BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e20492

Article

Vitamin C as a shelf-life extender in liposomes

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The objective of this study was to evaluate the influence of vitamin C (VC) on the stability of stored liposomes under different climatic conditions. Liposomal formulations containing 1 mg/mL of VC (LIP-VC) and blank formulations (LIP-B) were prepared by the reverse-phase evaporation method. After preparation, they were characterized according to their refractive index, average vesicle diameter, polydispersity index (PDI), zeta potential, pH, content, encapsulation efficiency (EE%), morphology, stability and antioxidant activity. For stability, LIP-VC and LIP-B were stored in different climatic conditions (4 °C, 25 °C and 40 °C) for 30 days. The LIP-VC presented 1.3365 refractive index, 161 nm of mean diameter, 0.231 PDI, -7.3 mV zeta potential, 3.2 pH, 19.4% EE%, spherical morphology, 1 mg/mL of VC content, and antioxidant activity of 12 and 11.4 µmol of TE/mL for the radical DPPH and $ABTS^{+}$, respectively. During stability, the LIP-B stored in 40 °C showed an instability in the parameters: PDI, vesicle size and zeta potential after 15 days, while the LIP-VC remained stable in its size and PDI for 30 days. After that, it is shown that VC can be used as an antioxidant and stabilizer in liposomes to increase the stability and shelf-life of vesicles.

Keywords: Ascorbic acid. Antioxidant. Nanoliposomes. Antioxidant activity. Reverse-phase evaporation.

INTRODUCTION

Liposomes are spherical vesicles with one (unilamellar) or more (multilamellar) bilayers of phospholipids, involved in an aqueous nucleus (Brannon-Peppas, 1993). The structure of the liposomes is organized according to the interactions of polarity (Winterhalter, Lasic, 1993). Liposomes are also non-toxic, non-immunogenic and biodegradable, as well as being naturally amphipathic, encapsulating hydrophobic, hydrophilic and amphiphilic compounds (Daudt *et al.,* 2013; Brannon-Peppas, 1993; Galvão *et al.,* 2016; Jiao *et al*., 2019).

The major problem that limits the wide use of liposomes is their stability, be that physical and chemical. These vesicles can be affected by several factors that impact their stability, and these factors are divided into three categories: physical, chemical, and biological (Sharma, Sharma, 1997). The chemical instability of these vesicles can be solved by the addition of antioxidants in the formulation (Cacela, Hincha, 2006). Antioxidants are substances capable of sequestering or preventing the formation of free radicals, thereby reducing or retarding the oxidation of another substance, and the main concern regarding the stability of liposomes is self-oxidation (Halliwell, Gutteridge, 1990; Hunt, Tsang, 1981).

Antioxidants are a diverse group of compounds, such as vitamins, minerals, natural pigments and other plant compounds (Food Ingredients Brasil, 2016). Vitamin C (VC) and alpha-tocopherol (vitamin E) are examples of the most commercially important natural antioxidants (Reische, Lillard, Eitenmiller, 2008). Vitamin C is also known as ascorbic acid, its chemical formula is $C_6H_8O_6$, molecular weight 176 g/mol, melting point 192 °C, and

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is a white, odorless, stable and hydrophilic solid crystal (Mann, Truswell, 2011; Bobbio, Bobbio, 1995). It is considered an acid of medium strength (pK1 = 4.04 and pK2 = 11.4 at 25 ºC) and its absorption in the ultraviolet region depends on its pH (Belitz, Grosch, 1999).

Vitamin C is normally used as a food preserving agent because of its antioxidant capacity and it is currently used in a variety of food products (Food Ingredients Brasil, 2016). Vitamin E is a hydrophobic antioxidant widely used to prevent lipid oxidation of liposomes (Urano *et al*., 1987; Hunt, Tsang, 1981, Roggia *et al*., 2020). Although vitamin E is widely used, it is hydrophobic, and therefore it is among the phospholipid bilayer present in liposomes, what can affect the encapsulation of hydrophobic assets (Hunt, Tsang, 1981).

Vitamin C, on the other hand, is a hydrophilic antioxidant, and is found in the aqueous nucleus of the liposomes. Thus, it does not affect the encapsulation of hydrophobic compounds, and may be a new alternative to increase the stability, shelf life and antioxidant activity of liposomes containing hydrophobic compounds. The aim of this study was to investigate the influence of vitamin C (as an antioxidant) on the physical and chemical characteristics of liposomes stored in different climatic conditions for 30 days.

MATERIAL AND METHODS

Material

Vitamin C (ascorbic acid), cholesterol, 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonate) (ABTS) and 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH$^{\bullet}$) were purchased from Sigma-Aldrich®. Polysorbate 80, ethyl alcohol, methanol, potassium persulphate, anhydrous sodium acetate, and glacial acetic acid were purchased from Synth®, and Lipoid S-100 from Lipoid®. HPLC-grade acetonitrile was acquired from J.T. Baker®, phosphoric acid P.A from Nuclear®, and Milli-Q® water.

Liposome preparation

Liposomes were prepared according to the reverse phase evaporation method developed by Mertins and collaborators (2005) and Oliveira and collaborators (2014), with modifications. Liposomes containing vitamin C (LIP-VC) and blank liposomes (LIP-B) were prepared in triplicate. The liposome suspensions were produced according to the formulation described in Table I and the liposomes were prepared according to Figure 1.

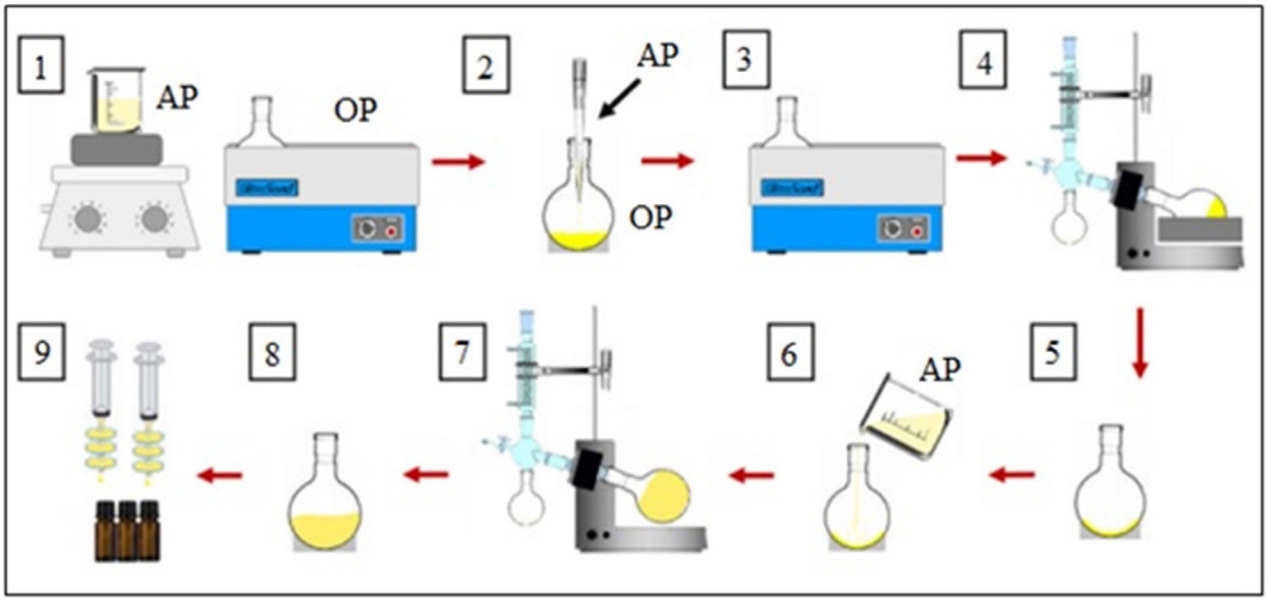

FIGURE 1 - Steps preparation of liposomes by the reverse phase evaporation method.

TABLE I – Composition of LIP-VC and LIP-B

Components	LIP-VC	LIP-B
Organic phase (OP)		
Phospholipid – Lipoid S100®	0.80 g	0.80 g
Cholesterol	0.15 g	0.15 g
Ethanol	40 mL	40 mL
Aqueous phase (AP)		
Polysorbate 80	0.15 g	0.15 g
Buffer – pH 3.65	100 mL	100 mL
Vitamin C	0.10 g	---

First, the aqueous phase (AP) components were added in a beaker, homogenized with the help of a magnetic agitator, with temperature of 35 ºC, until its complete solubility.Then, the organic phase (OP) components were added in a round-bottom balloon, and put in an ultrasound bath for 20 minutes for homogenization. After the homogenization of both phases, 4 mL of AP was added in the OP and the mixture was taken again to the ultrasound bath for 10 minutes, for the formation of reverse micelle. After that, the formulation was taken to the rotary evaporator (80 rpm, temperature of 35ºC) for the complete organic solvent evaporation and formation of the organogel. Subsequently, the rest of the AP was added and put again in the rotary evaporator without vacuum for 30 minutes (120 rpm/35ºC) for agitation, and the formation of liposomal vesicles. Lastly, the liposomal formulation underwent an extrusion process to standardize vesicle size using membranes of 0.45 μm and 0.22 μm porosity.

Vitamin C determination during the preparation of liposomal formulations

During the preparation of LIP-VC, 75 μL of the formulations to determine the vitamin C content by HPLC were removed in three separate steps, in order to examine whether vitamin C was lost during the production of the formulations, which were:

1) After homogenization of AP;
2) After the formulation had been stirred in the rotary evaporator for 30 minutes;
3) After the extrusion of the formulation.

The vitamin C content was determined by HPLC.

Liposome characterization

The liposome characterization was done through the refractive index, vesicle size, polydispersity index, zeta potential, pH, vitamin C content, encapsulation efficiency, morphology, stability and antioxidant activity. The analysis followed the recommendations of the manufacturers of the equipment used. All analysis were performed in triplicate.

Refractive index

The refractive index was determined using the refractometer, for LIP-VC and LIP-B. The reading was performed in a graduated scale, through an optical system. First of all, the equipment was calibrated with distilled water. After that, a drop of the sample was placed in the equipment, in which a limit line between light and dark parts was observed through the focusing eyepiece. After this adjustment, the scale was verified, which corresponds to the refractive index of the sample.

Vesicle size and polydispersity index (PDI)

The vesicle size and polydispersity index were determined by dynamic light scattering on the Zetasizer® Nano-ZS model ZEN 3600 (Malvern, England), diluted 500 times (v/v) in deionized water, which was filtered using a 0.45 μm diameter porous membrane.

Zeta potential

Zeta potential was determined by electrophoresis on the Zetasizer®, diluted in NaCl 10 mM solution (500 times, v/v) previously filtered through a 0.45 μm membrane.

pH

The determination of the pH was carried out in a potentiometer (Digimed®) previously calibrated with standard solutions.

Vitamin C quantification by HPLC

The vitamin C content in the formulations was performed by HPLC based on the method previously described by Scherer and collaborators (2012), with adaptations. The system consisted of a chromatograph Prominence (Shimadzu, Japan) equipped with a CBM-20A system controller, LC-20AT pump, DGU-20A 5R degasser, SIL-20A HT auto-sampler and SPD-M20A detector (UV/VIS). Analytical separation was performed on a Phenomenex C18 (2) column (Torrance, USA) (150 mm × 4.6 × 5 m). The mobile phase was composed by 0.05 M KH2PO4 and acetonitrile (99:1 v/v), with 0.095% phosphoric acid (v/v), which was pumped at a flow rate of 0.6 mL/min. The volume injected was 20 µL and vitamin C was detected at 243 nm. The determination of the vitamin C content occurred by the co-validation method from the analytical parameters: linearity, limits of detection and quantification, specificity and precision, according to official validation guides (ICH, 2005; Brasil, 2017).

Encapsulation Efficiency

Encapsulation efficiency was determined by an ultrafiltration-centrifugation technique described by Ourique and collaborators (2014). The free drug was separated from liposomes using a filter unit (Ultrafree-MC® 10 000 MW, Millipore, Bedford, USA) submitted to a centrifugation at 5000 rpm for 10 minutes. Afterwards, the drug content was determined in the ultrafiltrate by HPLC. Encapsulation efficiency (%) was calculated by the difference between the total (C_{total}) and free drug (C_f) concentrations.

$$EE\% = \frac{C_{total} - C_f}{C_{total}} \text{ x } 100$$

Morphology

The morphology of the LIP-VC was performed by Atomic Force Microscopy (AFM) using the Agilent Technologies 5500 equipment. The images were obtained at room temperature using non-contact high-resolution SSS-NCL tips (Nanosensors, force constant 48 N/m, resonance frequency 154 kHz). The images were captured using PicoView 1.14.4 software (Molecular Imaging Corporation) and analyzed using PicoImage 5.1 software.

Liposomes stability

Three batches of LIP-VC, three batches of LIP-B and three batches of vitamin C solution in buffer (prepared with sodium acetate and acetic acid) were prepared for the stability determination. Each batch was divided into three vials, and each vial was stored under different conditions. The conditions were: climatic chamber (CC - 40 ° C), refrigerator (RE - 4 ° C) and room temperature (RT - 25 ° C). At the 0, 24, 48, 72, 96 hours, and 7, 10, 15, 20, 30 days after preparation the following parameters of the sections were evaluated: determination of mean diameter, PDI, zeta potential, pH and vitamin C content for liposome formulations and the parameter content of vitamin C for free vitamin C solutions.

Antioxidant activity

The antioxidant activity analysis were performed with the same formulations of LIP-VC and free vitamin C used for stability (2.4) and on the same days of stability, for a better monitoring of the data. The LIP-B formulations were analyzed only at an initial time to verify whether any constituent of the formulation would exhibit activity or influence the assay. For better understanding the results of the antioxidant activity, computational simulations were performed. All analysis were done in triplicate.

Free radical sequestration DPPH·

The determination of the antioxidant activity was performed according to the method described by Brand-

Williams and collaborators (1995) and updated by Roesler and collaborators (2007). A solution of DPPH$^{\bullet}$ (0.004% m/v), with an absorbance range between 0.8 and 1.2 at a wavelength of 517 nm, and stored in the refrigerator and in the dark up to the time of analysis, was prepared for the analysis. For the samples, in one tube, an aliquot of the sample was mixed with methanol to a final volume of 400 µL, and then, 2 mL of the DPPH$^{\bullet}$ solution was added. The control (blank) was prepared with 400 µL of methanol plus 2 mL of DPPH$^{\bullet}$ solution. Each tube was incubated for 30 minutes at room temperature in the dark. A standard curve with 8 points (range 10 - 175 µg/mL) was prepared for the analysis. A Trolox solution at the concentration of 1500 µmol was used in this curve. It was withdrawn 400 µL from each point of this standard curve, and mixed to 2 mL of DPPH$^{\bullet}$ solution. After that, the curve was incubated for 30 minutes at room temperature in the dark (Roesler *et al*., 2007).

After 30 minutes, the samples blank and curve were measured at 517 nm in UV/VIS-UV-1650 PC spectrophotometer (Shimadzu®). The decrease in absorbance percentage was recorded for each sample, and the percentage of DPPH$^{\bullet}$ was calculated based on the observed decrease in absorbance of the radical. The antioxidant activity was expressed as µmol of Trolox equivalents (TE) per µl of sample (µmol TE/µL) and was calculated using three equations (Yen, Duh, 1994).

Free radical sequestration ABTS$^{\bullet+}$

The determination of the antioxidant activity by ABTS$^{\bullet+}$ was performed using the method described by RE and collaborators (1999). The free radical cation ABTS$^{\bullet+}$ was generated by 5.0 mL of ABTS solution (7mmol) with 88.0 µL of potassium persulfate solution. The system was kept in the dark for 16 hours at room temperature. The ABTS$^{\bullet+}$ solution was diluted in deionized water until an absorbance of 0.700 ± 0.02 nm was obtained at a wavelength of 734 nm, read in a UV/VIS spectrophotometer. For the samples, an aliquot of the sample was mixed with deionized water to a final volume of 400 µL, and then 2 mL of the ABTS$^{\bullet+}$ solution was added. The (blank) control was prepared with 400 µL of methanol plus 2 mL of ABTS$^{\bullet+}$ solution. Each tube was incubated for 6 minutes at room temperature in the dark. A standard curve with 8 points (range 10 - 175 µg/mL) was prepared for the analysis, and a Trolox solution at the concentration of 1500 µmol was used in this curve. It was withdrawn 400 µL, from each point of this standard curve, and mixed to 2 mL of ABTS$^{\bullet+}$ solution. Then the curve was incubated for 6 minutes at room temperature in the dark (Re *et al*., 1999).

Beyond 6 minutes, the samples, blank and curve were measured at 734 nm in UV/VIS spectrophotometer. The percentage decrease in absorbance was recorded for each sample and the percentage of ABTS$^{\bullet+}$ was calculated based on the observed decrease in absorbance of the radical [25]. The antioxidant activity was expressed as µmol of Trolox equivalents (TE) per µl of sample (µmol TE/µL) and was calculated using three equations (Yen, Duh, 1994).

Computer simulation

The theoretical study is based on the Density Functional Theory (DFT) (Hohenberg, Kohn, 1964) with the Local Density Approximation (LDA) of Perdew and Zunger (1981) parameterization for the exchange and correlation term. The computational procedures were performed using the SIESTA (Spanish Initiative for Electronic Simulations with Thousands of Atoms) code (Soler *et al*., 2002). A double zeta basis set including polarization orbitals was used, and an energy cutoff of 150 Ry was applied. Residual forces were smaller than 0.05 eV/ Å for all the atomic coordinates. The binding energy (E_B) is given by the following equation:

$$E_B = -\{ E_{(VitC+FR)} - (E_{VitC} + E_{FR})\}$$

where $E_{(VitC+FR)}$ corresponds to the total energy of the interacting system, when Vitamin C interacts with one free radical (FR) molecules (ABTS or DPPH). E_{VitC}/ E_{FR} is the total energy corresponding to isolated Vitamin C/ FR molecule. Positive values of binding energies represent attractive interactions.

Statistical analysis

Data analysis were expressed through the medium followed by the standard deviation (SD), or mean ± SD. Data were submitted to analysis of variance (ANOVA) and Dunnett's post-test. The Dunnett post-test considered values of $p < 0.05$ as statistically significant. The analysis were performed with the aid of GraphPad Prism® software and the graphs were created using Excel software.

RESULTS AND DISCUSSION

The first analysis was the determination of the refractive index, which is a necessary parameter for the Zetasizer® equipment, and it is in this equipment that the average vesicle diameter, polydispersity index and zeta potential are analyzed. This parameter is necessary for more reliable results, and the value obtained for LIP-VC and LIP-B was 1.3365. This value corroborates with the one found by Estes and Mayer (2005) for liposomes filled with glycerol solution, which was 1.3365. During the production of liposomes, the vitamin C content of the LIP-VC formulation was quantified in different steps, in order to analyze if during the production of LIP-VC there would be a loss of vitamin C. Figure 2 shows the vitamin C content during LIP-VC preparation steps.

Results in Figure 2 presented no significant loss of vitamin C in any of the three steps analyzed. Figure 3 shows the vesicle size, PDI, zeta potential and pH of the LIP-VC and LIP-B for 30 days at different temperatures.

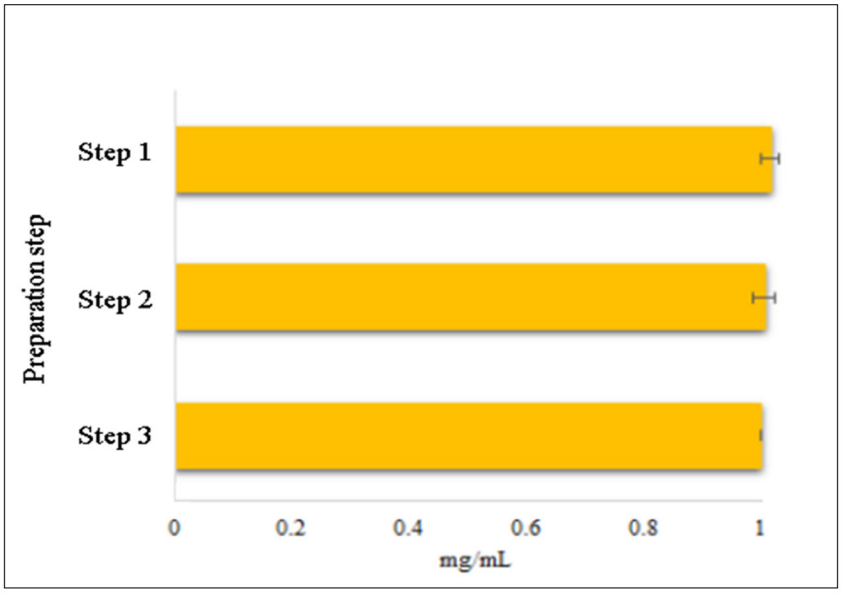

FIGURE 2 - Vitamin C content during LIP-VC preparation steps.

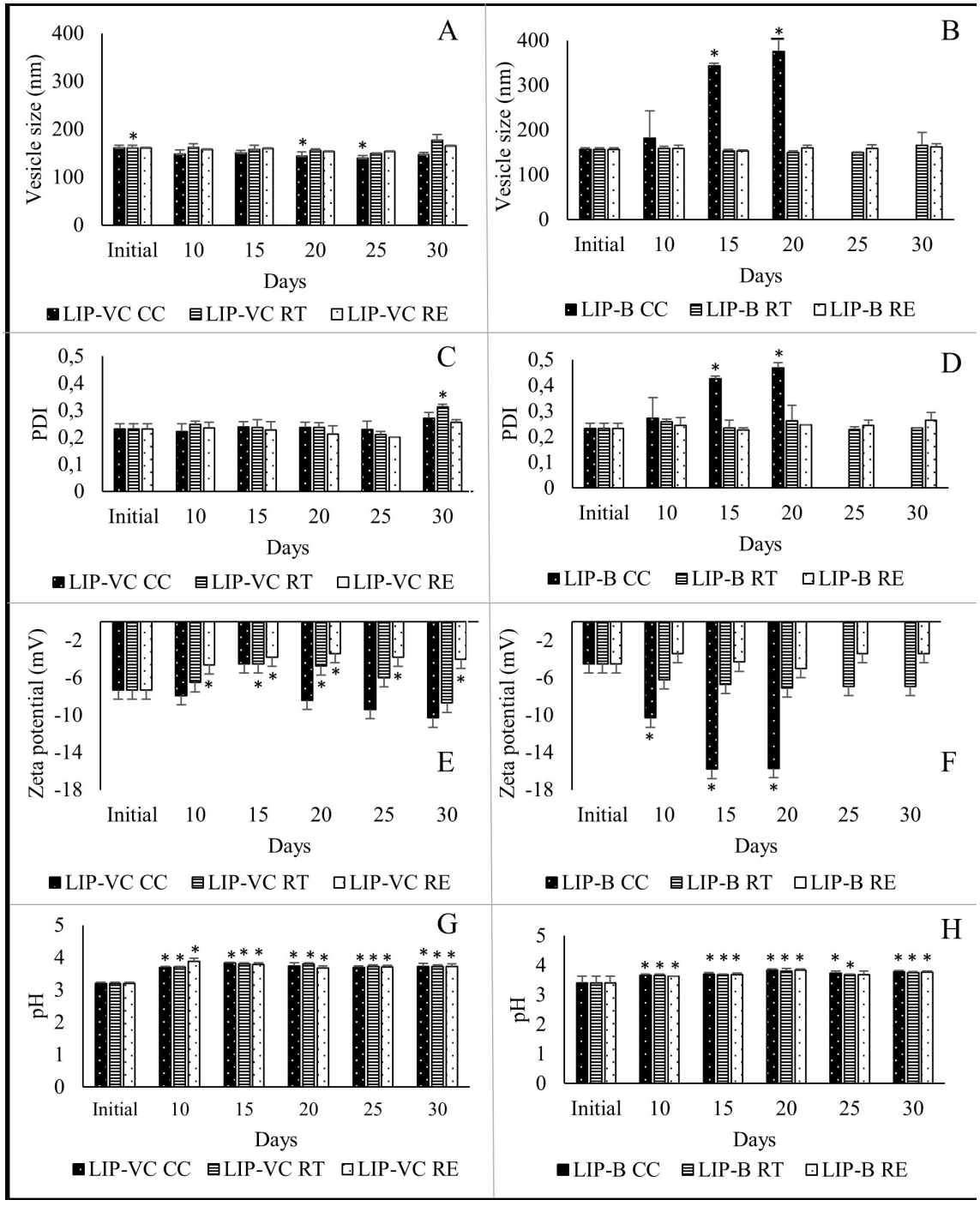

FIGURE 3 - Stability of vesicle size, IPD, zeta potential and pH of the LIP-VC and LIP-B for 30 days under different storage conditions: room temperature (RT), climate chamber (CC) and refrigeration (RE). The results were expressed as the tail moment (number = 3, mean ± standard error of mean, *p<0.05 compared to time 0 - analysis of variance and Dunnett's post-test).

According to Figure 3, it can be observed that the LIP-VC and LIP-B produced on the nanoscale presented a vesicle diameter of ± 160 nm and PDI of 0.23. LIP-VC or LIP-B showed no significant difference in diameter or PDI, therefore, vitamin C had no influence on these parameters. The LIP-VC during the 25-day stability analysis maintained its diameter and PDI stable in all three conditions studied, while LIP-B was only stable in

these parameters for 10 days in all conditions. LIP-VC different from LIP-B has vitamin C as an antioxidant, and vitamin C has the function of preventing oxidation by neutralizing free radicals, so, vitamin C was probably the reason for the formulation to remain stable for a longer time since the formulation did not have any other antioxidant as a constituent.

Alves and collaborators (2016) developed liposomes-associated cocoa extracts also by the reverse phase evaporation method followed by extrusion and obtained a vesicle size of 196 nm and PDI of 0.26. The size and PDI found by Alves and collaborators (2016) are similar to that found in this study. However, they used vitamin E instead of vitamin C as an antioxidant in the formulation.

The initial zeta potential of both formulations was negative, and shortly after the preparation, LIP-VC obtained a potential of - 7.3 mV and the LIP-B of - 4.5 mV. LIP-VC constantly varied its zeta potential in the three temperature conditions, while the LIP-B remained stable under the conditions of RT and RE at different times, during the analysis. The fact that LIP-VC has a more negative zeta potential than LIP-B can be the result of the pKa_1 (4.04) of vitamin C, that is present in LIP-VC and absent in LIP-B (Marsanasco *et al*., 2015).

Because this pKa_1 causes the vitamin C to become ionized and release hydrogen atoms, thus increasing the number of negative charges, this increase made the zeta potential more negative. LIP-B presented changes in its diameter, PDI and zeta potential in the condition of CC after 10 days, which according to Schaffazick and collaborators (2003) and Müller and collaborators (2011) can be a demonstration of instability.

The initial pH of the LIP-VC and LIP-B formulations was 3.20 and 3.40, respectively. LIP-VC has a lower pH than LIP-B, possibly due to the acidic characteristics of vitamin C, demonstrating that vitamin C influenced the pH of the formulation, making it more acid. Over the course of 30 days both formulations, LIP-VC and LIP-B, showed an increase in their pH. Figure 4 shows the vitamin C content of the LIP-VC for 20 days at different temperatures.

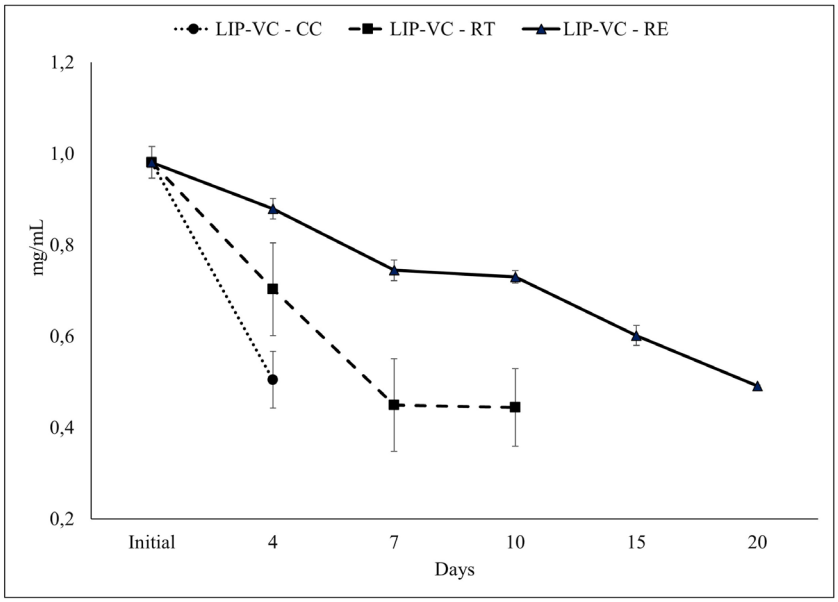

FIGURE 4 - Vitamin C content of LIP-VC for 30 days under different storage conditions: room temperature (RT), climate chamber (CC) and refrigeration (RE).

The initial vitamin C content of LIP-VC was 1 mg/mL, after this, as can be seen in Figure 4, the vitamin C content of the liposomes decreased in all conditions. LIP-VC, which was stored in the CC, showed lower content

stability than the LIP-VC stored in the RE. This might be because vitamin C generally increases its stability as its temperature decreases (Ordóñez *et al.,* 2007). With this, the condition of the refrigerator was the condition that best kept the vitamin C content in liposomes, with vitamin C content (0.06 mg/mL) for 20 days.

It is noticed that the vitamin C content of the liposomes decreases while the physical-chemical parameters of the liposomes (vesicle size, zeta potential and PDI) remain stable for longer than the parameters of the liposome without the vitamin (LIP-B). The decrease in the vitamin C content may be an indication that the vitamin is degrading to maintain the physical-chemical stability of the liposomes.

The efficiency of encapsulation of vitamin C in LIP-VC was 19.4%. Depending on the method used for the liposome preparation and polarity of the active, this efficiency can increase or decrease. For example, Farhang and collaborators (2012) prepared liposomes with phospholipids containing vitamin C, following the microfluidization method and obtained an encapsulation efficiency of 26% for vitamin C. Figure 5 shows the morphology of liposomes containing vitamin C (LIP-VC) performed by Atomic Force Microscopy (AFM).

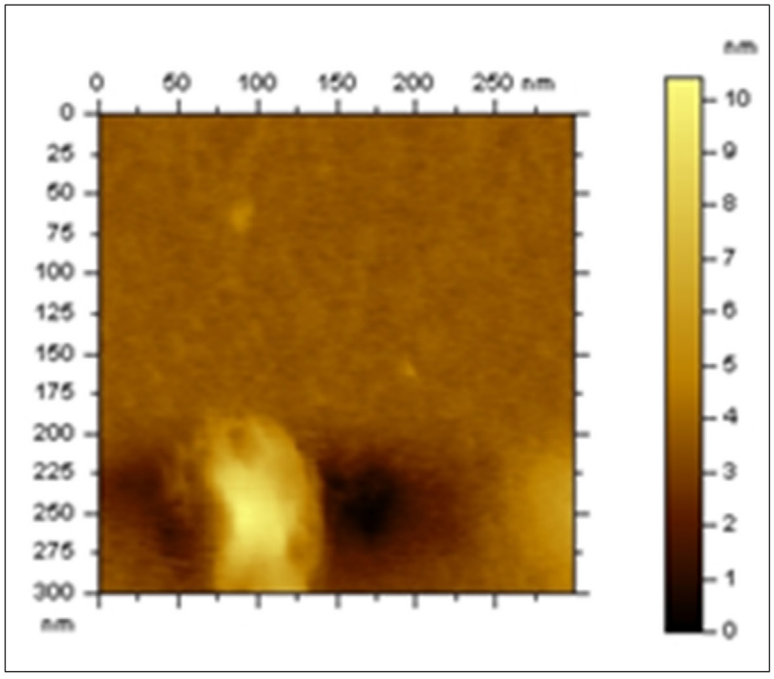

FIGURE 5 - Image referring to the morphological analysis of LIP-VC by AFM.

According to Figure 5, it is possible to observe the LIP-VC morphology and the fact that liposome had a spherical shape and size similar to that found by the dynamic light scattering technique. Roggia and collaborators (2020) produced liposomes by the same method as this paper, and analyzed the morphology and size of the liposomes by cryo-transmission electron microscopy. Liposomes showed spherical shape and average diameter around 200 nm. Results that corroborate with those found in this work. Through the cryo-transmission electron microscopy, Roggia and collaborators (2020) demonstrated that liposomes produced by the reverse phase evaporation method form multilamellar (MLV) and large unilamellar (LUV) vesicles, with a higher amount of unilamellar systems. Figure 6 shows the antioxidant activity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) of the LIP-VC for 20 days at different temperatures, because after 20 days LIP-VC did not show more antioxidant activity.

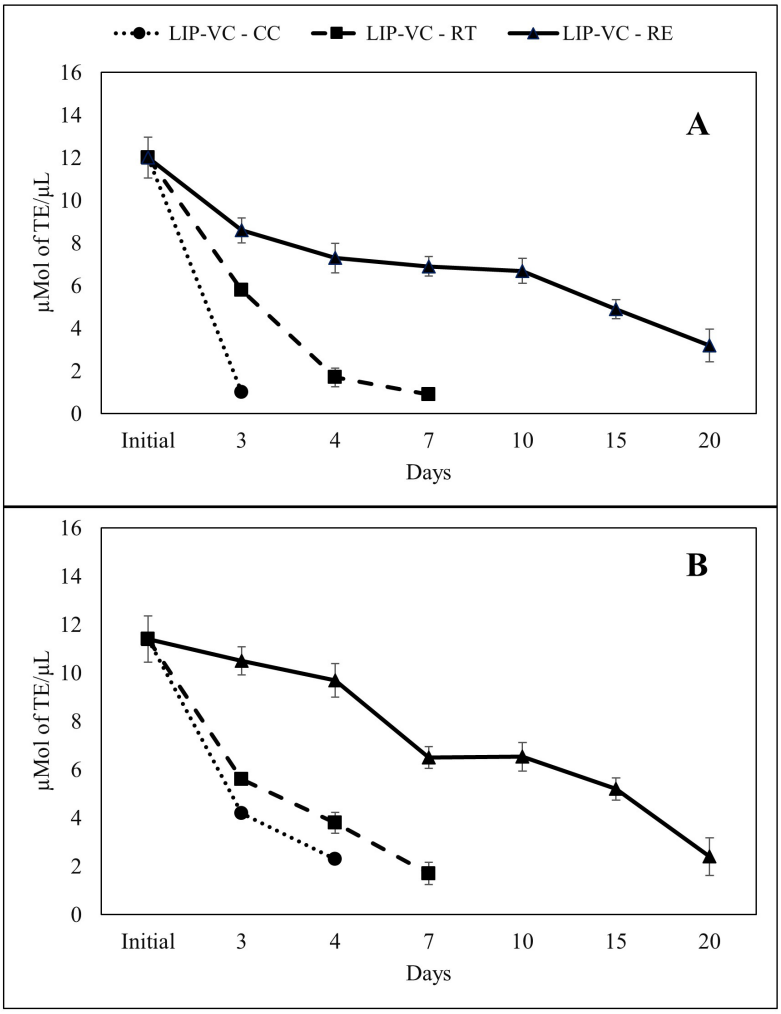

FIGURE 6 - Antioxidant activity of LIP-VC for 30 days under different storage conditions: room temperature (RT), climate chamber (CC) and refrigeration (RE) against the free radical DPPH$^{\bullet}$ (A) and ABTS$^{\bullet+}$ (B).

The initial activity of LIP-VC for the DPPH$^{\bullet}$ radical was 12.0 µmol TE/mL and for ABTS$^{\bullet+}$ 11.4 µmol TE/mL. LIP-B showed no sequestering activity. As in vitamin C content, the sequestering activity of LIP-VC relative to free radical's DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ decreased in CC and RT conditions. As an antioxidant, vitamin C has the function of sequestering free radicals. So, when the vitamin C begins to degrade, it becomes 2,3-diketogulonic acid and its sequestering activity begins to decrease, because 2,3-diketogulonic acid is not an antioxidant, therefore, it does not present a sequestering activity.

The difference in the results obtained in the antioxidant activity of two free radicals (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) for the same active compound has been demonstrated by several authors since distinct free radicals interact differently with the same active (Apak *et al*., 2013). In this study, a computational simulation was performed to complement the experimental results related to antioxidant activity. This methodology can help to elucidate the difference in the analysis of antioxidant activity for the studied radicals. The vitamin C, ABTS$^{\bullet+}$, DPPH$^{\bullet}$ isolated molecules and the ABTS@Vitamin C, DPPH@Vitamin C were simulated through the DFT methodology and the optimized arrangements are presented in the Figure 7 (a-e). We also analyzed the energy levels for all the studied systems as can be observed in Figure 7 (f). In order to verify the interacting systems' HOMO (Highest

Occupied Molecular Orbital) and LUMO (Lowest Unoccupied Molecular Orbital) levels, we performed LDOS (Local Density of States) calculations, which are presented in Figure 7 (g and h). Table II shows the main electronic and structural properties of the isolated and interacting systems.

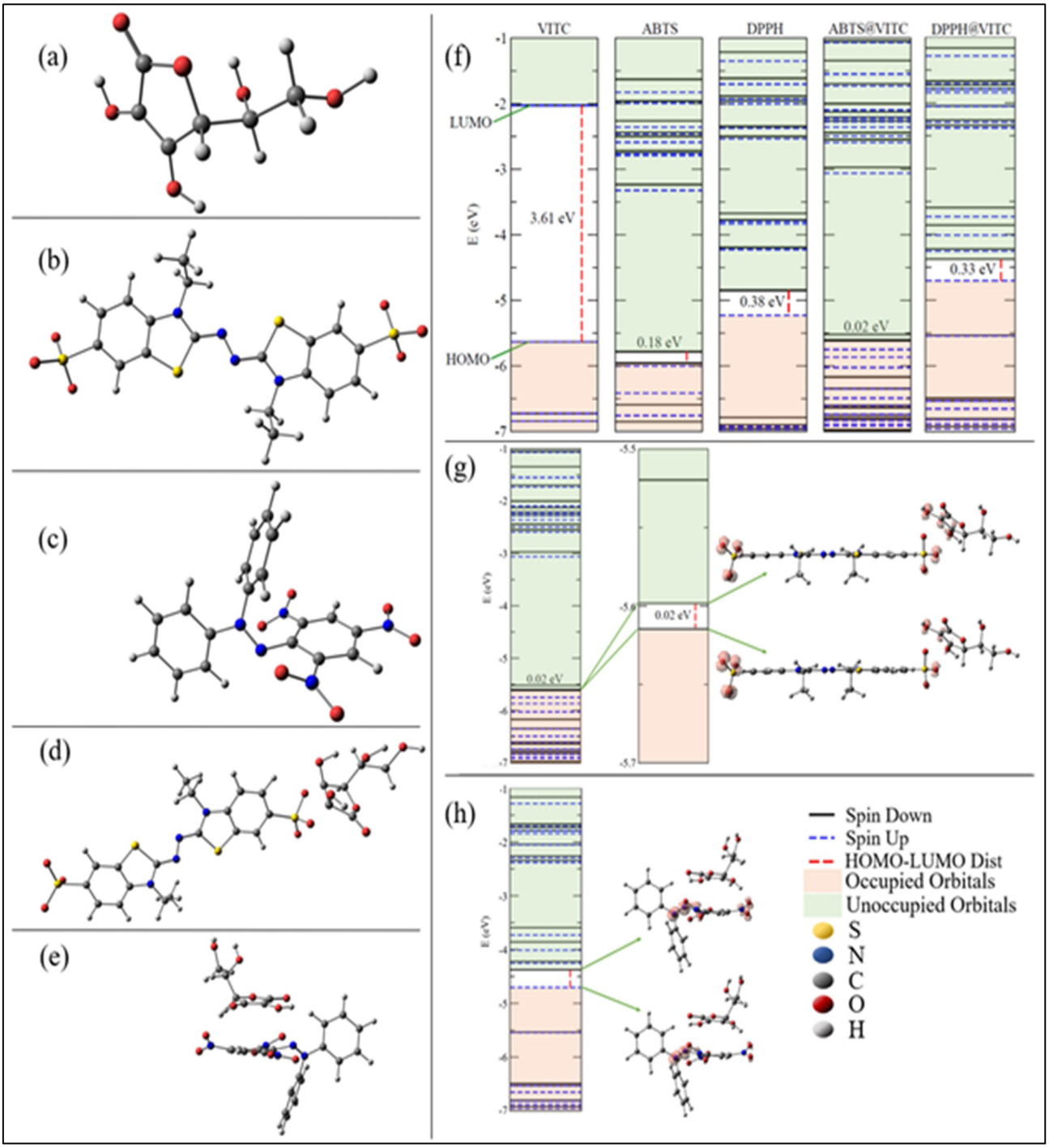

FIGURE 7 - Representation of the molecule of (a) vitamin C, (b) $ABTS^{\bullet+}$, (c) $DPPH^{\bullet}$, (d) DPPH@Vitamin C and (e) ABTS@Vitamin C. Energy levels for isolated and interacting systems (f). Corresponding LDOS to (g) ABTS@Vitamin C and (h) DPPH@Vitamin C (0.0054 $e^{-}/Å^{3}$ charge density).

TABLE II – Binding distance, binding energy, spin polarization, HOMO-LUMO difference and charge transfer for all the systems. Negative values for the charge transfer represent that the electronic charge flow from the vitamin C to $ABTS^{\bullet+}$/$DPPH^{\bullet}$ molecules

System	Binding Distance (Å)	Binding Energy (eV)	Spin Polarization (μB)	HOMO-LUMO difference (eV)	Charge Transfer (e^-)
VITC	-	-	0.00	3.61	-
$ABTS^{\bullet+}$	-	-	1.84	0.18	-
$DPPH^{\bullet}$	-	-	1.00	0.38	-
ABTS@Vitamin C	2.31	0.82	2.00	0.02	-0.27
DPPH@Vitamin C	1.59	0.81	1.00	0.33	0.00

According to Table II, it can be noticed that both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ molecules interact with vitamin C with binding energy values up to 0.82 eV, which can be considered as a weak interaction, so-called physisorbed, agreeing with the values of previous studies (Jauris *et al*., 2016). Binding distance is given by the shorter distance between closest atoms from each molecule. ABTS@Vitamin C [DPPH@Vitamin C] has shown a 2.31 [1.59] Å for this parameter.

The $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ present spin polarization when they are isolated. After the interaction with vitamin C, the values of the spin polarization are basically with the same order. Moreover, the charge transfer occurred only for vitamin C interacting with $ABTS^{\bullet+}$ molecule, with a value of 0.27 e^-. This charge transfer can also corroborate the increase in the spin polarization of the ABTS@Vitamin C compared with the isolated $ABTS^{\bullet+}$ molecule.

The red dashed lines represent the distance between HOMO and LUMO levels in Figure 7 (f). Black continuous [blue dashed] lines represent spin down [up] levels, and orange [green] background represents occupied [unoccupied] orbitals. The different positions of the up and down electronic levels indicate spin polarization of the systems. In addition, we verified that a HOMO-LUMO distance decrease for both free radical molecules after interactions with vitamin C molecule, due to the hybridization levels derived from the adsorption.

Figure 7 (g) shows that the HOMO and LUMO levels are in only 0.02 eV energy difference. Thus, we considered a look closer to a 0.2 eV energy range. Now we can verify that both HOMO and LUMO have contributions from free radical's and weak contribution from vitamin C's five atoms ring. Fig. 7 (h) indicates that vitamin C has no contribution for HOMO and LUMO energy levels, without electronic charge density localized in its atoms. $DPPH^{\bullet}$ exhibits charge density on nitrogen and on oxygen atoms of its structure.

The difference in the charge transfer of the systems (DPPH@Vitamin C and ABTS@Vitamin C) performed by computer simulation, may explain the fact that antioxidant activity of vitamin C is higher against the free radical $ABTS^{\bullet+}$, and consequently lower against the $DPPH^{\bullet}$, as seen in Figure 7. There was only charge transfer to the ABTS@Vitamin C (0.27 e^-) system. This increase in charge transfer shows more interactions between the molecules of vitamin C (antioxidant) and $ABTS^{\bullet+}$ (free radical), which agrees with the experimental results of antioxidant activity (LIP-VC showed higher activity against the radical $ABTS^{\bullet+}$). This charge transfer can also corroborate with the increase in the spin polarization of the ABTS@Vitamin C compared with the isolated $ABTS^{\bullet+}$ molecule.

Therefore, it demonstrates that the results experimentally obtained through the in vitro tests for antioxidant activity corroborate with the theoretical results obtained by the ab initio computer simulation, a higher antioxidant activity of vitamin C against $ABTS^{\bullet+}$.

CONCLUSION

In this study, it was possible to investigate and confirm that the vitamin C can increase the liposome stability and shelf-life. This increase is due to the vitamin C degradation (demonstrated through antioxidant activity and content) to preserve vesicle stability in terms of size, PDI and zeta potential. The storage temperature of LIP-VC influences the parameters described above, and this influence occurs because vitamin C degrades differently at different temperatures, be that under refrigeration the best stability of vitamin C and LIP-VC, consequently. In addition to improving the stability and shelf-life of liposomes, vitamin C also adds antioxidant activity to these vesicles, since liposomes without vitamin C (LIP-B) did not show antioxidant activity for both free radicals ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$) that were tested. The initial antioxidant activity of LIP-VC for the $DPPH^{\bullet}$ was 12.0 µmol TE/mL and for $ABTS^{\bullet+}$ 11.4 µmol TE/mL. The results obtained in the experimental analyzes of antioxidant activity (higher antioxidant activity of LIP-VC on $ABTS^{\bullet+}$) were explained through a computer simulation performed between the vitamin C, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ molecules. Along with this work, it was concluded that vitamin C can be used as a hydrophilic alternative to improve the stability and shelf-life of liposomes, mainly in the encapsulation of hydrophobic compounds, in addition to joining antioxidant activity to this particle.

ACKNOWLEDGEMENTS

This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS - Project 17/2551-0000830-8), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Universidade Franciscana. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 (number 88887.153004/2017-00). They are also grateful to CENAPAD-SP and UFN for the computer support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Received for publication on 17th August 2020
Accepted for publication on 05th April 2021