Taylor & Francis
Taylor & Francis Group

Journal of Microencapsulation
Micro and Nano Carriers

ISSN: 0265-2048 (Print) 1464-5246 (Online) Journal homepage: https://www.tandfonline.com/loi/imnc20

Design and development of vitamin C-encapsulated proliposome with improved *in-vitro* and *ex-vivo* antioxidant efficacy

Elahehnaz Parhizkar, Marzieh Rashedinia, Maryam Karimi & Shohreh Alipour

To cite this article: Elahehnaz Parhizkar, Marzieh Rashedinia, Maryam Karimi & Shohreh Alipour (2018) Design and development of vitamin C-encapsulated proliposome with improved *in-vitro* and *ex-vivo* antioxidant efficacy, Journal of Microencapsulation, 35:3, 301-311, DOI: 10.1080/02652048.2018.1477845

To link to this article: https://doi.org/10.1080/02652048.2018.1477845

Accepted author version posted online: 21 May 2018.
Published online: 06 Jun 2018.

JOURNAL OF MICROENCAPSULATION
2018, VOL. 35, NO. 3, 301–311
https://doi.org/10.1080/02652048.2018.1477845

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Design and development of vitamin C-encapsulated proliposome with improved *in-vitro* and *ex-vivo* antioxidant efficacy

Elahehnaz Parhizkar[a], Marzieh Rashedinia[b], Maryam Karimi[c] and Shohreh Alipour[c,d]

[a]Department of Pharmaceutics, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran; [b]Department of Toxicology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran; [c]Department of Quality Control, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran; [d]Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Vitamin C, as an antioxidant additive in pharmaceutical and food products, is susceptible to environmental conditions, and new design strategies are needed to enhance its stability. The aim of this study is to prepare vitamin C proliposome using film deposition on the carrier by applying different factors, and optimise the characteristics of the obtained powder using the design expert® software. The optimised formulation demonstrated acceptable flowability with 20% vitamin C loading. This formulation released about 90% vitamin C within 2 h and showed higher (1.7-fold) *in-vitro* antioxidant activity. *Ex-vivo* antioxidant activity was 1.9 and 1.6 times higher in brain and liver cells, respectively. A 27% reduction in malondialdehyde (MDA) level of liver cell was obtained comparing free vitamin C. Therefore, this study results suggest that the vitamin C-encapsulated proliposome powder might be an appropriate carrier for oral drug delivery of vitamin C with better antioxidant efficacy.

ARTICLE HISTORY
Received 9 January 2018
Accepted 24 April 2018

KEYWORDS
Vitamin C; proliposome; *ex-vivo* antioxidant activity; *in-vitro* antioxidant activity

1. Introduction

Vitamin C is an essential hydrophilic nutrient that is used as an antioxidant and additive in many pharmaceutical and food products (Yang *et al.* 2013, Comunian *et al.* 2014, Matos-Jr *et al.* 2015). It is a reactive oxygen-derived species, and a free radical scavenger and deactivator (American Pharmacists Association 2012). Intravenous vitamin C demonstrated oxidative stress reduction and physiological function improvement such as vascular conductance and endothelial performance in adults. Vitamin C infusion has a low acceptance rate and carries a risk of infection, discomfort, and phlebitis. The alternative route, i.e. oral vitamin C, however, has less bioavailability than infusion (Davis *et al.* 2016). Vitamin C is a chemically active and highly unstable agent against environmental factors such as temperature, pH, light, and oxygen, which shows wide instability in aqueous solutions (Matos-Jr *et al.* 2015, Alvim *et al.* 2016, Fong *et al.* 2016). To improve stability and bioavailability of vitamin C, different formulation strategies such as microparticles, nanoparticles, and liposomes have been reported (Comunian *et al.* 2013).

Liposomes – spherical lipid bilayer vesicles – are natural, biodegradable and non-toxic carriers that encapsulate and transport both hydrophilic and hydrophobic agents (Moraes *et al.* 2013, Toniazzo *et al.* 2014, Silva *et al.* 2017). Ingested liposomes' pharmacokinetic properties override the usual absorption pattern of the typical encapsulated drug by regulating the absorption pattern and increasing the absorption rate (Kosaraju *et al.* 2006). Proliposome is a free flowing solid dispersion formulation of phospholipid, which forms liposomal systems in contact with aqueous phase (Yan-Yu *et al.* 2006, Piao *et al.* 2011, Shaji and Bhatia 2013). Proliposome was introduced to eliminate physicochemical instability of liposome (fusion, aggregation and sedimentation) and improve the lowered bioavailability of their encapsulated drugs (Shaji and Bhatia 2013, Khan *et al.* 2015, Fong *et al.* 2016). Previous studies reported different routes of administration for proliposomes, such as parenteral, oral, pulmonary and mucosal routes (Shaji and Bhatia 2013). Oral delivery of proliposomes is an interesting approach because liposomes can be formed in the

CONTACT Shohreh Alipour alipour_sh@sums.ac.ir Department of Quality Control, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

contact of gastric fluid at the site of absorption. Thereafter, the instability of the liposomes diminishes over the time, causing a more predictable release profile (Yan-Yu *et al.* 2006, Shaji and Bhatia 2013). The aim of this study is to prepare and optimise the vitamin C-encapsulated proliposome powder and evaluate its *in-vitro* and *ex-vivo* antioxidant efficacy.

2. Materials and methods

2.1. Materials

Chloroform, methanol, ethanol, cholesterol, sorbitol, mannitol, 2, 4, 6-tripyridyl-s-triazine (TPTZ), sodium acetate, acetic acid, $FeCl_3$ $6H_2O$, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), thiobarbituric acid and H_2O_2 purchased from Merck (Germany). Vitamin C purchased from Samchun chemical (Korea). L-α-Lecithin ((3-*sn*-Phosphatidylcholine) from Soybean, Type IV-S, ≥30 enzymatic) obtained from Sigma (USA). Other materials and reagents were of analytical grade.

2.2. Vitamin C analysis

For vitamin C quantification, T80 UV-Vis spectrophotometer (Germany) was used at maximum absorbance wavelength. Analytical curves were plotted in water and HCl 0.1 N as medium solutions.

2.2.1. Analytical curve validation

Analytical curves were validated by linearity, inter-day and intra-day precision and accuracy. All concentrations were prepared in three different days. Each concentration was tested triplicate. Vitamin C recovery process was calculated by comparing the amount of free vitamin C with vitamin C in the presence of blank proliposome. Considering vitamin C stability in ethanol solution during preparation process was determined within 4 h.

2.3. Proliposome powder preparation

2.3.1. Carrier preparation by wet granulation method

Considering preliminary studies, different amounts of vitamin C (0.5 and 1 g) were mixed with 1 g sorbitol/mannitol as carrier and ethanol was added to the mixture as granulating agent and mixed. To get a homogenous powder, the mixture was passed through 35 mesh and obtained granules were transferred to a flask. Subsequently, the flask was attached to a rotary evaporator (80 rpm, 40 °C, IKA, Germany) connected to a vacuum pump for 30 min to facilitate the ethanol evaporation. Finally, prepared granules were gathered and used for proliposome preparation. Ethanol was chosen as a proper solvent because vitamin C had no destruction in the contact with ethanol (Jeney-Nagymate and Fodor 2008).

2.3.2. Film deposition on carrier method

Determined amounts of prepared granules were transferred into a flask and connected to the rotary evaporator under vacuum. Different lipid phases (Table 1) solubilised in chloroform were added drop wise to the flask until chloroform was evaporated completely. The obtained solid particles were passed 35 meshes and maintained in desiccator for complete drying (Tunsirikongkon *et al.* 2014).

2.3.3. Formulation design

According to preliminary studies, three factors of carrier type, vitamin C amount and lipid phase amount were chosen for experiment. Powders were prepared applying 2-level full factorial experimental design using Design expert 10® software (Table 1).

Three different responses of yield%, flowability and compressibility index for proliposome powders and three different responses of size, drug loading and encapsulation efficiency% for hydrated liposomes were examined to obtain the best formulation.

Table 1. Factors, factor levels and responses used in 2-level full factorial experimental design for proliposome preparation based on three different factors (carrier type, vitamin C amount and lipid phase amount) in two levels and three different responses for powders and hydrated liposomes.

Factors	Type of factors	Factors level		Response	
X1	Carrier type	Mannitol	Sorbitol	Y1	Powder yield%
X2	Vitamin C (g)	0.5	1	Y2	Powder flowability
X3	Lipid phase (g)	0.3	0.6	Y3	Powder compressibility index
				Y4	Hydrated liposome size
				Y5	Hydrated liposome drug loading%
				Y6	Hydrated liposome encapsulation efficiency%

2.4. Proliposome powder evaluation

2.4.1. Proliposome powder yield

Final powders of each formulation were collected and their weight was calculated. Powder yield was calculated and compared with theoretical sum of excipients weight.

2.4.2. Proliposome powder flowability

According to USP guidelines, defined weight of proliposome formulations powders were transferred to Erweka® granule flow tester and angle of repose was measured (Shah *et al.* 2008). All tests were repeated three times. Flowability of proliposome powder was defined using USP guidelines tables.

2.4.3. Proliposome powder compressibility index

According to USP guidelines, defined weight of proliposome formulations powders were transferred to Erweka® tap density tester and powder compressibility index was obtained using following equation:

$$\text{Compressibility index} = \frac{\text{first volume} - \text{final volume}}{\text{first volume}} \times 100 \quad (1)$$

2.4.4. Hydrated liposome characterisation

2.4.4.1. Hydrated liposomes morphology. Liposomes were hydrated in water and their shape was examined by transmission electron microscope (TEM). The sample was added on a formvar-coated grid and stained by 2% (w/w) uranyl acetate. The shapes of the hydrated liposome were observed using a LEO 906E TEM (Philips, Germany).

2.4.4.2. Hydrated liposomes size. The volume median diameter (VMD, 50% undersize) of produced liposomes was evaluated by particle size analyser (Shimadzu, SALD-2101, Japan).

2.4.4.3. Hydrated liposomes drug content. Drug content uniformity was defined by measuring total amount of vitamin C (free and encapsulated) in proliposome. Definite amounts of proliposome formulations were dissolved in chloroform. Then, water was added to the solution in order to form two-phased system. Vitamin C would resolved into aqueous phase and total content of vitamin C in proliposome particle evaluated by analysis method. This process performed three times for selected formulations.

2.4.4.4. Hydrated liposomes drug loading (DL%) and encapsulation efficiency (EE%). Determined amounts of proliposome were dispersed in distilled water and shaked to obtain a colloidal solution. Produced solution was centrifuged 15 min at 4000 rpm at 4 °C. The vitamin C amount in supernatant was determined by analysis method. The absorbance demonstrates the un-encapsulated drug content. So, amount of trapped drug was calculated indirectly. Drug loading means the amount of drug that has been encapsulated in hydrated liposomes. Drug loading encapsulation efficiency was assessed using following equations (Afrooz *et al.* 2017, Vakilinezhad *et al.* 2018):

$$\%\,\text{DL} = \frac{\text{Drug in liposomes}}{\text{Weight of liposomes}} * 100 \quad (2)$$

$$\%\,\text{EE} = \frac{\text{total drug} - \text{uncapsulated drug}}{\text{total drug}} * 100 \quad (3)$$

2.4.4.5. In-vitro release of hydrated liposomes. Twenty milligrams of selected proliposome formulations were dispersed in water and centrifuged at 4000 rpm at 4 °C in order to sediment the performed liposomes. The sediment liposomes were dispersed in to release media (HCl 0.1 N) and incubated in shaker incubator at 50 rpm for 8 h at 37 ± 1 °C. Samples were taken at 0.5, 1, 2, 4, 6 and 8 h ($n = 3$) and were analysed using analysis method. Vitamin C aqueous solution profile was evaluated similarly.

2.5. Proliposome solid state

2.5.1. Proliposome powder differential scanning calorimetry (DSC)

The DSC method was used to investigate the solid state and stability of vitamin C in the proliposome particles by the BAHR thermoanalyser (Gmbh, Germany). Free vitamin C, vitamin C-encapsulated proliposome and blank proliposome were placed in aluminium pans and heated up to 300 °C with a heating rate of 10 °C per minute. Void aluminium pan was sealed as the reference control.

2.5.2. Proliposome powder FT-IR

FT-IR spectra of free vitamin C, blank proliposome and vitamin C-encapsulated proliposome were obtained using a FT-IR spectrometer VERTEX 70 (Bruker, Germany). Samples were previously ground and mixed thoroughly with potassium bromide (KBr), an infra-red transparent matrix. The KBr discs were prepared by compressing the powders, under force of 5 t for 5 min

in a hydraulic press. Thirty scans were obtained at a resolution of 4 cm^{-1}, from 4500 to 400 cm^{-1}.

2.6. Proliposome powder in-vitro antioxidant efficacy test

Ferric Reducing Antioxidant Power (FRAP) assay was performed to determine the total antioxidant activity of vitamin C-encapsulated and blank proliposome. FRAP stock solution was prepared as reported previously (Kumar *et al.* 2014, Sadeghi *et al.* 2015); 2.5 ml acetate buffer (300 mM, pH 3 containing 3.1 g sodium acetate and 16 ml acetic acid), 0.25 ml TPTZ (10 mM in 40 mM HCl) and 0.25 ml $FeCl_3$ $6H_2O$ (20 mM) were mixed and kept in 37 °C for 5 min. Samples were reacted with FRAP solution in the dark condition and studied at 593 nm. Results expressed in μM Fe (II)/g (dry mass) were compared with vitamin C as standard sample.

2.7. Proliposome powder ex-vivo antioxidant efficacy test

2.7.1. Animals

Male wistar rats (average weight 220 ± 20 g) obtained from the centre of comparative and experimental medicine (Shiraz University of Medical Sciences, Shiraz, Iran) were used for *ex-vivo* experiments. Animals were kept at temperature 22 ± 2 °C with a 12 h light/dark cycle. Standard diet and water were provided to the animals *ad libitum*. The experiments were carried out in accordance with the standard guidelines of laboratory animal care and use, Animal Care and Use Committee, Shiraz University of Medical Sciences.

2.7.2. Preparation of PNS

Post nuclear supernatant (PNS) is biological fraction of preserved cell organelles, including mitochondria that were prepared using previously described method (Govil *et al.* 2012). Animals were killed by decapitation and their brain and liver were rapidly removed, washed in a cold phosphate buffer, weighted and kept on ice until homogenisation. Tissues were homogenised in cold phosphate buffer (0.1 M, pH = 7.4) and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was PNS, which was immediately separated and incubated at 37 °C for further evaluation.

According to several previous studies on PNS (Fernandes *et al.* 2010, Moraes *et al.* 2010, Govil *et al.* 2012), for comparison of antioxidant and protective effect against H_2O_2 toxicity, the brain and liver PNS were incubated with various concentrations of H_2O_2 (5, 10, 20 and 25 mM) as toxic agent and vitamin C, vitamin C-encapsulated proliposome and blank proliposome (50, 100, 200 and 250 μM) as antioxidant agent. After that, selected concentration (200 μM) of vitamin C, vitamin C-encapsulated proliposome and blank proliposome pre-incubated for 30 min at 37 °C in a temperature controlled water bath. Then H_2O_2 with final concentration of 10 mM was added and incubated for 1 h at 37 °C and was used for MTT and malondialdehyde (MDA) level assay in the separated repeated samples.

2.7.3. MTT assay

The MTT assay is a measuring method to evaluate the mitochondrial activity. After incubation time, MTT was added to all samples at a final concentration of 0.2 mg/mL and incubated for 30 min at 37 °C. Then DMSO was added into each tube and shaked for 10 min to dissolve the precipitation completely. The absorbance was detected at 540 nm with a Microplate Reader spectrophotometer (BioTek, ELX800 ELISA Reader, USA). The results were expressed as percentage of control, untreated group.

2.7.4. Lipid peroxidation determination

Malondialdehyde (MDA) was determined as an index of lipid peroxidation. The samples were mixed with (TCA 20%, HCl 0.5 N and thiobarbituric acid 0.6%) and heated in boiling water bath for 40 min. The absorbance of produced pink colour was measured at a wavelength of 532 nm. The results were expressed as percentage of control, untreated group.

2.7.5. Statistical analysis

Statistical analysis of data was carried out using GraphPad Instat 3.3 software. Results are expressed as mean ± SD for ($n = 4$–5) independent experiments performed in duplicate. One-way ANOVA and Tukey–Kramer Multiple Comparisons Test were used to test the difference between groups. $p < 0.05$ was considered as statistically significant difference in all groups [*Significant difference $p < 0.05$, **Significant difference $p < 0.01$ and ***Significant difference $p < 0.001$ (*compared to the control group, #compared to the H_2O_2 group)].

3. Results

3.1. Vitamin C analysis

3.1.1. Analytical curve validation

Analytical curve data were gathered in the range of the expected concentrations in water and HCl 0.1 N at

Table 2. Regression equation, correlation coefficient (R^2), precision and accuracy for different analytical curves of vitamin C in water and HCl (as release media) ($n = 3$).

Solvent	Equation	R^2	Precision% (intra-day)	Precision% (inter-day)	Accuracy%
Water	$y = 0.0457x + 0.0348$	0.9999	96.3 ± 4.4	94.4 ± 1.7	96.3 ± 5.3
HCl 0.1 N	$y = 0.0464x + 0.0598$	0.9999	95.2 ± 1.2	98.2 ± 1.3	95.5 ± 3.2

Table 3. Proliposome powder and hydrated proliposome characterisation results ($n = 3$).

	Formulation properties			Proliposome powder			Hydrated liposome			
	Carrier type	Vitamin C (g)	Lipid (g)	Yield% (w/w)	Flowability (angle of repose)	Compressibility index	Size (μm)	Drug content% (w/w)	DL%	EE%
F1	Mannitol	1	0.3	90 ± 1.7	31 ± 1.4	18 ± 0.2	32.8 ± 0.2	95 ± 0.6	1.5 ± 0.2	3.8 ± 0.4
F2	Mannitol	0.5	0.6	80 ± 1.5	35.5 ± 0.7	20 ± 0.4	38.6 ± 0.3	97 ± 3.3	0.6 ± 0.08	1.1 ± 0.2
F3	Mannitol	1	0.6	81 ± 0.9	28.5 ± 0.7	20.2 ± 0.8	34.2 ± 0.5	101 ± 1.4	1.2 ± 0.1	3.2 ± 0.5
F4	Mannitol	0.5	0.3	87 ± 1.1	38.5 ± 2.1	33.3 ± 0.6	34.2 ± 0.4	95 ± 1.1	0.7 ± 0.05	1.4 ± 0.06
F5	Sorbitol	1	0.6	83 ± 1	32.5 ± 0.5	20 ± 0.9	30.5 ± 0.3	101 ± 1.5	19.8 ± 0.6	51.7 ± 1.6
F6	Sorbitol	0.5	0.3	89 ± 2.1	32.6 ± 0.6	20.2 ± 0.6	48.8 ± 0.5	93 ± 1.1	2.9 ± 0.4	11.3 ± 1.7
F7	Sorbitol	0.5	0.6	80 ± 1.7	31.6 ± 0.6	33 ± 0.4	38.2 ± 0.5	102 ± 0.8	3.7 ± 0.5	8.6 ± 1.1
F8	Sorbitol	1	0.3	95 ± 1.2	34.5 ± 0.7	20.1 ± 0.4	46.6 ± 0.2	96 ± 1.6	11.3 ± 2.3	23.8 ± 3.5

λ_{max} 275 nm. The regression equation, the correlation coefficient (r^2) of the standard curve and the precise and accurate validation of the results of analytical curves are reported in Table 2. Vitamin C recovery was 98.5 ± 0.7%, indicating that the extraction process was complete. The average amount of vitamin C in an ethanol solution after 4 h was 99 ± 1.1%, suggesting that the vitamin C amount was intact during the preparation process.

3.2. Proliposome powder preparation

Three factors, namely carrier type, vitamin C amount and lipid phase amount, were selected to prepare eight different formulations, by applying a 2-level full factorial experimental design. Factors, responses and formulation details are presented in Tables 1 and 3.

3.3. Proliposome powder evaluation

3.3.1. Proliposome powder yield

The final process yield of the dried proliposome powder is presented in Table 3. Proliposome yield contour plot is shown in Figure 1.

3.3.2. Proliposome powder flowability

The flow properties of the prepared proliposome powder were determined by angle of repose and compared with mentioned USP indexes (United States Pharmacopeial Convention (USP) 1984), which possessed proper and acceptable flow attributes. The results are reported in Table 3. The proliposome flowability contour plot is illustrated in Figure 1.

3.3.3. Proliposome powder compressibility index

The compressibility properties of the prepared proliposome powder were compared using the mentioned USP indexes (USP 1984), which possessed proper and acceptable compressibility attributes (Table 3 and Figure 1).

3.4. Hydrated liposome characterisation

3.4.1. Hydrated liposomes morphology

TEM technology showed the hydration of vitamin C-encapsulated proliposome (Figure 2(A)). The oily droplets of hydrated proliposome were clearly seen in TEM micrographs.

3.4.2. Hydrated liposome size

The hydrated liposome size was evaluated using a laser diffraction particle size analyser after the hydration of the proliposome. The results are presented in Table 3. The volume median diameter of the hydrated liposome was in the range of 30.5–48.8 μm.

3.4.3. Hydrated liposome drug content

The vitamin C content in hydrated liposomes, as shown in Table 3, confirms a reproducible preparation process for the proliposome powder. The vitamin C content in hydrated liposomes was in the range of 95–102%.

3.4.4. Hydrated liposome drug loading and encapsulation efficiency

As seen in Table 3, drug loading and encapsulation efficiency of hydrated liposomes were in the range of 1.1–51.7% and 0.55–19.8%, respectively. Hydrated

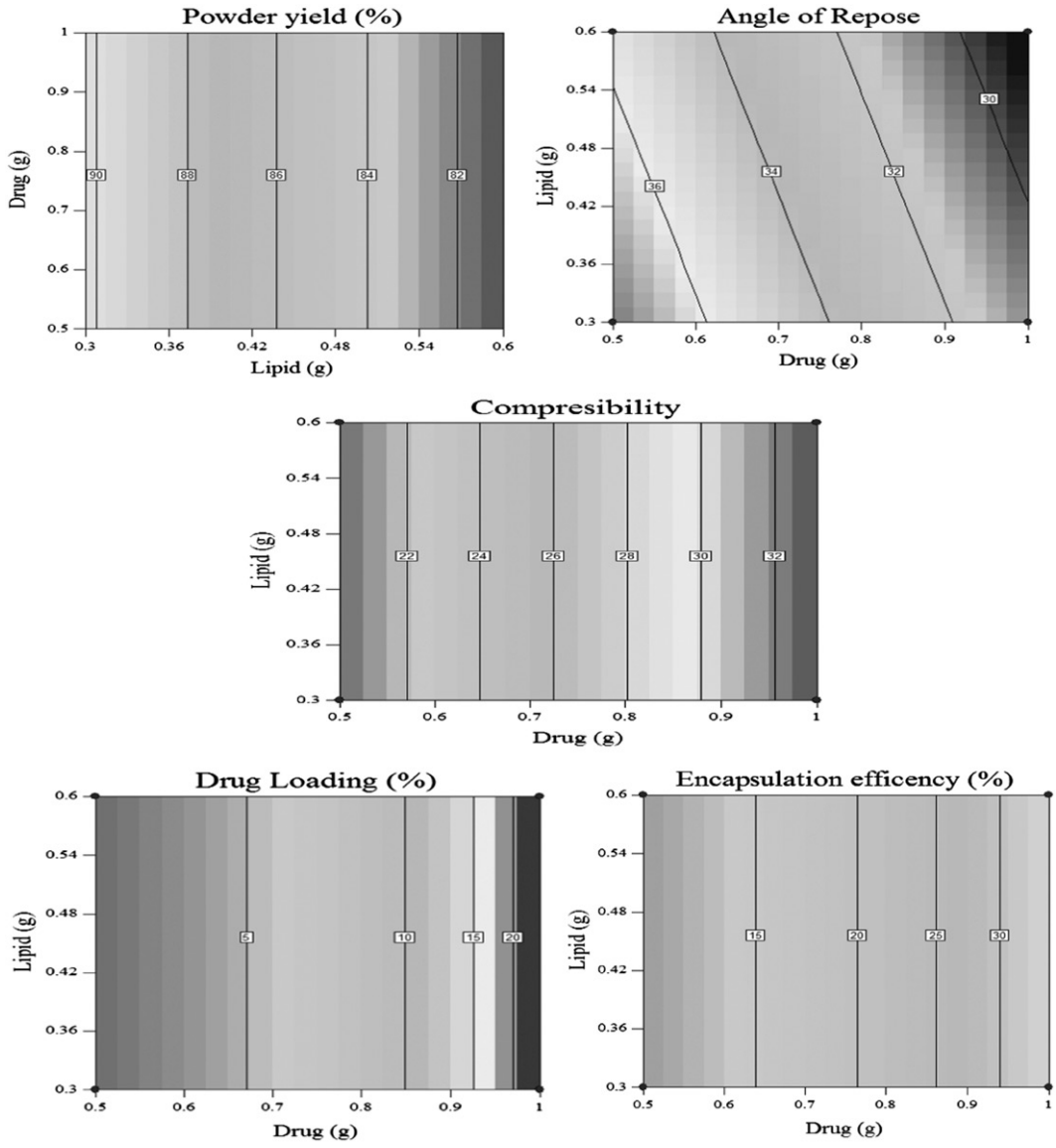

Figure 1. The contour plot of proliposome powder yield%, flowability (angle of repose), compressibility index, drug loading and encapsulation efficiency. Straight lines indicate the effect of a specific factor on the desired response, while the diagonal lines show the effect of both factors on the response.

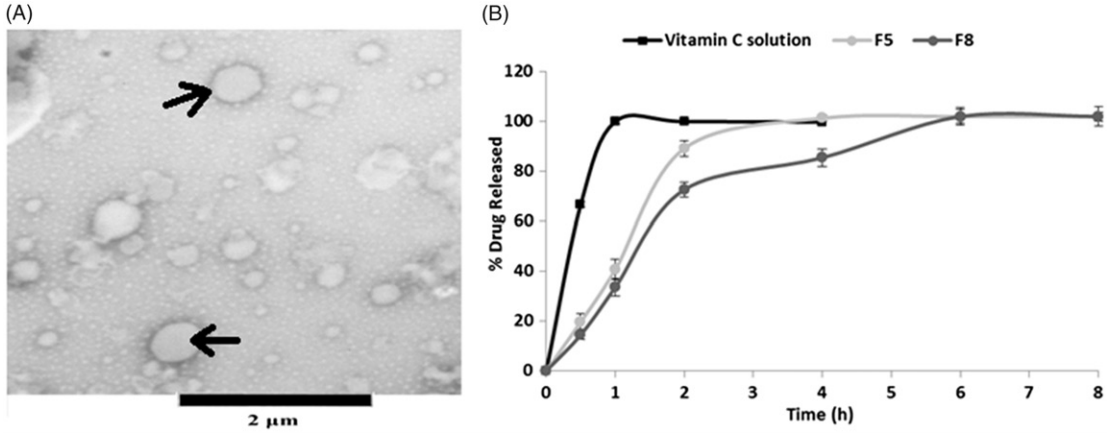

Figure 2. (A) TEM of obtained hydrated liposome from vitamin C-encapsulated proliposome (liposomes are pointed as specified oily droplets), (B) *In-vitro* release of vitamin C solution and optimised hydrated liposomes in release media (HCl 0.1 N) that showed faster release from F5 sample.

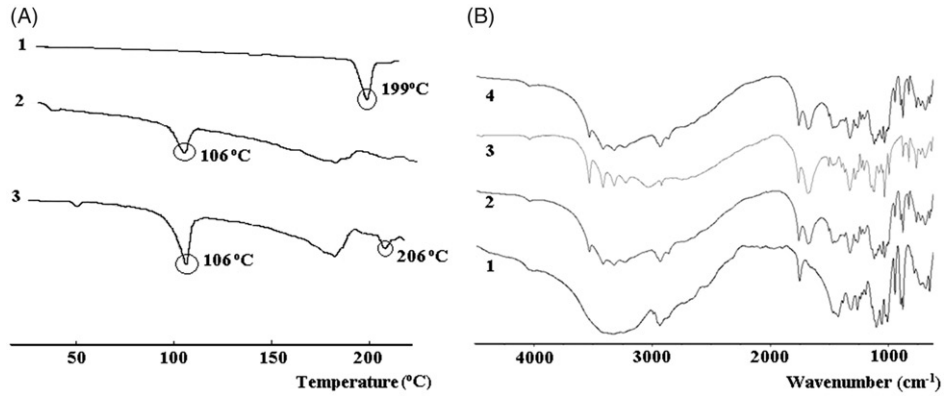

Figure 3. (A): DSC thermograms of free vitamin C (1) with melting point at 199 °C, blank proliposome (2) with obvious peak of sorbitol at 106 °C and vitamin C-encapsulated proliposome (3) with peaks of sorbitol and vitamin C (B): FT-IR of (1) free vitamin C, (2) blank proliposome, (3) vitamin C-encapsulated proliposome, (D) vitamin C-encapsulated proliposome–blank proliposome subtraction.

liposome loading and encapsulation efficiency contour plot are presented in Figure 1.

3.4.5. In-vitro *release profile of hydrated liposomes*

Considering powder control results, two formulations F5 and F8 were examined for *in-vitro* release test. As shown in Figure 2(B), vitamin C release from hydrated liposome of F5 was similar to F8 within early hours, while it was significantly faster in F5 than F8 at 2 ($p = 0.017$) and 4 h ($p = 0.002$). Therefore, F5 was selected for further studies.

3.5. Proliposome powder solid state

3.5.1. Proliposome powder DSC analysis

Figure 3(A) shows the DSC thermograms of free vitamin C, blank proliposome and vitamin C-encapsulated proliposome powder (F5). Free vitamin C thermograms showed a transition at 199 °C, which was related to its melting point. The melting point of sorbitol at 106 °C was seen in blank and F5 proliposome powder thermograms. F5 proliposome powder thermograms showed vitamin C melting point with transition at 206 °C. Since there was no appearance of new peaks or existing peak disappearance, it was confirmed that vitamin C was stable during the preparation process and its structure was not destroyed.

3.5.2. Proliposome powder FT-IR

As it can be seen it Figure 3(B), vitamin C main peak are presented at 1026, 1321, 1674, 1755, 3030, 3218, 3316, 3411 and 3526 cm^{-1} that are similar to previous reports (Lohmann *et al.* 1984, Desai and Park 2005). Blank proliposome FT-IR main peaks were in 1016, 1096, 1186, 1211, 1229, 1746 and 2931 cm^{-1}. The vitamin C-encapsulated proliposome contains vitamin C main peaks at 1026, 1319, 1673, 1752, 3226, 3317, 3410 and 3526 cm^{-1}. The subtraction of vitamin C-encapsulated proliposome and blank proliposome FT-IR confirmed the presence of vitamin C main peaks.

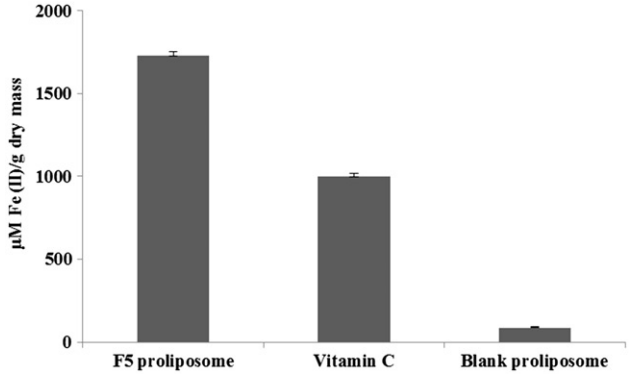

Figure 4. *In-vitro* antioxidant assay of vitamin C, vitamin C-encapsulated proliposome (F5) and blank proliposome by FRAP assay. Increase in the amount of reduced Fe (Fe(II)) shows higher antioxidant activity.

3.6. Proliposome powder in-vitro *antioxidant efficacy test*

As shown in Figure 4, the FRAP values for F5 proliposome was significantly higher than that of free vitamin C ($p < 0.05$). Furthermore, the blank proliposome powder showed low antioxidant activity, which was in line with previous studies (Andorn *et al.* 1996).

3.7. Proliposome powder ex-vivo *antioxidant efficacy test*

3.7.1. MTT assay

As presented in Figure 5, the preservation from H_2O_2 in F5 proliposome powder was significantly higher

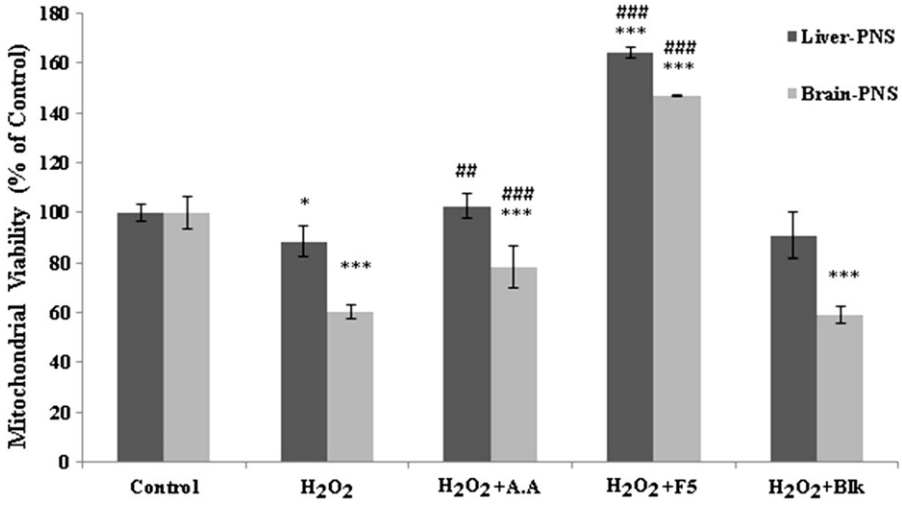

Figure 5. MTT assay of free vitamin C (A.A), blank proliposome powder (Blk) and vitamin C-encapsulated proliposome powder (F5) in the presence of H_2O_2. *Compare with control. #Compare with H_2O_2. Significant difference in mitochondrial activity in F5 in comparison with control and H_2O_2 groups approved proper antioxidant properties.

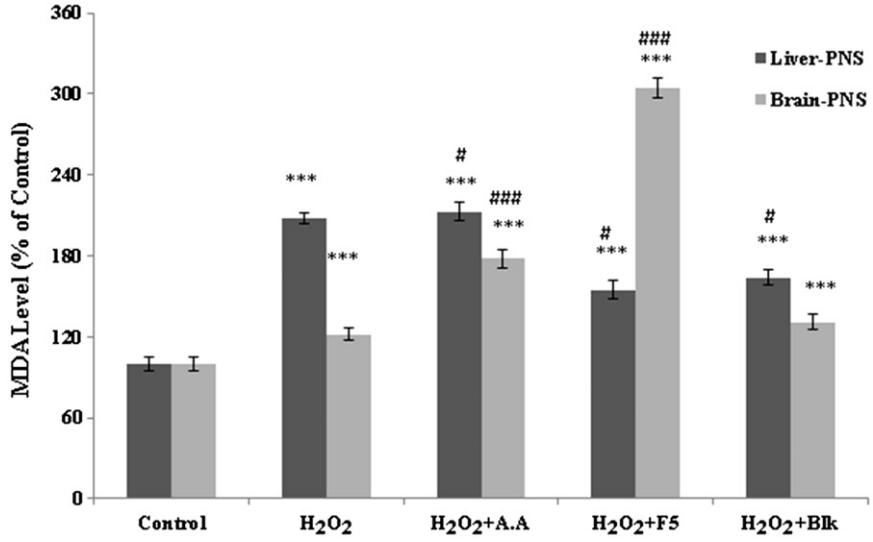

Figure 6. MDA assays of free vitamin C (A.A), blank proliposome powder (Blk) and vitamin C-encapsulated proliposome powder (F5) in the presence of H_2O_2. *Compare with control. #Compare with H_2O_2. F5 proliposome powder had proper antioxidant properties in liver cells compared to H_2O_2 and free vitamin C but was not effective in brain tissue (higher MDA level showed higher peroxidation).

($p < 0.001$) than with free vitamin C in both brain (1.9-fold) and liver (1.6-fold) organs. However, the blank proliposome showed negative effects especially in brain samples.

3.7.2. Lipid peroxidation determination

The lipid peroxidation test indicated that F5 proliposome powder reduced the MDA level up to 27% compared to H_2O_2 and free vitamin C in liver cells (Figure 6). In contrast, in brain tissue, F5 proliposome powder increased MDA level significantly in comparison with control and H_2O_2 samples.

4. Discussion

4.1. Vitamin C analysis

The validation results of the analytical curves in Table 2 imply acceptable correlation coefficient, inter- and intra-day precision and accuracy.

4.2. Proliposome powder evaluation

Powders were prepared applying a 2-level full factorial experimental design using Design-Expert 10® software. As an explanation, R-square is a proof of correlation coefficient; the results become better as the mentioned

Table 4. Proliposome powder and hydrated liposomes significant responses analysis of variance obtained from factorial experimental design.

	Powder yield% (w/w)	Powder flowability	Powder compressibility index	Liposome DL%	Liposome EE%
R^2	0.8077	0.9569	0.9924	0.9780	0.9676
Adj. R^2	0.7756	0.9246	0.9867	0.9692	0.9547
Pred. R^2	0.6581	0.8277	0.9697	0.9437	0.9171
Adeq. Precision	7.099	15.570	27.8	23.292	19.059

parameters reach 1. Adj. R^2 is an adjustment of the number of model parameters relative to the number of runs. Pred. R^2 is a measurement of the predictive capability of the model. The difference between these two parameters should be less than 0.2, indicating the reasonable agreement of the results. The value of Adeq Precision shows a comparison between the ranges of predicted values with the average prediction error, in which ratios greater than 4 indicate adequate model discrimination. Results of significantly different responses ($p < 0.05$) are shown in Figure 1 (the contour plot) and Table 4 (the analysis of variance).

Proliposome powder yield contour plot in Figure 1 indicated that the lipid amounts can significantly ($p = 0.0024$) affect the final proliposome powder yield. The Pred. R^2 and Adj. R^2 difference is less than 0.2. Adeq Precision of 7.099 indicates an adequate signal (Figure 1, Table 4). With the increase in the lipid phase amount, the powder yield decreased due to the higher adherence of the proliposome powder to the processing instrument.

The angle of repose is the powder characteristic parameter describing the flow property and particle friction of the powders. As seen in Table 3, F2 and F4 showed fair (36–40) flow properties, while in other formulations flowability was good (31–35). With the increase in drug and lipid, the angle of repose decreased, leading to better powder flowability. The Pred. R^2 is in reasonable agreement with the Adj. R^2 of 0.9246, and the Adeq Precision is greater than 4 (15.570) (Figure 1, Table 4).

4.3. Hydrated liposome characterisation

Hydrated liposome morphology was evaluated using TEM technique. Figure 2(A) suggesting a progressive conversion of vitamin C-encapsulated proliposome to liposomes with the number size range of 500–1200 nm (data were not shown) on contact with water. The presence of the oily droplets in TEM micrographs confirms the number size of hydrated liposomes.

Particle size analysis indicated that the volume median diameter of the liposomes was in the range of 30.5–48.8 micron. According to statistical analysis, there was no significant difference ($p = 0.075$) between the volume median diameter hydrated liposomes in different formulations. Drug content results may suggest lower drug content in powders containing a higher lipid phase. This phenomenon was expected due to the hydrophilic characteristic of vitamin C that causes less interaction with the lipid phase (Marsanasco *et al.* 2011).

As seen in Figure 1, carrier type ($p < 0.0001$) and drug amount ($p = 0.0009$) were significantly effective factors on drug loading. While sorbitol was the carrier, increasing drug amount caused higher drug loading. For drug loading results, the Pred. R^2 difference from the Adj. R^2 is in reasonable agreement (less than 0.2). The Adeq Precision is greater than 4 and in the acceptable range (Figure 1, Table 4).

For encapsulation efficiency results, Pred. R^2, Adj. R^2 and Adeq Precision are in an acceptable range (Figure 1, Table 4). This model can be used to navigate the design space. While sorbitol is a carrier, increasing drug amount causes higher encapsulation efficiency. Results indicate that carrier type ($p = 0.0001$) and the amount of vitamin C ($p = 0.0023$) can significantly affect encapsulation efficiency.

Sorbitol and mannitol are six-carbon polyhydric alcohol isomers that differ in the molecular orientation of the hydroxyl group in the second carbon atom. Physical properties of these isomers are different, especially in terms of solubility aspects; sorbitol is hygroscopic and mannitol is non-hygroscopic. Furthermore, sorbitol melting point is lower than mannitol (106 °C compared to 164 °C). These aspects caused sorbitol to form a melted mixture during the proliposome-forming process, which could mix with the organic phase in a more efficient manner than mannitol and led to higher loading percentage (Rowe *et al.* 2009, O'Brien-Nabors 2011).

The optimised formulations F5 (with higher drug loading and encapsulation efficiency% of hydrated liposomes) and F8 (with higher powder yield% and better flowability) were selected by software for *in-vitro* release assay. Faster vitamin C release in F5 samples at 2 and 4 h could be related to the smaller size of hydrated liposomes. According to obtained results, formulation F5 with better powder features, higher drug loading and encapsulation efficiency and faster vitamin C release was selected for further experiments.

4.4. Proliposome powder solid state

The presence of vitamin C melting peak in DSC thermogram of vitamin C-encapsulated proliposome (Figure 3(A)) indicated that vitamin C was intact during the preparation process. Also, comparing the subtraction FT-IR peaks of vitamin C-encapsulated proliposome and blank proliposome with free vitamin C FT-IR (Figure 3(B)) confirmed that vitamin C structure was not destroyed.

4.5. Proliposome powder in-vitro antioxidant efficacy test

The FRAP antioxidant assay is constructed on the *in-vitro* ability of samples to reduce the TPRZ-Fe (III) complex to TPTZ-Fe (II). The results showed that the proliposome structure could not only save the antioxidant activity of vitamin C and prevent it from destruction, but it could also increase the antioxidant characteristics due to the presence of lecithin and cholesterol.

4.6. Proliposome powder ex-vivo antioxidant efficacy test

According to the obtained results, vitamin C in free and liposomal form increases the lipid peroxidation in brain. This finding is in line with previous studies, where, ascorbate-stimulated lipid peroxidation in the human brain depends on iron. One of the reasons for this could be the penetration enhancement of vitamin C to the brain due to the presence of liposome as the carrier. We chose the PNS of liver and brain tissues to show that there was a difference between the protective effects of our formulation in liver as an organ with high enzymatic and non-enzymatic antioxidant contents, relative to brain tissue with a low level of antioxidant capacity and high levels of polyunsaturated fatty acids vulnerable to oxidant damage. The present study has shown that brain PNS is much less capable of tolerating H_2O_2-induced oxidative stress, whereas liver PNS is much more resistant than the brain, which is like the previously observed difference between different organs (Dasuri *et al.* 2009).

5. Conclusions

Proliposome powders are acceptable alternatives that can reduce the disadvantages of liposomal carriers such as physical instability, non-predictable release profile and aggregation of vesicles. Proliposome was prepared using film deposition on the carrier method. Optimised formulation (F5) produced liposomes with acceptable size, drug loading and drug release. Vitamin C was stable during the preparation process and showed higher antioxidant activity – in FRAP assay (*in-vitro*) and in brain and liver cells (*ex-vivo*), respectively. Therefore, this could suggest that the proliposome powder is an efficient carrier for oral drug delivery of vitamin C with enhanced *in-vitro* and *ex-vivo* antioxidant efficacy.

Acknowledgment

The authors thank Dr. Saeed Daneshamouz for his beneficial comments in this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was part of the Pharm.D project of Maryam Karimi and was financially supported by Shiraz University of Medical Sciences [Grant No. 94-01-103-10903].

References

Afrooz, H., *et al.*, 2017. Design and characterization of paclitaxel-verapamil co-encapsulated PLGA nanoparticles: potential system for overcoming P-glycoprotein mediated MDR. *Journal of drug delivery science and technology*, 41, 174–181.

Alvim, I.D., *et al.*, 2016. Comparison between the spray drying and spray chilling microparticles contain ascorbic acid in a baked product application. *LWT - food science and technology*, 65, 689–694.

Andorn, A.C., Britton, R.S., and Bacon, B.R., 1996. Ascorbate-stimulated lipid peroxidation in human brain is dependent on iron but not on hydroxyl radical. *Journal of neurochemistry*, 67 (2), 717–722.

American Pharmacists Association. 2012. *Drug information handbook: with international trade names index*. London: Lexicomp.

Comunian, T.A., *et al.*, 2013. Microencapsulation of ascorbic acid by complex coacervation: protection and controlled release. *Food research international*, 52 (1), 373–379.

Comunian, T.A., *et al.*, 2014. Fabrication of solid lipid microcapsules containing ascorbic acid using a microfluidic technique. *Food chemistry*, 152, 271–275.

Dasuri, K., *et al.*, 2009. Comparison of rat liver and brain proteasomes for oxidative stress-induced inactivation: influence of ageing and dietary restriction. *Free radical research*, 43 (1), 28–36.

Davis, J.L., *et al.*, 2016. Liposomal-encapsulated ascorbic acid: influence on vitamin C bioavailability and capacity to protect against ischemia–reperfusion injury. *Nutrition and metabolic insights*, 9, 25–30.

Desai, K., and Park, H., 2005. Encapsulation of vitamin C in tripolyphosphate cross-linked chitosan microspheres by spray drying. *Journal of microencapsulation*, 22 (2), 179–192.

Fernandes, C.G., *et al.*, 2010. Experimental evidence that phenylalanine provokes oxidative stress in hippocampus and cerebral cortex of developing rats. *Cellular and molecular neurobiology*, 30 (2), 317–326.

Fong, S.Y.K., *et al.*, 2016. Solid phospholipid dispersions for oral delivery of poorly soluble drugs: investigation into celecoxib incorporation and solubility-in vitro permeability enhancement. *Journal of pharmaceutical sciences*, 105 (3), 11113–11123.

Govil, N., *et al.*, 2012. Postnuclear supernatant: an in vitro model for assessing cadmium-induced neurotoxicity. *Biological trace element research*, 146 (3), 402–409.

Jeney-Nagymate, E., and Fodor, P., 2008. The stability of vitamin C in different beverages. *British food journal*, 110 (3), 296–309.

Khan, I., *et al.*, 2015. Proliposome powders prepared using a slurry method for the generation of beclometasone dipropionate liposomes. *International journal of pharmaceutics*, 496 (2), 342–350.

Kosaraju, S.L., Tran, C., and Lawrence, A., 2006. Liposomal delivery systems for encapsulation of ferrous sulfate: preparation and characterization. *Journal of liposome research*, 16 (4), 347–358.

Kumar, S., Sandhir, R., and Ojha, S., 2014. Evaluation of antioxidant activity and total phenol in different varieties of *Lantana camara* leaves. *BMC research notes*, 7 (1), 560.

Lohmann, W., Pagel, D., and Penka, V., 1984. Structure of ascorbic acid and its biological function. *European journal of biochemistry*, 138 (3), 479–480.

Marsanasco, M., *et al.*, 2011. Liposomes as vehicles for vitamins E and C: an alternative to fortify orange juice and offer vitamin C protection after heat treatment. *Food research international*, 44 (9), 3039–3046.

Matos-Jr, F.E., *et al.*, 2015. Development and characterization of solid lipid microparticles loaded with ascorbic acid and produced by spray congealing. *Food research international*, 67, 52–59.

Moraes, T.B., *et al.*, 2010. Lipoic acid prevents oxidative stress in vitro and in vivo by an acute hyperphenylalaninemia chemically-induced in rat brain. *Journal of the neurological sciences*, 292 (1), 89–95.

Moraes, M., *et al.*, 2013. Liposomes encapsulating beta-carotene produced by the proliposomes method: characterisation and shelf life of powders and phospholipid vesicles. *International journal of food science & technology*, 48 (2), 274–282.

O'Brien-Nabors, L., 2011. *Alternative sweeteners*. New York: CRC Press.

Piao, H., *et al.*, 2011. Preparation of a solid-in-oil nanosuspension containing L-ascorbic acid as a novel long-term stable topical formulation. *International journal of pharmaceutics*, 420 (1), 156–160.

Rowe, R. C., Sheskey, P. J., and Quinn, M. E., 2009. *Handbook of pharmaceutical excipients*. Washington, DC: Pharmaceutical Press.

Sadeghi, Z., *et al.*, 2015. Antioxidant activity and total phenolic content of *Boerhavia elegans* (choisy) grown in Baluchestan, Iran. *Avicenna journal of phytomedicine*, 5 (1), 1.

Shah, R.B., Tawakkul, M.A., and Khan, M.A., 2008. Comparative evaluation of flow for pharmaceutical powders and granules. *AAPS PharmSciTech*, 9 (1), 250–258.

Shaji, J., and Bhatia, V., 2013. Proliposomes: a brief overview of novel delivery system. *International journal of pharmacy and biological sciences*, 4 (1), 150–160.

Silva, G.S., *et al.*, 2017. Characterisation of curcumin-loaded proliposomes produced by coating of micronised sucrose and hydration of phospholipid powders to obtain multilamellar liposomes. *International journal of food science & technology*, 52 (3), 772–780.

Toniazzo, T., *et al.*, 2014. β-carotene-loaded liposome dispersions stabilized with xanthan and guar gums: physico-chemical stability and feasibility of application in yogurt. *LWT - food science and technology*, 59 (2), 1265–1273.

Tunsirikongkon, A., Charernruttanakul, A., and Sarisuta, N., 2014. Physical properties of proliposome for industrial quality control and reconstitution of proliposome in porcine intestinal mucosa. *International journal of pharmacy and pharmaceutical sciences*, 6 (9), 546–551.

United States Pharmacopeial Convention, 1984. Committee of Revision. The United States Pharmacopeia. United States Pharmacopeial Convention, Incorporated.

Vakilinezhad, M.A., Alipour, S., and Montaseri, H., 2018. Fabrication and in vitro evaluation of magnetic PLGA nanoparticles as a potential methotrexate delivery system for breast cancer. *Journal of drug delivery science and technology*, 44, 467–474.

Yan-Yu, X., *et al.*, 2006. Preparation of silymarin proliposome: a new way to increase oral bioavailability of silymarin in beagle dogs. *International journal of pharmaceutics*, 319 (1–2), 162–168.

Yang, S., *et al.*, 2013. Preparation and characterization of nanoliposomes entrapping medium-chain fatty acids and vitamin C by lyophilization. *International journal of molecular sciences*, 14 (10), 19763–19773.