effects on human health, besides their basic nutritional properties [1]. They are known to exhibit antioxidant, antimicrobial and anti-inflammatory activities, which vary according to their chemical structure and amount [2]. Bioactive components can be extracted from wastes and can be further used for the development of functional foods. Due to the hydrophobicity and low bioavailability of some bioactive compounds, there is a need for adequate delivery systems, which may help to increase their functionality and to perform specific functions into the body. In this context, nanoemulsions have gained great attention and have been used as a delivery system not only for enhancing the stability, solubility, as well as the bioavailability of bioactive compounds in the body, but also to protect them from adverse environmental conditions (pH, light, moisture, temperature, etc).

Because of their plethora of health benefits, polyphenols are a good source of bioactive compounds for the development of functional foods, dietary supplements or in the cosmetic industry. However, due to the presence of multiple hydroxyl groups in their structure, polyphenols are highly unstable when are exposed to different conditions, such as light, heat or high pH. Moreover, many polyphenols have poor solubility into the aqueous solutions used for the preparation of nutraceutics or cosmetic products. This feature represents a technological drawback and restricts polyphenols incorporation into several nutraceutic products.

The astringency is another specific polyphenols property, which limits their use to enrich functional foods. Polyphenols astringency is related to their capacity to precipitate salivary proteins, especially salivary α-amylase, in the mouth of animals and humans and thus, to generate an unpleasant taste of teeth and tongue drying [3]. However, α-amylase inhibition function is beneficial in the case of supportive treatment of type-2 diabetes or metabolic syndrome [4]. Another limitation of polyphenols use in functional foods is related to the gastric barrier. In the stomach, some polyphenols suffer

modifications, which impair their absorption and bioactivity [5]. Therefore, it is of considerable interest to develop delivery systems to overcome these problems related to the use of polyphenols.

Among the latest developed delivery systems are those based on different types of biopolymers, such as polysaccharides, which can form together with polyphenols a complex system with emergent properties [6, 7]. The most known emergent property is the synergism, i.e. reciprocal enhancement of the biological activity of different bioactive ingredients. Previous clinical studies have showed that chia seeds and polyphenols from forest berries could lower the postprandial blood glucose level [8-10].

Forest fruit pomace is the waste obtained after juice production and it is, generally, not further processed. It is known that ~30% of the initial quantity of forest fruits is represented by the dried residue of pomace [11]. Most of the water-soluble antioxidants from forest fruits are already extracted during the juice extraction process. However, the skins and the seeds remain in pomace, which includes the majority of the polyphenols associated with the plant lignocellulosic cell wall. It was reported that the polysaccharides were easier extracted after juice extraction process, due to partial depolymerization and reduced viscosity [12]. Polyphenols were known for their antioxidant activity [13], but in the last decades, evidences were accumulated probing the antioxidant effect of polysaccharides [14].

The aim of this work was to develop a functional food based on a combination of polyphenols and polysaccharides from forest fruit pulp and chia seeds with emergent properties useful as adjuvant for hypocaloric diet (Fig. 1). According to the best of our knowledge, such combination was not yet studied. Our approach was to select the synergistic combination of polyphenols and polysaccharides, to test its capacity to inhibit α-amylase activity and the antioxidant capacity, to prepare its nanoformulation and to include it into the composition of a functional food.

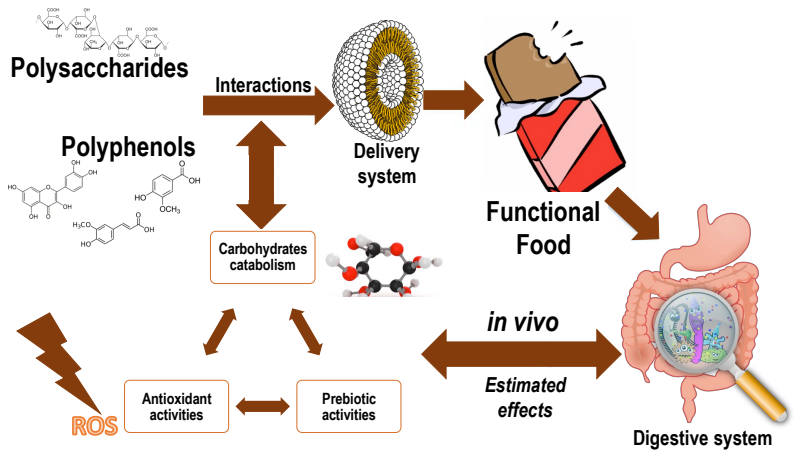

Fig. 1. Scheme of functional food development and characterization in the current study

2. MATERIAL AND METHODS

2.1. Materials

Berries (*Vaccinium myrtillus, Vaccinium vitis-idaea*, *Hippophaea rhamnoides* and *Ribes sp.*) pomace, in the form of dry powder of seeds, skin and residual pulp resulted after juice production, together with chia (*Salvia hispanica* L.) seeds were provided by the local company of food supplements, SC Santo Raphael SRL.

Chemicals: high purity, thermostable α-amylase (E.C. 3.2.1.1) from Bacillus sp. (3000 U/mL) was purchased from Megazyme (Ireland). Folin-Ciocalteu reagent, anthrone, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), HPLC-grade acetonitrile 99.9%, 3,5-dinitrosalicylic acid (DNS), acarbose and other analytical grade reagents were purchased from Sigma-Aldrich (Germany), unless otherwise specified.

2.2. Methods

Extraction of polyphenols and polysaccharides: The extraction of polyphenols from berries pomace and chia seeds, respectively, was conducted in ethanol:water 70:30 (v/v), in a ratio of 1:10 (w/v) under magnetic stirring, at room temperature, for 24 h. The extract was centrifuged at 9000 g, for 20 min and then, the procedure was repeated. The supernatants were reunited, evaporated in a rotary evaporator (Heidolph, Germany) and stored in an exicator, until further analyses. The polyphenolic extract from berries pomace was further noted PP-BR, while that from chia seeds was noted PP-CS.

The remaining residue after polyphenols extraction from berries pomace and chia seeds, respectively, was further used for polysaccharides extraction. The method consisted in residue incubation in distilled water, in a ratio of 1:10 (w/v), in Soxhlet equipment operated at 100°C, for 1 h. After filtration, the procedure was repeated. The reunited supernatants were precipitated using chilled ethanol solution, in a ratio of 1:3 (v/v) and incubated at 4°C. After centrifugation at 9000 g, for 20 min, the precipitate was washed with distilled water, lyophilized and then, stored in an exicator, until further analyses. The polysaccharidic extract from berries pomace was noted PS-BP, while that from chia seeds was noted PS-CS. The extraction yield was calculated as percentage from the initial dry weight (d.w.).

Determination of total phenolic content of bioactive extracts was determined using the Folin-Ciocalteu assay, as previously described [15]. Briefly, a volume of 150 µL sample was mixed with 750 µL Folin-Ciocalteu reagent and incubated in the dark, for 5 min. Then, 2 mL of sodium carbonate solution (12%, w/w) were added and distilled water to reach 15 mL. The mixture was vortexed and incubated at room temperature, for 30 min. The optical density (OD) was read at 765 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). For the standard curve we used different concentrations of gallic acid in the range of 0-500 µg/mL. The results were expressed as gallic acid equivalents per g d.w.

Determination of total carbohydrate content was carried out by phenol-sulfuric acid method, as previously described [16]. Briefly, the sample was vigorously mixed with 5% (w/v) phenol solution, in equal ratio and then, concentrated sulfuric acid was added in a ratio of 1:10 (v/v) and vigorously vortexed at 200 rpm (Velp Scientifica). The mixture was then incubated at room temperature, for 30 min and the OD was read at 490 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). A standard curve was built using D-(+)-glucose (Glu) solution in the range of concentrations 5-100 µg/mL. The results were expressed as mg glucose per g d.w.

Determination of the antioxidant activity was performed by Trolox equivalent antioxidant capacity (TEAC) and free DPPH radical inhibition assays [17]. For TEAC assay, a stock solution was obtained by mixing equal volumes of 7 mM ABTS and 2.45 mM potassium persulfate and incubation in the dark, at room temperature, for 16 h. Before use, the solution was diluted with distilled water to reach an OD of 0.7 ± 0.02 (blank) read at 734 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). Then, 100 µL sample were mixed with 1 mL ABTS reagent and the mixture's OD was read at 734 nm after 10 min of incubation, at room temperature.

The capacity to inhibit free DPPH radicals was determined by mixing 1.35 mL of 0.25 mM DPPH methanolic solution with 150 µL sample of different concentrations (10-500 µg/mL) and 0.9 mL of 0.1 M Tris-HCl buffer, pH 7.4. Then, the mixtures were incubated in the dark, at room temperature, for 30 min. The OD was read at 517 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). A blank was obtained by sample replacing with the same volume of buffer. A standard curve was built for each assay using Trolox in the range of concentrations 0-250 µM. The results were expressed as Trolox equivalents per g d.w. The sample concentration (µg/mL) that inhibited 50% free DPPH radicals (IC_{50}) was determined from the nonlinear regression curve of DPPH inhibition vs. concentration plot using Microsoft Excel 2018 software.

Determination of α-amylase activity inhibition was performed according to a previously described method [18], with minor modifications. A reaction mixture consisting of 50 µL phosphate buffer (100 mM, pH 6.8), 10 µL α-amylase (2 U/ml) and 20 µL sample was made in the wells of a 96-well microplate and incubated at 37°C, for 20 min. Then, 20 µL of 1% starch solution was added and incubation continued at 37°C, for 30 min. Then, 100 µL DNS solution was added and the mixture was boiled in a water bath, for 10 min. The OD of the mixture was read at 540 nm using a Spectrostar nano microplate

reader (BMG Labtech, Germany). A mixture containing distilled water in place of sample served as control. A solution of 0.5 mg/mL acarbose, known as α-amylase inhibitor, served as positive control. The α-amylase inhibition was calculated using the following equation:

$$\alpha\text{-amylase activity inhibition (\%)} = (OD_{control} - OD_{sample} / OD_{control}) \times 100$$

where: As is the absorbance in the presence of test compounds and Ac is the absorbance of the control.

Preparation of the liposomal formulations: The selected combination of polyphenols and polysaccharides (24 mg) having the highest antioxidant activity was dissolved into a phosphate buffer solution (0.1 M, pH 7) by vortexing at 300 rpm, for 10 min. Two variants of liposomal formulations were prepared by adding 250 mg of soy lecithin (LPP1) and a mixture of soy phospholipids with a content of 25% phosphatidylcholine (LPP2), respectively, over the polyphenols-polysaccharides solution (10:1 ratio of phospholipids:bioactive compounds). The mixture was incubated in a water bath at 80 °C, with gentle stirring at 100 rpm, for 1 h. Glycerol (0.75 mL) was then added to cryoprotect the liposomal formula and incubation continued in a water bath at 80°C, with gentle shaking at 100 rpm, for 1 h. In order to obtain lipid vesicles, the mixture was diluted with 15 mL phosphate buffer and then, incubated at 60°C, 100 rpm, for 1 h. Formation of small lipid vesicles took place by mixture sonication in a 550 W sonication bath, using 60 s cycles, for 10 min and then, incubation at room temperature, for 24 h. The obtained liposomal suspension was then dried by lyophilization in glass Petri dishes using a freeze-dryer equipment (Martin Christ, Germany) operated at a freezing program at -35^{0}C, one step at 0^{0}C, 0.26 mbar, for 17 h and then, the drying program at +30^{0}C. The liposomal powder containing the selected combination of bioactive compounds was stored at -20 °C, until use. For rehydration, LPP1 and LPP2 lyophilized variants were vortexed with distilled water, at a concentration of 1%, 100 rpm, at room temperature, for 3 h. The mixture is sonicated as described above and incubated at room temperature, for 24 h.

Characterization of the liposomal formulations:

Determination of encapsulation efficiency: The liposomal suspension was centrifuged at 8000 rpm, 25^{0}C. The amount of polyphenols present in the supernatant (not encapsulated) was determined by Folin-Ciocalteu method. The encapsulation efficiency (EE) was calculated using the following equation:

$$EE\ (\%) = (\text{total amount - not encapsulated amount / total quantity}) \times 100$$

Solubility in water: The lyophilized liposomal formulation was dissolved in distilled water at a concentration of 1%, with stirring at 100 rpm, 25^{0}C, for 3 h. After centrifugation at 3500 rpm, for 5 min, the supernatant was dried at 110^{0}C, for 4 h and weighted. The solubility in water was calculated using the following equation:

$$\text{Solubility (\%)} = (\text{solubilized amount / total amount}) \times 100$$

Determination of moisture: The lyophilized liposomal formulation was weighted (10 mg) in a Petri dish and dried in an oven at 110^{0}C, for 4 h, to reach constant weight. The moisture content was gravimetrically determined and expressed as percent from initial weight.

Transmission electron microscopy (TEM): The lyophilized liposomal formulation was rehydrated at a concentration of 40 mg/mL in phosphate buffer by magnetic stirring, at 20^{0}C, for 30 min and then, the suspension was sonicated, as described above. The morphology and diameter size of particles in the liposomal suspensions before and after lyophilization were observed by TEM analysis, as previously described [19]. Briefly, two drops of liposomal suspension were deposited on 200 mesh copper grids covered with formvar, dried and then, contrasted with uranyl acetate, for 10 min. The observations were carried out at a Philips/FEI 208S equipment, operated at a voltage of 80 kV.

3. RESULTS AND DISCUSSION

Polyphenols and polysaccharides extracted from berries pomace resulted after juice extraction and chia seeds were characterized, in order to be further used in a new functional food formula. The berries pomace contained a mixture of *Vaccinium myrtillus* (blueberries), *Vaccinium vitis-idaea* (cranberries),

Hippophaea rhamnoides (seabuckthorn) and *Ribes sp*. (currants). The highest polyphenols extraction yield was registered for berries pomace as a source (17.05%), while the polysaccharides extraction yield from chia seeds (1.52%) had the lowest value (Table 1). The bioactive extracts were quantitatively analysed and the results are presented in Table 1.

Sample	Dry matter (mg/mL)	Yield (%)	Total phenolic content (mg gallic acid/g d.w.)	Total carbohydrate content (mg glucose/g d.w.)
PP-BR	69.44 ± 3.12	17.05 ± 0.75	304.01 ± 17.23	-
PP-CS	11.35 ± 0.51	4.42 ± 0.32	39.27 ± 1.67	-
PS-BR	10.18 ± 0.56	1.65 ± 0.17	-	222.9 ± 11.54
PS-CS	2.22 ± 0.14	1.52 ± 0.05	-	129.3 ± 5.05

Table 1. Determination of dry matter, total phenolic content and total carbohydrate content in extracts of polyphenols from berries pomace (PP-BR) and chia seeds (PP-CS) and polysaccharides from berries pomace (PS-BP) and chia seeds (PS-CS). The results are expressed as mean ± standard deviation (n=3)

The antioxidant capacity of the extracted polyphenols and polysaccharides was determined as TEAC (ABTS cation radical) and free DPPH anion radical scavenging capacity. The results are presented in Table 2. To determine the antioxidant synergism between polyphenols and polysaccharides from berries pomace and chia seeds, a screening of several combinations of extracts in different weight ratios were also analysed for antioxidant activity, as presented in Table 2.

Sample	Antioxidant activity as TEAC (mM Trolox equivalents/mg d.w.)	Antioxidant activity as free DPPH radical scavenging capacity expressed as IC_{50} (µg/mL)
PP-BR	1.32 ± 0.05	1056.17 ± 42.24
PP-CS	0.30 ± 0.02	2248.56 ± 85.44
PS-BR	0.23 ± 0.01	1409.04 ± 45.08
PS-CS	0.04 ± 0.01	ND
PP-BR:PS-BR 1:1	1.52 ± 0.07	1029.50 ± 33.96
PP-BR:PS-BR 1:2	0.87 ± 0.04	1598.16 ± 67.12
PP-BR: PS-BR 2:1	1.94 ± 0.11	804.94 ± 42.26
PP-BR:PS-CS 5:1	**2.37 ± 0.12**	**603.12 ± 2.78**
PP-CS:PS-CS 1:1	0.06 ± 0.01	ND
PP-CS:PS-CS 1:2	0.04 ± 0.01	ND
PP-CS:PS-CS 2:1	0.08 ± 0.01	ND
PS-BR:PP-CS 2:1	0.81 ± 0.05	1624.60 ± 69.82
PS-CS:PP-BR 5:1	0.83 ± 0.05	1653.44 ± 57.86

ND – not determined

Table 2. Determination of the antioxidant activity of polyphenols from berries pomace (PP-BR) and chia seeds (PP-CS) and polysaccharides from berries pomace (PS-BP) and chia seeds (PS-CS), in comparison to that of their combinations in different weight ratios. The results are expressed as mean ± standard deviation (n=3)

The obtained results have shown that the combination of polyphenols from berries and polysaccharides from chia seeds PP-BR:PS-CS in the ratio of 5:1 had the highest antioxidant activity. Because the value of the antioxidant activity of this mixture was greater than the sum of the values of the two components, it was considered that the polysaccharides from chia seeds have potentiated the antioxidant activity of polyphenols from berries pomace, obtaining a synergistic antioxidant mixture.

Given the importance of α-amylase inhibition for carbohydrate metabolism, it was further conducted the determination of α-amylase inhibition by polyphenols from berries pomace, polysaccharides from chia seeds and their combination in the selected ratio of 5:1, to verify the synergism of these compounds.

α-Amylase is a key enzyme in the digestion of starch, playing an important role in determining the amount of glucose released into the blood from food. Inhibition of its activity by polyphenols was only recently suggested, as a potential approach in controlling starch digestion and regulating postprandial hyperglycemia. The obtained results are presented in Table 3.

Sample	Optical density at 540 nm	α-Amylase activity inhibition (%)
PP-BR	0.36 ± 0.02	44.04 ± 1.54
PS-CS	0.58 ± 0.03	9.73 ± 0.03
PP-BR: PS-CS 5:1	**0.26 ± 0.01**	**59.96 ± 2.64**
Acarbose (0.5 mg/mL)	0.23 ± 0.01	65.07 ± 3.51
Blank	0.65 ± 0.03	0

Table 3. α-Amylase activity inhibition by polyphenols from berries pomace (PP-BR), polysaccharides from chia seeds (PS-CS) and their combination in a weight ratio of 5:1. A solution of acarbose served as positive control. The results are expressed as mean ± standard deviation (n=3)

The results have indicated that the combination of PP-BR:PS-CS in a ratio of 5:1 had the highest α-amylase inhibition effect (59.96%). The value was higher than the sum of the values obtained for each component, which demonstrated that this mixture had a synergistic effect. Previous studies have shown that α-amylase activity was inhibited by tea polyphenols, flavonoids, phenolic acids and tannins through key moieties involved in the binding process (20).

The bioavailability of bioactive compounds can be increased by the use of carrier systems, such as microemulsions, microencapsulation, solid-lipid nanoparticles or liposomes. In this study, it was developed an experimental model of liposomal formulation of the polyphenols-polysaccharides combination PP-BR:PS-CS in the ratio 5:1 and its lyophilization to a dry powder form for improved stability. The liposomes were prepared using soy lecithin and a mixture of soy phospholipids, respectively, which had the advantage of improving the nutritional value of the final product, due to the high content of polyunsaturated fatty acids with a beneficial role in lipid metabolism. Being natural compounds, they could easily interact, as active ingredients, with the phospholipids of the cell membrane and also did not raise legislation issues regarding the food product composition.

The results of the physicochemical analyses of LPP1 and LPP2 liposomal formulation variants, conditioned in the form of suspension and liposomal powder are presented in Table 4.

Sample	Lyophilized form	Encapsulation efficiency (%)	Solubility in water (%)	Moisture (%)
LPP1	-	80.46 ± 4.56	-	-
LPP1	+	-	32.02 ± 1.62	31.11 ± 1.30
LPP2	-	75.71 ± 3.51	-	-
LPP2	+	-	28.98 ± 1.39	57.24 ± 2.56

Table 4. Physicochemical characteristics of the liposomal formulation variants using soy lecithin (LPP1) and soy phospholipids (LPP2), encapsulating the selected synergistic combination, before and after lyophilization. The results are expressed as mean ± standard deviation (n=3).

The encapsulation efficiency varied between 80.46% in the case of LPP1 liposomal formulation with soy lecithin and 75.71% in the case of LPP2 soy phospholipids variant. The lyophilized liposomal variants have presented a degree of solubility in water of 32% for LPP1, slightly higher compared to 29% for LPP2. Previous studies have shown that solubility in water could be modulated by varying the phospholipids:bioactive compounds ratio [21, 22]. The moisture content of the lyophilized formulations varied between 31% for LPP1 and 57% for LPP2. The value of LPP1 soy lecithin liposomal formulation was close to that reported in previous similar studies (34-36%) using glycerol as a cryoprotective agent. The glycerol content of the lyophilized liposomal formulations did not allow totally drying of the mixture and gave a paste appearance of the obtained variants.

The morphological characterization of the liposomal formulations encapsulating the synergistic combination was carried out by TEM. The obtained images of LPP1 and LPP2 liposomal formulation variants, in the form of suspension before lyophilization are shown in Figure 2, while those of the lyophilized and rehydrated liposomal formulation are shown in Figure 3.

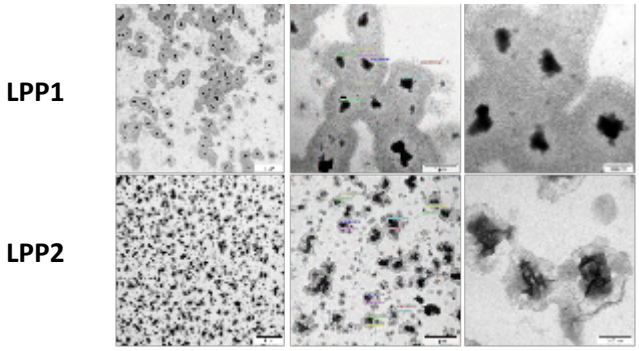

Fig. 2. Transmission electron micrographs showing the morphology and vesicle size of LPP1 and LPP2 liposomal formulation variants before lyophilization

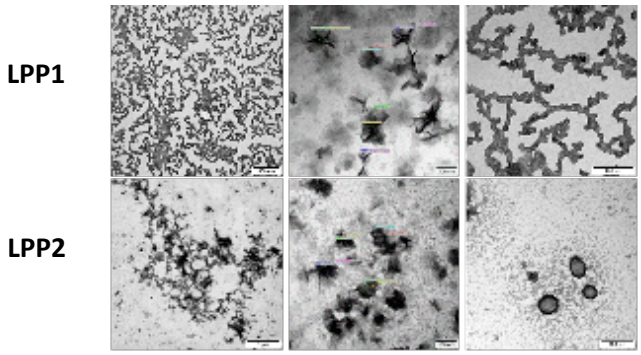

Fig. 3. Transmission electron micrographs showing the morphology and vesicle size of LPP1 and LPP2 liposomal formulation variants after lyophilization and rehydration

LPP1 soy lecithin liposomal formulation showed spherical vesicles with a diameter ranging from 294-1273 nm, while smaller values in the range of 139-761 nm diameter size were registered for LPP2 variant. It was observed a higher degree of aggregation in the case of LPP1, which implicitly indicated lower stability, probably due to the use of unpurified soy lecithin. The lyophilized LPP1 and LPP2 formulation variants showed after rehydration a different morphology from that of the liposomal suspension. Thus, the LPP1 and LPP2 variants have presented more homogeneous vesicles in terms of diameter size, which varied between 115-300 nm in both variants. However, the degree of vesicle association was higher, in particular for LPP1 variant.

Thus, the liposomal formulation of the selected combination of bioactive compounds in the form of dried powder (paste) could represent an alternative approach to the microencapsulation process using proteins. Previous studies have reported that lyophilization of the liposomal formulation could provide a number of advantages, such as reduced vesicle size and prevention of lipid oxidation, as well as increased stability over a long storage period [23, 24].

A composition of functional food was prepared as a mixture of berries (blueberries, cranberries, seabuckthorn, currants) pomace obtained by cold pressing and Himalayan salt, which was further dried and then ground. The resulting powder was extracted in coconut oil, then mixed with a powder of grape seeds, apricot kernels and hazelnuts and further mixed with chia seeds, cocoa butter, malt syrup and honey, in specific conditions and proportions [25]. A lyophilized liposomal powder encapsulating the synergistic combination of PP-BR and PS-CS was added. The final mixture was conditioned by cold pressing, for 48 h, portioned and packaged in sterile conditions. The obtained functional food had the following nutritional values per 100 g of product: 405 kcal energy value, 35.4% carbohydrates, 15.7% fat, 8% fiber, 3% protein and 0.15% salt.

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