Encapsulation of antioxidant sea fennel (*Crithmum maritimum*) aqueous and ethanolic extracts
in freeze-dried soy phosphatidylcholine liposomes

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Abstract

Soy phosphatidylcholine liposomes encapsulating increasing concentrations of two sea fennel extracts (aqueous and ethanolic) prepared by ultrasonication were freeze-dried, using glycerol as lyoprotectant. Particle properties, water dispersibility, colour, thermal properties and antioxidant capacity (radical scavenging capacity, ferric ion reducing power, Folin-reactive substances) of the liposomal preparations were determined. The freeze-drying process caused an overall increase in particle size and polydispersity index, while the zeta-potential became more electronegative. Both sea fennel extracts were rich in chlorogenic acid (42.61 and 58.48 mg/g for the aqueous and ethanolic extracts, respectively) and showed great antioxidant activity. Vitamin C was identified in the aqueous extract, whereas rutin and rosmarinic acid in the ethanolic one. The entrapment efficiency, determined in the liposomes prepared at the highest extract concentration, was 65.6% and 49.1% for the aqueous extract and the ethanolic extract, respectively. The liposomal antioxidant activity and total phenolic content followed a linear increasing tendency as a result of increasing the extract concentration, irrespective of the type of extract. Higher antioxidant activity was found in the liposomes loaded with the ethanolic extract, in a clear relationship to the greater amount of highly antioxidant phenolic compounds extracted, and also to their lower entrapment efficiency, which caused a greater amount of extract to remain outside the liposome. Both extracts were suitable for producing liposomes with antioxidant properties which could be dried and used to design functional foods.

Keywords: halophyte plant, chlorogenic acid, soy phosphatidylcholine, nanovesicles, lyophilisation, antioxidant activity.

1. Introduction

The needs and preferences of consumers are constantly changing and nowadays there is a growing demand for functional food products, prioritizing beneficial effects for health and prevention of diseases over the convenience of ready-to-eat products. The preferential use of healthy bioactive compounds from natural sources as food ingredients is therefore a challenging goal for the current food industry. In addition, the use of underutilized or waste materials as sources of biologically active molecules provides high added value and contributes to environmental sustainability.

Crithmum maritimum, commonly called sea fennel or marine parsley, is an edible halophile plant that is very abundant on the Mediterranean and Atlantic Sea coasts. This species is very rich in phenolic compounds with recognized antioxidant properties (Siracusa et al., 2011), due to the presence of chlorogenic acid (major compound) and hydroxycinnamic acids (Nabet et al., 2017). It has also a high amount of vitamin C; in fact, in ancient times this plant was used by sailors to avoid scurvy. Sea fennel is considered as an underutilized and undervalued plant, whose commercial cultivation has not yet been exploited (Pavela et al., 2017; Renna et al., 2017).

Natural antioxidant compounds can be incorporated in the design of functional foods to prevent numerous diseases related to oxidative stress, such as cardiovascular and neurodegenerative diseases, diabetes and cancer (Pham-Huy, He, & Pham-Huy, 2008). However, their effectiveness may be impaired by interaction with the food components or degradation during industrial processing. Their encapsulation in liposomes may offer a potential solution to enhance their stability by protecting them from extreme pH, temperature and high ion concentrations (Mozafari, Khosravi-Darani, Borazan, Cui, & Pardakhty, 2008). It also enables effective delivery of encapsulated compounds to target sites while minimizing systemic toxicity (Sercombe et al., 2015). Liposomes are spherical colloidal vesicles consisting of one or more phospholipid bilayers around an aqueous core. This type of structure gives an amphipathic behaviour, allowing entrapment of both lipophilic compounds (within the acyl chains of the bilayer) and hydrophilic compounds (in the inner aqueous core). The effects of phenolic compounds on vesicle properties and structural changes in the membrane may be

influenced by the type of phenolic and the phospholipid composition (Popova & Hinch, 2016; Lopes de Azambuja et al., 2015).

The use of partially purified soybean phospholipids for the preparation of liposomes offers an economically more profitable source than synthetic phospholipids and does not raise any food legislation concerns (Laye et al., 2008). Residual impurities could be an impediment for parenteral liposome-based drug administration, but they would be admissible for most food applications. Furthermore, the high amount of polyunsaturated fatty acids in soy lecithin plays a beneficial role in lipid metabolism (Ramdath et al., 2017) and can act as an active ingredient against various diseases, such as cancer, cardiovascular risk, neurological disorders, etc. (Küllenberg et al., 2012). The main drawback of highly unsaturated phospholipids is that they typically give more permeable and less stable bilayers (Biltoen & Lichtenberg, 1993).

Aqueous liposomal suspensions may lose stability with time, causing vesicle fusion, aggregation and sedimentation and the release of entrapped compounds (Sharma & Sharma, 1997). Freezing or freeze-drying could be alternative ways of maintaining the stability of liposomes and preserving their shelf life (Stark et al., 2010). In addition, the availability of liposomes in the dry state may facilitate industrial processing in the same way as more conventional food ingredients. However, the lipid bilayer may be damaged by ice crystal formation, vesicle aggregation and changes in phase transition (Chen et al., 2010). These changes normally lead to an increase in particle size that can cause liposomes to lose their essential nano-sized condition. The addition of cryoprotectants, such as carbohydrates or polyalcohols, has been shown to attenuate the detrimental effect of these treatments (Stark et al., 2010). Furthermore, the storage stability of freeze-dried liposomal preparations and their technological suitability as food ingredients can also be affected by the chemical nature of the entrapped compounds (Marín, Alemán, Montero, & Gómez-Guillén, 2018b).

The aim of this work was the exploitation of underutilized, low-cost raw materials with great functional properties to obtain a natural, healthy food ingredient with high added value. For this purpose, two types of sea fennel extracts (aqueous and ethanolic) were characterized in terms of chemical composition and antioxidant capacity, as an example of potential functional activity. Increasing concentrations of both extracts were encapsulated in partially purified soy phosphatidylcholine liposomes in combination with glycerol and were then freeze-dried. The liposomal preparations were characterized in terms of encapsulation efficiency, and physico-chemical, structural and antioxidant properties.

2. Materials and Methods

2.1. Materials

Sea fennel (*Crithmum maritimum*) was kindly provided by Porto-Muñños S.L. (Cereda, A Coruña, Spain). Soybean lecithin was purchased from Manuel Riesgo S.A. (Madrid, Spain). The standards of chlorogenic acid, rosmarinic acid, coumaric acid, ferulic acid, syringic acid, vitamin C, gallic acid, ellagic acid, caffeic acid, punicalagin, quercetin, rutin, epigallocatechin, epigallocatechin-3-gallate, epigallocatechin gallate, hyperoside and kaempferol-3-O-glucoside were acquired from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, methanol, acetonitrile and Triton X-100 were acquired from Panreac Química S.L.U. (Barcelona, Spain).

2.2. Preparation of sea fennel extracts

Sea fennel leaves and stems were crushed in a Thermomix until a homogeneous paste was obtained. For preparation of the ethanolic extract (Et), the paste was mixed (1:20, w/v) with an ethanol/water solution (70/30) and heated in a water bath at 60 °C for 30 min. Then it was probe-tip sonicated (model Q700, Qsonica sonicators, Newton, CT, USA) at 114 W for 5 min in pulse mode with a 1 min stop for each 2.5 min. After tempering the solution, it was centrifuged (Sorvall RC-5B, Sorvall Instruments) at 9000 rpm and 4 °C for 15 min, and the supernatant was filtered through Whatman No. 1 paper. The resulting extract was rota-evaporated (R-300, BÜCHI, Switzerland) at 85 °C to eliminate the solvent, freeze-dried (VirTis BenchTop 6K) and stored at –20 °C. The extraction yield on a dry weight basis was 31%. The aqueous extract (Aq), with an extraction yield (dry weight basis) of 35.5%, was prepared following the same procedure, using distilled water instead of ethanol/water and without the rota-evaporation step.

2.3. Cytotoxic effect of sea fennel extracts

The cytotoxic effect of the fennel extracts was evaluated in differentiated Caco-2 cells. The cells were grown in 75 cm² flasks and maintained in minimum essential media (MEM) supplemented with 10% foetal bovine serum in a humidified incubator (5% CO₂) at 37 °C. For cytotoxic determination, the cells were seeded in 96-well plates at a density of 5000 cells/well and incubated for 24 h before they were treated with various concentrations of fennel extracts. Serum-free MEM was used as a negative control and ethanol was used as a positive control. The treated cells were incubated again for 24 hours and cell viability was assessed using the Dojindo Cell Counting Kit-8 (Dojindo, Rockville, MD), following the manufacturer's protocol.

2.4. Preparation of liposomes

Partially purified soy phosphatidylcholine (PC) was prepared according to the method of Taladrid et al. (2017) by applying five washes with acetone. The liposomes loaded with sea fennel extracts were prepared according to Marín et al. (2018a). The plant extract and phosphatidylcholine were suspended in phosphate buffer at pH 7.0 and heated at 80 °C for 1 h. Glycerol (0.6 mL/g PC) was then incorporated and it was heated again at 80 °C for 1 h. The suspension was vortexed at 60 °C to form multilamellar vesicles and was then probe-tip sonicated (model Q700, Qsonica) at a measured power of 120 W for 5 minutes, with a 60 s stop every min to avoid sample overheating, and stored at 4 °C. In parallel, both sea fennel extracts, in the absence of phosphatidylcholine and glycerol, were subjected to the same heating and sonication procedure in order to check for eventual thermal degradation. Different liposomes were prepared by using increasing concentrations (8, 16, 32 and 64% with respect to PC weight) of the aqueous (Aq) and ethanolic (Et) extracts, giving L-Aq8, L-Aq16, L-Aq32, L-Aq64, L-Et8, L-Et16, L-Et32 and L-Et64. Newly obtained liposomal dispersions were freeze-dried and stored at –20 °C until use. For freshly prepared liposome determinations, the liposomes were stored at 4 °C for no more than 2 days.

2.5. Moisture and protein content

The water content was determined by method 950.46 (A.O.A.C., 2005), at least in triplicate. The total protein content of the sea fennel extracts (5 mg/mL) was determined from the amino acid composition after acid hydrolysis, employing a Biochrom 30 amino acid analyser (Pharmacia, Barcelona, Spain).

2.6. High Performance Liquid Chromatography (HPLC)

To identify the main phenolic compounds, the dried extracts and liposomal preparations (with and without Triton X-100) were dispersed in Milli-Q water and analysed by reverse phase high performance liquid chromatography (RP-HPLC; model SPE-MA10AVP, Shimadzu, Kyoto, Japan) on a C18 analytical column (Tracer Excel 120 ODS-A 5 µm 25 × 0.46, Teknokroma, Barcelona, Spain). The samples were eluted with a gradient system consisting of solvent A (MilliQ water) and solvent B (methanol:acetonitrile, 60:40), both containing 1% acetic acid, at a flow rate of 0.6 mL/min. The temperature of the column was maintained at 25 °C, and the injection volume was 20 µL. The gradient system started at 10% solvent B and increased to 60% B over 60 min, followed by a further decrease to 10% solvent A over 10 min. The final conditions were held for 10 min. The peaks of the phenolic compounds were monitored by absorbance at 253 nm

and 368 nm. The identification of phenolic compounds was carried out by comparison of the retention times of the pure external standards listed above. Chlorogenic acid, vitamin C, rutin and rosmarinic acid were quantified using a calibration curve of the corresponding standard compound. Analyses were carried out at least in duplicate.

2.7. Particle characterization

Particle size (expressed as z-average in % intensity), polydispersity index and zeta-potential of the liposomes were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted in 0.2 M phosphate buffer at pH 7.0 to avoid particle aggregation. At least 10 replicates were measured per sample. Dried liposomes were previously rehydrated by suspending in distilled water under magnetic stirring at a concentration of 0.77 mg/mL for 30 min at 20 °C.

2.8. Water dispersibility

The dried liposomal preparations were dispersed in distilled water (1%, w/v) at room temperature and 100 rpm for 150 min, and centrifuged (Multifuge 3L-R, Heraeus, Madrid, Spain) at 3500 rpm for 5 min. The supernatant was dried at 105 °C for 24 h, and the results, obtained by weight differences, were expressed as percentages. The determinations were performed at least in triplicate.

2.9. Entrapment efficiency

The entrapment efficiency (EE) of fennel extracts in the freeze-dried liposomal preparations after rehydration was determined by the equation:

$$\% \text{ EE} = \text{encapsulated extract in liposomes} / \text{total extract in liposomes} * 100$$

The encapsulated extract was calculated by difference between the total and the non-encapsulated material, considering as non-encapsulated material all of the free phenolic extract in the liposome suspension.

To separate the non-encapsulated material, liposomes loaded with fennel extracts were placed in an Amicon Ultra-15 centrifugal filter device with molecular weight cut-off of 10 KDa (10000 MWCO, Millipore Corp.) and were centrifuged at 4000 *g* for 30 min. The non-encapsulated extract penetrated through the membrane while the liposomes remained in the filter. The ultrafiltrate was then collected. To determine the amount of total extract, the liposomes were mixed (1:1) with Triton X-100 (20% v/v) and were homogenized in a vortex until complete solubilization of the phosphatidylcholine. The non-encapsulated extract and the total extract

were quantified by measuring the phenolic content by the Folin–Ciocalteu method. The amount of extract encapsulated in the inner core of the liposome was determined by comparison of the phenolic content in the liposomal suspension before and after disrupting the liposome membrane with Triton X-100.

2.10. Colour

A Konica Minolta CM-3500d colorimeter (Konica Minolta, Madrid, Spain) with D65 illuminant and D10 as standard observer was used to determine the colour parameters L (luminosity, 0–100), a^* (red-green range) and b^* (yellow-blue range) in the freeze-dried liposomal preparations. Chroma (C, 0–100) or colour saturation and Hue (H, 0–360°) or tonality values were also obtained. At least 10 replicates were performed for each sample.

2.11. Differential Scanning Calorimetry (DSC)

DSC analyses of freeze-dried liposomal preparations (5 mg) were performed using a model TA-Q1000 differential scanning calorimeter (TA Instruments, New Castle, DE, USA). They were placed in hermetic aluminium capsules and subjected to a heating ramp from –30 °C to 95 °C at a heating rate of 10 °C/min under dry nitrogen purge (50 mL/min). Endothermic phase transition temperatures (°C) were determined for each sample at least in duplicate.

2.12. Antioxidant activity

The ferric ion reducing power (FRAP) and the ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging assays were used to determine the antioxidant activity of freeze-dried sea fennel extracts and liposomes, which were dissolved in distilled water, shaken until totally homogeneous and filtered (Whatman No. 1). The filtrate was used for the antioxidant activity determination. The methods used for the FRAP and ABTS assays were previously described (Alemán, Giménez, Pérez-Santín, Gómez-Guillén, & Montero, 2011). Results were expressed as mg Fe^{2+} equivalents/g of sample for FRAP and mg of Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of sample for ABTS, based on standard curves of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and Vitamin C, respectively. All determinations were performed at least in triplicate and expressed as a function of the freeze-dried sample weight.

2.13. Folin-reactive substances determination

Folin-reactive substances content was determined spectrophotometrically, in triplicate, using gallic acid as a standard, according to a modified method of Slinkard and Singleton (1977) with

the Folin–Ciocalteu reagent. The absorbance of the resulting blue colour was measured at 765 nm (UV-1601, model CPS-240, Shimadzu, Kyoto, Japan). Folin-reactive substances content was expressed as mg gallic acid (GA) equivalent/g of freeze-dried sample.

2.14. Statistical analyses

Analysis of variance (ANOVA) was performed using the SPSS® computer program (IBM SPSS Statistics 22 Software, Inc., Chicago, IL, USA). Differences between means were assessed on the basis of confidence intervals using the Tukey test, with a significance level set at $p \leq 0.05$.

3. Results and Discussion

3.1. Properties of sea fennel extracts

Residual moisture, total protein, Folin-reactive substances and antioxidant properties of the dried sea fennel aqueous (Aq) and ethanolic (Et) extracts are presented in Table 1. Both extracts had relatively high residual moisture content ($>15\%$) after freeze-drying, probably owing to high hygroscopicity, which was slightly higher in the ethanolic (Et) extract. In spite of this, both had the appearance of a fine powder. The Aq extract presented a yellow-brownish coloration with total protein content of 4.1%, while the Et extract had 2.2% protein with a characteristic greenish colour. As could be expected, the total amount of Folin-reactive substances (FRS) extracted in Et was noticeably higher than in Aq. Meot-Duros and Magne (2009) reported levels of phenolic compounds by the Folin method up to 33 mg gallic acid eq./g sample during different seasons of the year for sea fennel methanol extracts. Great variations in extraction efficiency could be attributed to the different feedstock as well as to the extraction procedure. In agreement with the FRS content, the antioxidant activity determined by the ABTS method (radical scavenging capacity) and the FRAP method (ferric ion reducing power) were, respectively, 1.4-fold and 2.1-fold higher in the Et extract than in the Aq extract (Table 1).

The RP-HPLC profile (Figure 1) revealed that chlorogenic acid was the main phenolic constituent in both types of extract, being more abundant in the ethanolic extract (58.48 mg/g) than in the aqueous one (42.61 mg/g). Meot-Duros and Magne (2009) determined the chlorogenic acid content in methanol extracts from sea fennel growing in sand hills and below cliffs, and reported amounts between 3 and 27.9 mg/g. Other minor compounds were also identified (Figure 1), their presence and relative abundance varying according to the nature of

the solvent. Thus, highly water-soluble vitamin C was a prominent compound in the Aq extract (1.46 mg/g), while larger phenolic molecules of rutin and rosmarinic acid were possible to be quantified only in the Et extract (4.52 mg/g and 2.81 mg/g, respectively). Pereira et al. (2017) also reported chlorogenic acid as the most abundant phenolic compound present in infusions and decoctions of *Crithmum maritimum* leaves and stems. However, other compounds reported by those authors, such as hydroxycinnamic acids (ferulic acid or coumaric acid), were not found in the present extracts.

As shown in Figure 2, the Aq extract did not have significant ($p>0.05$) cytotoxic effects. In contrast, a slight reduction ($p\leq 0.05$) in cell viability was found with the Et extract, probably attributable to a residual presence of ethanol. Considering that concentrations as high as 1 mg/mL led to cell viability values above 80%, and that no differences were observed at concentrations ranging from 0.01 to 1 mg/mL, the possible cytotoxic effect of the Et extract was almost negligible at the conditions used in the present work.

3.2. Fresh liposomes

The mean particle size (z-average), polydispersity index (PDI) and membrane surface charge (zeta-potential) of fresh liposomes loaded with the two sea fennel extracts at increasing concentrations are shown in Table 2. No significant differences ($p>0.05$) in particle size were found between L-Aq8 and the empty (L-E) liposome. The progressive increase in extract concentration increased ($p\leq 0.05$) the mean liposomal size from 89.5 to 137.1 nm for Aq-liposomes and from 96.3 to 150.1 nm for Et-liposomes. The increasing amounts of extract compounds located in the inner aqueous core, as well as inside or adhering to the outside of the bilayer might have caused the expansion of the liposome, with the consequent increase in z-average. Furthermore, the Et-liposomes showed significantly greater particle size ($p\leq 0.05$) than their Aq-liposome counterparts. The difference could be attributed to the larger molecular size of the phenolic compounds found in the Et extract (rutin and rosmarinic acid) and their higher affinity for the interior of the acyl chains compared to the Aq extract, where hydrophilic and low molecular weight compounds, such as vitamin C, predominated. The accumulation of lipophilic compounds in the hydrophobic part of the membrane would disturb interactions between the acyl chains of phospholipids, leading to swelling of the bilayer (Sebaaly et al., 2015). An increase in liposome size as a result of increasing the concentration of quercetin was also reported to be probably caused by excess flavonoid that was no longer

soluble in the system and might therefore have formed aggregates (Frenzel & Steffen-Heins, 2015).

No significant differences ($p>0.05$) were found in relation to extract type or concentration in the polydispersity index (PDI) or zeta-potential, which ranged between 0.295 and 0.370 and between -27.7 and -36.7 mV, respectively. Furthermore, these values were close to that of the empty liposome (L-E). In general, all the formulations kept a reasonable nanometric size typical of this kind of vesicle. The largest size was recorded in L-Et64 (150 nm), the liposome with the highest concentration of ethanolic extract, which represented 22.7% (expressed as dry matter) with respect to the total liposome weight. Feng et al. (2016) obtained a similar z-average (132.1 nm) for soybean liposomes containing chlorogenic acid extracted in ethanol ($\approx 16.7\%$ of bioactive with respect to lecithin). The PDI was very close to the expected range for systems prepared from biological materials (0.2–0.3) (Malheiros et al., 2011). All the liposomes had high particle stability, with strong electronegative surface charge (Müller et al., 2001). Dag and Oztop (2017) reported a very similar zeta-potential, with values of -35 mV for empty soy lecithin liposomes and -30 mV for green tea-loaded liposomes. A similar zeta-potential was also determined in soy phosphatidylcholine liposomes loaded with quercetin (Frenzel & Steffen-Heins, 2015).

3.3. Freeze-dried liposomal preparations

3.3.1. Particle properties

To improve the storage stability and avoid possible liposome aggregation or sedimentation, freshly prepared liposomes were frozen at -80 °C and dried by lyophilization. This stabilization process caused an overall increase in z-average and PDI after rehydration, while the zeta-potential became more electronegative (Table 3). These changes were much more pronounced in the case of empty liposomes than in loaded liposomes, indicating that the phenolic extracts might to some extent prevent freeze-drying-induced vesicle damage. The size of freeze-dried Aq-liposomes varied ($p\leq 0.05$) from 171.1 nm for L-Aq8 to 311.4 nm for L-Aq64, the latter having a z-average significantly higher ($p\leq 0.05$) than the other loaded liposomes and similar to that of the empty one. In contrast, the freeze-dried Et-liposomes did not present significant changes ($p>0.05$) in z-average depending on extract concentration, with values ranging between 216.4 and 225.4 nm. The drying process helps to stabilize the vesicles, as denoted by the increase ($p\leq 0.05$) in zeta-potential following freeze-drying, which reflected higher stability in the corresponding rehydrated preparation. More vesicular stability was found in liposomes loaded at 8 and 16% of extract concentration for both kinds of extract

($p \leq 0.05$), and for Et-liposomes compared with Aq-liposomes. The range of zeta-potential values was from -34.2 mV to -48.2 mV, significantly lower than the value of -54.2 mV obtained for L-E. A possible explanation is that both the aqueous and the ethanolic extracts, having less electronegative charge (-14.8 and -17.1 mV, respectively) than the liposomal suspension (L-E), could slightly neutralize the electric charge by external adsorption to the membrane surface.

Variations in vesicle size and PDI could be due to many factors, such as the lyophilization conditions, the phospholipid:bioactive ratio or the presence of cryoprotectants (Sebaaly et al., 2016). Glycerol was incorporated in the liposomal formulation to provide stability against freezing and drying, maintaining the bilayer integrity and preventing sedimentation or leakage of the entrapped compounds after rehydration (Mozafari, 2005). This cryoprotectant was reported to increase the hydration level and fluidity of the lipid bilayer (Manca et al., 2013), and to produce a plasticizing effect that led to a pasty-like consistency with relatively high residual moisture content in the dried liposomal preparation (Marín et al., 2018b). In agreement with previous work, the water content of the freeze-dried samples was relatively high (Table 3), attributable to the hygroscopic nature of glycerol and its interference in the structure of the membrane bilayer, which might decrease the efficiency of water removal (Taladrid et al., 2017). The moisture level did not appear to follow any correlation depending on extract concentration or type of extract, with values ranging from 15 to 38% (Table 3). In general, the liposomal formulations with entrapped extracts had more ($p \leq 0.05$) residual water content than the empty liposome, which could be an indication that a significant amount of extract compounds remained outside the liposomes, contributing to an increase in the hygroscopicity. Freeze-dried loaded samples presented greater water dispersibility ($\geq 80\%$) than L-E (72.3%) (Table 2), L-Et64 being the preparation with the highest dispersibility ($\approx 100\%$). In agreement with the lower increase in particle size in loaded liposomes as compared to the empty one, the presence of phenolic compounds may have attenuated the freeze-drying-induced vesicle aggregation, producing higher water dispersibility. Marín et al. (2018a) obtained similar results for dried liposomes also made with the addition of glycerol.

3.3.2. Entrapment efficiency

The entrapment efficiency (EE) was determined in the freeze-dried preparations at the highest extract concentration (L-Aq64 and L-Et64). In L-Aq64, the proportion of free Folin-reactive substances (non-encapsulated) with respect to the total amount determined after disrupting the liposome membrane with Triton X-100 was found to be 34.4%. This means that 65.6% (EE) of the aqueous extract compounds was entrapped in the liposomes. When the amounts of FRS

measured in the L-Aq64 liposomal dispersion before and after treatment with Triton X-100 were compared, an increase of 11.1% was observed in the latter, attributed to the amount of FRS released from the broken vesicles. According to these results, only 11.1% of the extract would be included in the inner core of the liposome, whereas the remaining 54.5% of the 65.6% would remain associated with the membrane (embedded within the acyl chains or adsorbed to the membrane surface by interacting with the polar phosphate groups). In the case of L-Et64, following the same approach, the entrapment efficiency (EE) was found to be lower than in L-Aq64 (49.1% vs. 65.6%). The amount of FRS determined inside the liposome did not differ so much between the two preparations (11.9% in L-Et64 vs. 11.1% in L-Aq64). In contrast, the proportion of extract associated with the liposomal membrane was considerably lower in L-Et64 (37.2% vs. 54.5%). The present EE results were similar to the value reported by Feng et al. (2016) for liposomes encapsulating chlorogenic acid (53.1%). Slightly higher EE values were observed for L-Aq liposomes in comparison to their L-Et counterparts, probably owing to the different distribution of the phenolic compounds according to their different water dispersibility and molecular size. The predominant amount of chlorogenic acid in the ethanolic extract, together with other low hydrophilic high molecular weight phenolic compounds (rutin and rosmarinic acid), failed to be completely embedded inside the bilayer in Et-liposomes. In contrast, the lower amount of chlorogenic acid in the aqueous extract would favour better allocation within the fatty acid acyl chains, while the relative abundance of vitamin C, with high water solubility and simple phenolic structure, would have more affinity to remain in the inner aqueous core of the liposome.

The chromatographic profiles of L-Aq64 and L-Et64 suspensions are shown in Figure 1, in comparison with the corresponding sea fennel extracts before and after heating and sonication in the absence of phosphatidylcholine and glycerol (Figure 1 C and D). The prominent peak assigned to chlorogenic acid in both extracts was considerably smaller in the corresponding liposomal dispersions. Similarly to chlorogenic acid, smaller signal intensity was also observed for rutin and rosmarinic acid in L-Et64 liposomes, as well as for vitamin C and other relatively abundant non-identified compounds in L-Aq64, indicating that they were also largely encapsulated. The chromatograms of the liposomal suspensions showed an increase in the relative abundance of several unidentified compounds with both extracts, or even the appearance of new ones, which were also part of the non-encapsulated fraction. These changes could have arisen from thermal degradation or transformation of certain phenolics, including chlorogenic acid, during liposome preparation. The peak height corresponding to the chlorogenic acid in both sea fennel extracts decreased slightly in the processed extracts,

however, the decrease was much less evident than in the corresponding liposomal suspensions. Therefore, it could be assumed that, although some thermal degradation could occur, most of the extract was encapsulated. The amount of chlorogenic acid determined in the liposomal suspensions in the form of free compound (non-encapsulated) was considerably lower than in the corresponding Aq and Et extracts (Table 4). Furthermore, the decrease of chlorogenic acid resulting from heating and sonication was 10% and 15% for Aq and Et extract, respectively. Taking into account the thermal degradation of this compound during liposome preparation, the amount of encapsulated chlorogenic acid would represent 64.3% in L-Aq64 and 59.8% in L-Et64. This finding indicates that chlorogenic acid in the aqueous extract was more efficiently entrapped in the liposomal structure than in the case of the ethanolic extract. These results were in agreement with the lower entrapment efficiency of the whole extracts determined by the Folin method in L-Et64 (49.1%) as compared to L-Aq64 (65.6%). Differences in the entrapment degree associated to chemical and molecular properties of each specific compound in the whole extract, as well as possible interferences with other Folin reactive compounds would explain the slight divergence between EE values calculated either by the Folin method or by the chlorogenic acid concentration in the L-Et64 preparation.

3.3.3. Colour

The plot reflecting the colour parameters C* (Chroma, saturation) and H* (Hue, tonality) of the freeze-dried liposomal preparations is shown in Figure 2. The L-Et samples presented significantly higher Hue (66.0–77.1) and Chroma (5.8–9.9) values than the corresponding L-Aq samples (60.7–67.4 and 2.4–5.9, respectively). These results were in agreement with the different coloration of the respective extracts (greenish for the Et extract and yellow-brownish for the Aq extract), and could be attributed to the amount of non-encapsulated extract present in each preparation. As shown in Table 3, the L* parameter (luminosity) was in general slightly higher in the L-Et liposomes (29.3–32.1) than in the corresponding L-Aq liposomes (27.1–29.0). The empty liposomes, L-E, showed higher ($p \leq 0.05$) Chroma, Hue and L* values (8.3, 83.5, 32.5, respectively) than any loaded liposome, except L-Et64, which presented the highest Chroma value (9.9). Increasing the extract amount did not follow definite proportionality in either L-Aq or L-Et liposomes for any of the parameters studied, indicating that the phenolic concentration was not a decisive factor in the colour of the freeze-dried liposomal preparations.

3.3.4. Thermal properties (DSC)

Thermograms of freeze-dried empty liposomes and liposomes loaded with the two sea fennel extracts are shown in Figure 3. They reflect a single diffuse and not well-defined phase transition, with endothermic peaks ranging from 0.4 to 5.3 °C. The low transition temperatures are typical of liposomes made from soy phosphatidylcholine with a high content of polyunsaturated fatty acids (Biltonen & Lichtenberg, 1993). These temperatures are in agreement with those obtained by Marín et al. (2018b) for soy phosphatidylcholine liposomes entrapping different bioactive compounds, which varied between 1.8 °C and 11.1 °C. The empty liposome (L-E) was the sample with the lowest transition temperature (0.4 °C). A progressive increase in peak temperature was observed with the increase in phenolic concentration for both types of extract. Furthermore, L-Et liposomes showed higher transition temperatures than the corresponding L-Aq preparations, which was probably related to the effect of the total concentration of phenolic compounds, and also to the molecular properties of the predominant phenolic species of each type of extract.

These results indicate an increase in membrane rigidity and stability as a result of increasing the amount of bioactive added. The more pronounced membrane structural change in L-Et liposomes could be the result of the higher amount of large lipophilic compounds embedded in the membrane. An increase in phase transition temperature has been reported to occur in soy phosphatidylcholine liposomes as a result of the addition of catechin or epigallocatechin gallate (Rashidinejad, Birch, Sun-Waterhouse, & Everett, 2014). In contrast, a 1.5 °C decrease in T_m was ascribed to the interaction of DMPC liposomes with a phenolic extract from *Spirulina* (Pagnussatt et al., 2016). Liposomes made from synthetic phospholipids loaded with different single phenolic compounds at increasing concentrations showed a progressive decrease in T_m (Ota et al., 2011).

3.3.5. Antioxidant properties

The total phenolic content (FRS), ABTS radical scavenging capacity and ferric ion reducing power (FRAP) were measured in the freeze-dried liposomes loaded with either the aqueous (L-Aq) or the ethanolic (L-Et) extract at increasing concentrations (Figure 4).

The liposomal antioxidant activity and total phenolic content followed a linear increasing tendency ($R^2 > 0.90$) as a result of increasing the extract concentration, irrespective of the type of extract. Since the liposomes were not intentionally broken, the antioxidant activity measured would mostly correspond to the non-encapsulated compounds or those that continued to interact with the outer membrane surface through the polar phosphate groups. The contribution of the vesicle encapsulating material (empty liposomes) to the ABTS, FRAP

and FRS values measured was quite low in all cases: 20 mg VCEAC/g dried sample; 2.8 mg Fe^{2+} eq./g dried sample; 3.4 mg gallic acid/g dried sample, respectively (Figure 4).

In agreement with the higher amount of FRS measured in the liposomes loaded with the ethanolic extract (L-Et), in general they presented higher ($p \leq 0.05$) antioxidant activity than those loaded with the aqueous extract (L-Aq), especially at the higher extract concentrations (32 and 64%). This finding could be related to the greater amount of highly antioxidant phenolic compounds extracted in the ethanolic extract, and also to the higher amount of free chlorogenic acid located outside the liposome, remaining available for radical scavenging or iron reducing. There were only two exceptions regarding the ABTS values: L-Aq8 was very low but significantly higher than L-Et8; and L-Aq16 had the same activity ($p > 0.05$) as L-Et16. The lower concentration of non-encapsulated extract in these liposomal preparations was probably not enough to induce significant variations in their radical scavenging capacity.

4. Conclusions

Ultrasonication of crushed sea fennel stems and leaves in aqueous and ethanolic medium was used to obtain two extracts with high antioxidant capacity, in which chlorogenic acid was the main phenolic constituent. Increasing the extract:phosphatidylcholine ratio induced a progressive increase in the liposome particle size without changing the membrane surface charge. The liposomes could be loaded with a high extract concentration (64% with respect to phosphatidylcholine weight) with relatively high entrapment efficiency. The phenolic compounds were mostly entrapped at the membrane level rather than included in the inner core of the liposomes. Both the aqueous and the ethanolic sea fennel extracts were suitable for producing liposomes with antioxidant properties, which could be used to design functional foods and possibly also to preserve them from oxidative instability. Nevertheless, further work would be needed to explore the intestinal absorption of both bioactive extracts and/or liposomes, in order to confirm that they would be able to exert some beneficial effects in the organism.

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484 References

485 Alemán, A., Giménez, B., Pérez-Santín, E., Gómez-Guillén, M.C, & Montero, P. (2011).
486 Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide
487 sequences isolated from squid gelatin hydrolysate. *Food Chemistry*, 125, 334–341.

488 A.O.A.C. (2005). Official methods of analysis. Gaithersburg, MD, USA: Association of Official
489 Analytical Chemists.

490 Biltonen, R.L., & Lichtenberg, D. (1993). The use of differential scanning calorimetry as a tool to
491 characterize liposome preparations. *Chemistry and Physics of Lipids*, 64 (1–3), 129–142.

492 Dag, D., & Oztop, M.H. (2017). Formation and characterization of green tea extract loaded
493 liposomes. *Journal of Food Science*, 82 (2), 463-470.

494 Feng, Y., Sun, C., Yuan, Y., Zhu, Y., Wan, J., Firempong, C.K., ... & Xu, X. (2016). Enhanced oral
495 bioavailability and in vivo antioxidant activity of chlorogenic acid via liposomal formulation.
496 *International Journal of Pharmaceutics*, 501 (1-2), 342-349.

497 Frenzel, M., & Steffen-Heins, A. (2015). Impact of quercetin and fish oil encapsulation on
498 bilayer membrane and oxidation stability of liposomes. *Food Chemistry*, 185, 48-57.

499 Funes, L., Laporta, O., Cerdán-Calero, M., & Micol, V. (2010). Effects of verbascoside, a
500 phenylpropanoid glycoside from lemon verbena, on phospholipid model membranes.
501 *Chemistry and Physics of Lipids*, 163, 190-199.

502 Houta, O., Akrou, A., Najja, H., Neffati, M. & Amri, H. (2015). Chemical composition,
503 antioxidant and antimicrobial activities of essential oil from *Crithmum maritimum* cultivated in
504 Tunisia. *Journal of Essential Oil Bearing Plants*, 18 (6), 1459-1466.

505 Küllenberg, D., Taylor, L.A., Schneider, M. & Massing, U. (2012). Health effects of dietary
506 phospholipids. *Lipids in Health and Disease*, 11 (e3).

507 Laye, C., McClements, D.J. & Weiss, J. (2008). Formation of biopolymer-coated liposomes by
508 electrostatic deposition of chitosan. *Journal of Food Science*, 73 (5), N7-N15.

509 Malheiros, P.S., Sant'anna, V., Micheletto, Y.M.S., Da Silveira, N.P. & Brandelli, A. (2011).
510 Nanovesicle encapsulation of antimicrobial peptide P34: Physicochemical characterization and
511 mode of action on *Listeria monocytogenes*. *Journal of Nanoparticle Research*, 13, 3545–3552.

512 Manca, M.L., Zaru, M., Manconi, M., Lai, F., Valenti, D., Sinico, C. & Fadda, A.M. (2013).
 513 Glycosomes: A new tool for effective dermal and transdermal drug delivery. *International*
 514 *Journal of Pharmaceutics*, 455, 66–74.

515 Marín, D., Alemán, A., Sánchez-Faure, A., Montero, P. & Gómez-Guillén, M.C. (2018a). Freeze-
 516 dried phosphatidylcholine liposomes encapsulating various antioxidant extracts from natural
 517 waste as functional ingredients in surimi gels. *Food Chemistry*, 245, 525-535.

518 Marín, D., Alemán, A., Montero, P. & Gómez-Guillén, M.C. (2018b). Encapsulation of food
 519 waste compounds in soy phosphatidylcholine liposomes: effect of freeze-drying, storage
 520 stability and functional aptitude. *Journal of Food Engineering*, 223, 132-143.

521 Meot-Duros, L. & Magne, C. (2009). Antioxidant activity and phenol content of *Crithmum*
 522 *maritimum* L. leaves. *Plant Physiology and Biochemistry*, 47 (1), 37-41.

523 Mosquera, M., Giménez, B., Montero, P. and Gómez-Guillén, M.C. (2016). Incorporation of
 524 liposomes containing squid tunic ACE-inhibitory peptides into fish gelatin. *Journal of the*
 525 *Science of Food and Agriculture*, 96, 769–776.

526 Mozafari, M.R. (2005). Liposomes: An overview of manufacturing techniques. *Cellular and*
 527 *Molecular Biology Letters*, 10 (4), 711–719.

528 Mozafari, M.R., Khosravi-Darani, K., Borazan, G.G., Cui, J., Pardakhty, A. & Yurdugul, S (2008).
 529 Encapsulation of Food Ingredients Using Nanoliposome Technology. *International Journal of*
 530 *Food Properties*, 11, 833-844.

531 Müller, R.H., Jacobs, C. & Kayser, O. (2001). Nanosuspensions as particulate drug formulations
 532 in therapy: rationale for development and what we can expect for the future. *Advanced Drug*
 533 *Delivery Reviews*, 47 (1), 3–19.

534 Nabet, N., Boudries, H., Chougui, N., Loupassaki, S., Souagui, S., Biulo, F., ... & Larbat, R. (2017).
 535 Biological activities and secondary compound composition from *Crithmum maritimum* aerial
 536 parts. *International Journal of Food Properties*, 20 (8), 1843-1855.

537 Ota, A., Abramovič, H., Abram, V., & Poklar Ulrih, N. (2011). Interactions of p-coumaric, caffeic
 538 and ferulic acids and their styrenes with model lipid membranes. *Food Chemistry*, 125, 1256-
 539 1261.

540 Pagnussatt, F.A., de Lima, V.R., Dora, C.L., Costa, J.A.V., Putaux, J.L., & Badiale-Furlong, E. 2016.
 541 Assessment of the encapsulation effect of phenolic compounds from *Spirulina* sp. LEB-18 on
 542 their antifusarium activities. *Food Chemistry*, 211, 616-623.

543 Pavela, R., Maggi, F., Lupidi, G., Cianfaglione, K., Dauvergne, X., Bruno, M. & Benelli, G. (2017).
 544 Efficacy of sea fennel (*Crithmum maritimum* L., Apiaceae) essential oils against *Culex*
 545 *quinquefasciatus* Say and *Spodoptera littoralis* (Boisd.). *Industrial Crops & Products*, 109, 603-
 546 610.

547 Pereira, C.G., Barreira, L., Neng, N.D.R., Nogueira, J.M.F., Marques, C., Santos, T.F., ... &
 548 Custódio, L. (2017). Searching for new sources of innovative products for the food industry
 549 within halophyte aromatic plants: *In vitro* antioxidant activity and phenolic and mineral
 550 contents of infusions and decoctions of *Crithmum maritimum* L. *Food and Chemical Toxicology*,
 551 107, 581-589.

552 Pham-Huy, L.A., He, H. & Pham-Huy, C (2008). Free radicals, antioxidants in disease and
 553 health. *International Journal of Biomedical Science*. 4, 89-96.

554 Ramdath, D.D., Padhi, E.M.T., Sarfaraz, S., Renwick, S. & Duncan, A.M. (2017). Beyond the
 555 cholesterol-lowering effect of soy protein: a review of the effects of dietary soy and its
 556 constituents on risk factors for cardiovascular disease. *Nutrients*, 9 (4), 324.

557 Rashidinejad, A., Birch, E.J., Sun-Waterhouse, D., & Everett, D.W. (2014). Delivery of green tea
 558 catechin and epigallocatechin gallate in liposomes incorporated into low-fat hard cheese. *Food*
 559 *Chemistry*, 156, 176-183.

560 Renna, M., Gonnella, M., Caretto, S., Mita, G. & Serio, F. (2017). Sea fennel (*Crithmum*
 561 *maritimum* L.): from underutilized crop to new dried product for food use. *Genetic Resources*
 562 *and Crop Evolution*, 64, 205-216.

563 Sebaaly, C., Jraij, A., Fessi, H., Charcosset, C. & Greige-Gerges, H. (2015). Preparation and
 564 characterization of clove essential oil-loaded liposomes. *Food Chemistry*, 178, 52-62.

565 Sebaaly, C., Greige-Gerges, H., Stainmesse, S., Fessi, H. & Charcosset, C. (2016). Effect of
 566 composition, hydrogenation of phospholipids and lyophilisation on the characteristics of
 567 eugenol-loaded liposomes prepared by ethanol injection method. *Food Bioscience*, 15, 1-10.

568 Sercombe, L., Veerati, T., Moheimani, F., Wu, S. Y., Sood, A. K., & Hua, S. (2015). Advances and
 569 Challenges of Liposome Assisted Drug Delivery. *Frontiers in Pharmacology*, 6, 286.

570 Sharma, A., & Sharma, U.S. (1997). Liposomes in drug delivery: progress and limitations.
571 *International Journal of Pharmacology*, 154, 123-140.

572 Siracusa, L., Kulisic-Bilusic, T., Politeo, O., Krause, I., Dejanovic, B. & Ruberto, G. (2011).
573 Phenolic composition and antioxidant activity of aqueous infusions from Capparis spinosa L.
574 and Crithmum maritimum L. before and after submission to a two-step in vitro digestion
575 model. *Journal of Agricultural and Food Chemistry*, 59, 12453-12459.

576 Slinkard, K., & Singleton, V. L. (1977). Total phenol analysis: automation and comparison with
577 manual methods. *American Journal of Enology and Viticulture*, 28 (1), 49–55.

578 Stark, B., Pabst, G. & Prassl, R. (2010). Long-term stability of sterically stabilized liposomes by
579 freezing and freeze-drying: Effects of cryoprotectants on structure. *European Journal of*
580 *Pharmaceutical Sciences*, 41 (3–4), 546–555.

581 Taladrid, D., Marín, D., Alemán, A., Álvarez-Acero, I., Montero, P. & Gómez-Guillén, M.C.
582 (2017). Effect of chemical composition and sonication procedure on properties of food-grade
583 soy lecithin liposomes with added glycerol. *Food Research International*, 100 (1), 541-550.

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Figure captions

Figure 1. HPLC chromatographic profiles ($\lambda = 253$ nm) of the sea fennel extracts and the corresponding liposomes. (A) Top line: Aq extract; 2nd line: L-Aq64 liposome; 3rd line: vitamin C standard; 4th line: chlorogenic acid standard. (B) Top line: Et extract; 2nd line: L-Et64 liposome; 3rd line: chlorogenic acid standard; 4th line: rutin standard; 5th line: rosmarinic acid standard. (C) Top line: Aq extract; 2nd line: Aq extract heated at 80°C (2h) and sonicated; 3rd line: L-Aq64 liposome; (D) Top line: Et extract; 2nd line: Et extract heated at 80°C (2h) and sonicated; 3rd line: L-Et64 liposome

Figure 2. Caco-2 cell viability (% with respect to untreated control cell, C) incubated for 24 h with different concentrations (0.01–1 mg/mL) of sea fennel extracts. (A) aqueous extract; (B) ethanolic extract. Values presented are means $\pm$ SD of six determinations.

Figure 3. Polar plot representation of Hue and Chroma values of the freeze-dried liposomal preparations loaded with increasing concentrations of aqueous (L-Aq) and ethanolic (L-Et) sea fennel extracts.

Figure 4. Differential scanning calorimetry of freeze-dried liposomal preparations loaded with increasing concentrations of aqueous (L-Aq) and ethanolic (L-Et) sea fennel extracts.

Figure 5. Antioxidant activity of freeze-dried liposomal preparations loaded with increasing concentrations of aqueous (L-Aq) and ethanolic (L-Et) sea fennel extracts. (A) ABTS radical scavenging capacity. (B) Ferric ion reducing capacity (FRAP). (C) Folin-reactive substances content (FRS).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1. Moisture, protein content, Folin-reactive substances (FRS), ABTS radical scavenging activity and ferric ion reducing activity (FRAP) of freeze-dried aqueous (Aq) and ethanolic (Et) sea fennel extracts.

	Moisture g/100 g	Protein g/100 g	FRS mg gallic acid eq./g	ABTS mg vit C eq./g	FRAP mg Fe ²⁺ eq./g
Aq	15.67±1.79	4.12±0.21	75.95±3.97	556.8±4.26	273.6±7.06
Et	21.91±0.65	2.22±0.10	109.00±1.48	805.1±7.37	570.8±10.4

Table 2. Z-average (nm), polydispersity index (PDI) and zeta-potential (mV) of fresh phosphatidylcholine liposomes loaded with increasing concentrations of aqueous (L-Aq) and ethanolic (L-Et) sea fennel extracts.

	Z-average (nm)	PDI	Zeta-potential (mV)
L-Aq8	89.5 ± 0.9 ^{A/a/X}	0.316 ± 0.025 ^{A/a,b/X}	-31.8 ± 1.9 ^{A/a/X}
L-Aq16	97.3 ± 0.9 ^{B/b/X}	0.329 ± 0.039 ^{A/b/X}	-32.4 ± 3.7 ^{A/a/X}
L-Aq32	111.2 ± 0.8 ^{C/d/X}	0.345 ± 0.042 ^{A/b/X}	-36.7 ± 6.4 ^{A/a/X}
L-Aq64	137.1 ± 0.7 ^{D/f/X}	0.370 ± 0.018 ^{A/b/X}	-27.7 ± 2.8 ^{A/a/X}
L-Et8	96.3 ± 1.3 ^{A/b/Y}	0.309 ± 0.027 ^{A/a,b/X}	-34.4 ± 1.1 ^{A/a/Y}
L-Et16	106.0 ± 1.3 ^{B/c/Y}	0.305 ± 0.017 ^{A/a,b/X}	-33.0 ± 3.2 ^{A/a/X}
L-Et32	127.8 ± 0.2 ^{C/e/Y}	0.295 ± 0.018 ^{A/a,b/X}	-33.1 ± 3.2 ^{A/a/X}
L-Et64	150.1 ± 2.0 ^{D/g/Y}	0.324 ± 0.044 ^{A/b/X}	-30.1 ± 2.1 ^{A/a/X}
L-E	87.4 ± 0.8 ^a	0.240 ± 0.005 ^a	-35.5 ± 1.7 ^a

Different letters (A, B, C, D) indicate significance differences ($p \leq 0.05$) among different concentrations of sample for the same extract; (X, Y) among different extracts for the same concentration; and (a, b, c, d, e, f, g) among all samples, including the control, L-E.

Table 3. Physicochemical properties of freeze-dried liposomal preparations loaded with increasing concentrations of aqueous (L-Aq) and ethanolic (L-Et) sea fennel extracts.

	Z-average (nm)	PDI	Zeta-potential (mV)	Moisture (%)	Water dispersibility (%)	Luminosity (L*)
L-Aq8	171.1 ± 1.6 ^{A/a/X}	0.341 ± 0.004 ^{A/a/X}	-46.1 ± 1.1 ^{A/b,c/X}	38.1 ± 0.6 ^{D/e/X}	86.4 ± 6.8 ^{A/b/X}	29.0 ± 0.1 ^{C/c/X}
L-Aq16	249.4 ± 4.0 ^{C/d/X}	0.430 ± 0.010 ^{B/a/X}	-44.6 ± 1.3 ^{A/c/X}	30.5 ± 1.4 ^{C/d/X}	91.6 ± 1.4 ^{A/b,c/X}	29.0 ± 0.1 ^{C/c/X}
L-Aq32	230.2 ± 1.0 ^{B/c/X}	0.332 ± 0.046 ^{A/a/X}	-40.0 ± 1.3 ^{B/d/X}	14.8 ± 1.9 ^{A/a/X}	82.4 ± 2.7 ^{A/a,b/X}	28.0 ± 0.2 ^{B/b/X}
L-Aq64	311.4 ± 4.4 ^{D/e/X}	0.378 ± 0.025 ^{A,B/a/X}	-34.2 ± 0.9 ^{C/e/X}	20.9 ± 1.2 ^{B/b/X}	81.4 ± 4.7 ^{A/a,b/X}	27.1 ± 0.1 ^{A/a/X}
L-Et8	225.4 ± 1.8 ^{A/b,c/Y}	0.388 ± 0.067 ^{A/a/X}	-47.3 ± 1.2 ^{A/b,c/Y}	27.0 ± 0.8 ^{A,B/c,d/Y}	79.9 ± 2.5 ^{A/a,b/X}	30.9 ± 0.3 ^{C/e/Y}
L-Et16	219.3 ± 6.1 ^{A/b/Y}	0.371 ± 0.046 ^{A/a/X}	-48.2 ± 1.1 ^{A/b/X}	37.4 ± 2.5 ^{C/e/X}	88.2 ± 1.8 ^{B/b/X}	29.3 ± 0.5 ^{A/c/X}
L-Et32	225.1 ± 4.5 ^{A/b,c/X}	0.364 ± 0.039 ^{A/a/X}	-40.3 ± 2.3 ^{B/d/X}	22.8 ± 1.2 ^{A/b,c/Y}	82.5 ± 1.2 ^{A,B/a,b/X}	30.2 ± 0.4 ^{B/d/Y}
L-Et64	216.4 ± 2.1 ^{A/b/Y}	0.377 ± 0.010 ^{A/a/X}	-37.5 ± 0.5 ^{B/d/Y}	31.1 ± 2.6 ^{B,C/d/X}	100.0 ± 0.0 ^{C/c/X}	32.1 ± 0.2 ^{D/f/Y}
L-E	316.6 ± 6.7 ^e	0.374 ± 0.081 ^a	-54.2 ± 1.5 ^a	18.5 ± 2.2 ^{a,b}	73.6 ± 2.6 ^a	32.5 ± 0.2 ^g

Different letters (A, B, C, D) indicate significance differences ($p \leq 0.05$) among different concentrations of sample for the same extract; (X, Y) among different extracts for the same concentration; and (a, b, c, d, e, f, g) among all samples, including the control, L-E.

Table 4. Chlorogenic acid concentration in aqueous (Aq) and ethanolic (Et) sea fennel extracts, before and after heating and sonication (HS), and in the corresponding liposomal suspensions (L-Aq64 and L-Et64) in the free form (non-encapsulated).

	Chlorogenic acid (mg/g)
Aq	42.61 ± 0.94
Aq-HS*	38.31 ± 1.22
L-Aq64	13.67 ± 0.34
Et	58.48 ± 1.01
Et-HS*	49.94 ± 1.46
L-Et64	20.05 ± 0.57

*Aq and Et extracts heated at 80°C (2h) and sonicated