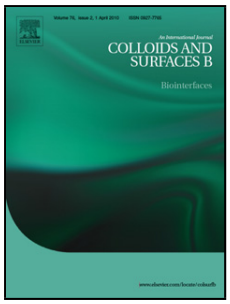
Journal Pre-proof



Protective effect of guarana-loaded liposomes on hemolytic activity

Isabel Roggia, Ana Júlia F. Dalcin, Aline F. Ourique, Ivana B.M. da Cruz, Euler E. Ribeiro, Montserrat Mitjans, Maria Pilar Vinardell, Patrícia Gomes

PII: S0927-7765(19)30780-5

DOI: https://doi.org/10.1016/j.colsurfb.2019.110636

Reference: COLSUB 110636

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received Date: 12 April 2019

Revised Date: 7 November 2019

Accepted Date: 9 November 2019

Please cite this article as: Roggia I, Dalcin AJF, Ourique AF, da Cruz IBM, Ribeiro EE, Mitjans M, Vinardell MP, Gomes P, Protective effect of guarana-loaded liposomes on hemolytic activity, *Colloids and Surfaces B: Biointerfaces* (2019), doi: https://doi.org/10.1016/j.colsurfb.2019.110636

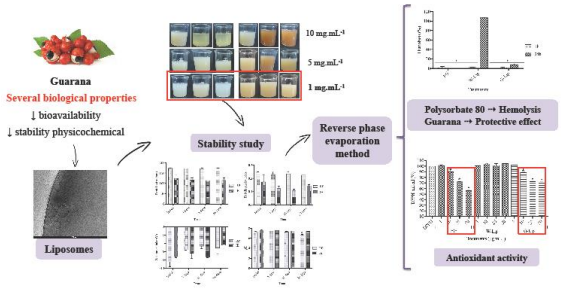
Protective effect of guarana-loaded liposomes on hemolytic activity

Isabel Roggia[a], Ana Júlia F. Dalcin[a], Aline F. Ourique[a], Ivana B. M. da Cruz[b], Euler E. Ribeiro[c], Montserrat Mitjans[d], Maria Pilar Vinardell[d] and Patrícia Gomes[a,*]

[a] Programa de Pós-graduação em Nanociências, Universidade Franciscana, Santa Maria, Brazil; isa_roggia@yahoo.com.br, anajuliadalcin@hotmail.com, alineourique@gmail.com, patriciagomes@ufn.edu.br
[b] Laboratory of Biogenomic, Federal University of Santa Maria, Santa Maria, Brazil; ibmcruz@hotmail.com
[c] Open University of the Third Age, State University of Amazonas, Manaus, Brazil; unatieuler@gmail.com
[d] Departament de Bioquimica i Fisiologia, Universitat de Barcelona, Barcelona, Spain; montsemitjans@ub.edu, mpvinardellmh@ub.edu

[*] Corresponding author: Universidade Franciscana, Programa de Pós-Graduação em Nanociências, Rua dos Andradas 1614, Santa Maria, RS, 97010-032, Brazil. E-mail address: patriciagomes@ufn.edu.br

Graphical abstract



Highlights

- Development of liposome using the guarana powder, an essential Amazonian species.
- The presence of polysorbate 80 induces a hemolytic process in the liposome.
- The addition of guarana to liposome helps to protect erythrocytes.
- Guarana-loaded liposome keeps the potent antioxidant activity of guarana.

Abstract: *Paullinia cupana* var. *sorbilis* (Mart.) Ducke, popularly known as guarana, is one of the most promising plants in Brazilian flora and has attracted considerable interest from the scientific community owing to its numerous therapeutic activities and less side effects. Hence, using nanotechnology is a viable alternative to primarily improve the physicochemical characteristics and bioavailability of guarana. The objective of the present study was to develop, characterize, and evaluate the stability of liposomes containing guarana powder and to evaluate their antioxidant and hemolytic activity *in vitro*. Three different concentrations of guarana powder and two methods of liposome preparation were tested. Liposomes were developed and characterized, and their stability was analyzed by evaluating physicochemical parameters. Hemolytic activity of guarana liposomal formulation (G-Lip) was compared with that of guarana in its free form (FG) and of liposome without guarana (W-Lip). Red blood cells from rats were exposed to these different formulations dissolved in phosphate buffer solution (PBS; pH 7.4). The best stability was achieved for the formulation containing 1 $mg.mL^{-1}$ guarana powder produced by the reverse phase evaporation method. FG showed dose-dependent antioxidant activity, which was maintained in G-Lip. W-Lip showed high hemolytic activity in PBS at pH 7.4 possibly because of the presence of polysorbate 80, and on addition of guarana to these structures, the hemolytic process was reversed. The same protective effect was observed for FG. It is believed that the

complex structure of guarana, primarily the presence of polyphenols, exerts a powerful antioxidant action, helping to protect erythrocytes.

Keywords: nanoparticle; *Paullinia cupana*; methylxanthines; polyphenols.

1. Introduction

Brazil houses the world's greatest biodiversity, containing more than 41,000 species of plants [1]. The Amazon region itself contains at least 10,000 species of plants containing active components for medicinal use [2]. Among these, we focused on guarana [*Paullinia cupana* var. *sorbilis* (Mart.) Ducke], an Amazonian species, which has been historically cultivated and has multiple uses [3]. Currently, guarana is also used worldwide for the production of caffeinated energy drinks [4].

The vast chemical composition of guarana guarantees its biological activity potentiality. The main compound is caffeine (CAF; 3.2%–7%), which can exceed up to three times the amount found in coffee [5]. Moreover, other methylxanthines, such as theobromine (TEOB) and theophylline (TEOF), as well as polyphenols, catechin (CAT) and epicatechin (EPICAT), have been reported as major active compounds of guarana [6-8]. Guarana chemical matrix found in roasted seeds has been reported to exert several biological properties, such as antioxidant [9-11], antimicrobial [12], hypocholesterolemiant [13], anti-inflammatory, immunomodulatory [14], genoprotective [15], and hepatoprotective [16] activities. Furthermore, guarana also modulates triglyceride levels in overweight individuals [17-18] by modulation of the purinergic system [19] and is used to reverse inflammatory alterations programmed by maternal obesity in adult mice [20].

Guarana also has important actions on cognition and memory [21-23], which seem to involve modulation of acetylcholinesterase activity [19, 24], suggesting the therapeutic use of guarana supplements. Furthermore, it also exhibits synergistic activity with chemotherapeutic drugs for the treatment of colorectal and breast cancer [25-27] and exhibits a hermetic effect on bladder cancer cells (T24) by reducing cell viability and proliferation [28]. Prior studies, such as that performed by Palma et al. [29], have also suggested that guarana intake could improve chemotherapy-related fatigue in women with breast cancer.

As for most products from plant extracts, one of the main problems of guarana is its physicochemical instability and vulnerability to possible degradation processes, which, in turn, may lead to a decrease in beneficial effects [30]. Furthermore, some bioactive molecules present in guarana matrix, such as polyphenols, could have low solubility and bioavailability [31-32]. Therefore, to improve the biopharmaceutical and technological properties of plant extracts, some strategies have been proposed based on nanotechnology, such as liposomes, nanoemulsions, and nanocapsules [33]. Liposomes are biocompatible and biodegradable vesicles formed essentially by phospholipids; they are amphiphilic molecules containing polar heads and hydrophobic hydrocarbon tails that have the ability to self-associate spontaneously and form bilayer vesicles dispersed in water, allowing the incorporation of hydrophilic and hydrophobic substances [34-35].

In this sense, the development of a safe nanotechnological product that contributes to the preservation of active compounds present in guarana is fundamental. Moreover, understanding its physicochemical characteristics, stability, and toxicity evaluation are essential steps to guarantee its safety and effectiveness [36].

Evaluation of hemolytic activity, using red cells as alternative tools, can be considered as a predictive cytotoxicity assay for different compounds to determine possible damage to the erythrocyte membrane [37-38]. These cells are devoid of internal membrane structures, are easy to isolate and handle, and have structures that are vulnerable to peroxidation, making them good biological membrane models for analyzing oxidative stress and lipid peroxidation of various compounds. Alterations in these cells may indicate the formation of reactive oxygen species, which cause oxidative damage in tissues [39].

Considering the potential therapeutic use of guarana, the present study was conducted with the aim to develop and characterize a liposomal system containing guarana powder with the purposes of improving its stability and guaranteeing its pharmacological activities in addition to evaluating the effect on red blood cell membrane by hemolytic analysis.

2. Materials and Methods

2.1 Materials

Analytical-grade acetonitrile, cholesterol, 2,2-diphenyl-1-picrilhydrazyl (DPPH), and polysorbate 80 were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Methanol and trifluoroacetic acid (TFA) were

acquired from J.T.Baker® (Mexico City, Mexico). Ethanol was acquired from Synth® (São Paulo, Brazil). Monobasic potassium phosphate was acquired from F Maia® (São Paulo, Brazil). Sodium chloride and dibasic sodium phosphate were obtained from Nuclear® (São Paulo, Brazil). Potassium chloride was obtained from Qhemis® (São Paulo, Brazil). Lipoid S100® was from Lipoid® (Ludwigshafen, Germany), and vitamin E was from Alpha Química® (Porto Alegre, Brazil). Guarana powder was kindly provided by Agropecuary Research Brazilian Enterprise (EMBRAPA Western Amazon - Manaus, Amazon, Brazil).

2.2. Pre-formulation study

Initially, formulations were prepared with different concentrations of guarana powder (10.0 $mg.mL^{-1}$, 5.0 $mg.mL^{-1}$, and 1.0 $mg.mL^{-1}$) using different methods of producing liposomes, viz., ethanol injection (EI) and reverse phase evaporation (RP) methods. Liposomes were produced and stored at room temperature (RT) and under refrigeration (RE), and their particle size and polydispersion index (PDI) were evaluated at different times.

The objective of the pre-formulation study was to evaluate the behavior of guarana at different concentrations when associated with liposomes produced by different methods to choose the formulation for further experiments.

2.3 Preparation of guarana-loaded liposomes

Liposomes containing guarana powder (G-Lip) were prepared by RP [40-41] and EI [42] methods. In the RP method, phospholipid, cholesterol, and vitamin E were solubilized in ethanol with the aid of ultrasound. Next, an aliquot of guarana powder and polysorbate 80 in phosphate buffer (pH 7.4) in the aqueous phase was mixed with this solution and sonicated again to produce a dispersion of reverse micelles. Subsequently, the organic solvent was removed by evaporation to form an organogel; this organogel was reverted into vesicles following the addition of the aqueous phase remainder under stirring (300 rpm) for 30 min using a rotary evaporator in a 40 °C water bath. In the EI method, the same constituents and quantities were used; however, in this method, the lipid and aqueous phases were solubilized separately, and after complete solubilization, the lipid phase was poured over the aqueous phase. The system was maintained on shaking for 10 min to form liposome dispersions. Subsequently, the organic solvent was evaporated using a rotary evaporator (300 rpm) in a 40 °C water bath.

For both methods, the vesicles were homogenized by sequential filtration using cellulose acetate filter membranes of 0.45 and 0.22 μm porosity (Millex®, USA). For the most appropriate concentration and production method, white liposomes (W-Lip) were prepared without guarana powder as mentioned above. All formulations were prepared in duplicates.

2.4 Physicochemical characterization of liposomes and evaluation of stability

2.4.1 Particle size and zeta potential analysis

Liposomes were analyzed for particle size by two different techniques, viz., laser diffraction (Microtrac S3500®, EUA) and dynamic light scattering (Zetaziser Nano-ZS®, Malverm, United Kingdom) using samples diluted in ultra-pure water (1:500 v/v). Moreover, dynamic light scattering was performed to determine the PDI.

Zeta potential values of the liposomes were evaluated by the determination of electrophoretic mobility (Zetaziser Nano-ZS®, Malverm, United Kingdom). Measurements were performed after dilution of the formulations in 10 mM NaCl aqueous solution.

2.4.2 Determination of pH

The pH values of formulations were determined directly from dispersions using a previously calibrated potentiometer (Digimed DM22®, Brazil).

2.4.3 Determination of the main active compounds and encapsulation efficiency (EE%)

Quantification of main active compounds present in guarana powder (TEOB, TEOF, CAF, CAT, and EPICAT) and EE% estimation was done by reversed-phase high-performance liquid chromatography (RP-HPLC) using Prominence chromatograph (Shimadzu®, Japan) equipped with a pump (model LC-20AT), photo diode array (PDA) detector (model SPD-M20A), and a RP-C18 chromatography column (250 × 4.60 mm, 5

mm) (Phenomenex®, Torrance, USA) and pre-column (4 × 3 mm, 5 mm) (Phenomenex®, Torrance, USA). The mobile phase consisted of water acidified with 0.1% TFA (phase A) and methanol:acetonitrile (25:75; v/v; phase B) in a ratio of 90:10 (v/v) of phase A:B followed by isocratic elution. The oven temperature was 30 °C, mobile phase flow of 1 mL.min^{-1}, and injection volume of 20 µL. Active compounds were detected at 280 nm.

The five major compounds in guarana powder sample were determined by quantifying the compounds from a guarana solution at a final concentration of 500 µg.mL^{-1} prepared by solubilization in a solution of deionized water and methanol (1:1, v/v). For liposomes containing 1 mg.mL^{-1} guarana powder, samples were also prepared at the concentration of 500 µg.mL^{-1} using a solution of methanol and mobile phase (1:1, v/v) as the solvent.

EE% was determined by ultrafiltration–centrifugation technique (Microcon®- Millipore 10,000 Å) according to the methodology adapted from Ourique et al. [43]. The ultrafiltrate was obtained by centrifugation of 500 µL of guarana liposome samples for 10 min at 500 rpm.

2.4.4 Stability studies

After selecting guarana powder concentrations with better characteristics and stability, liposomes were produced again (in duplicates) and characterized according to the parameters described previously in section 2.4 (2.4.1–2.4.3). Stability was evaluated over a period of 30 days for the samples produced in duplicates and stored under RE at 5 °C ± 2 °C.

2.4.5 Morphology

Based on physicochemical characterization and stability study, the best formulation was selected and liposomes were evaluated for their size and morphology by cryo-transmission electron microscopy (Cryo-TEM) using Tecnai F20 200kv FEG® (Thermo Fisher Scientific, Eindhoven, The Netherlands). Analyses were performed at the Scientific and Technological Center of the University of Barcelona, Spain.

Briefly, 3 µL of G-Lip and W-Lip liposomes were placed on a Lacey carbon film in 200-mesh copper grids. Films were vitrified by ethane immersion and maintained at their melting point with liquid nitrogen using Vitrobot Mark III® (Thermo Fisher Scientific - Eindhoven, The Netherlands) by holding the sample at 100% moisture before freezing. Samples were analyzed at a temperature between −170 °C and −175 °C and recorded at 200 kV with an Eagle CCD camera of 4096 × 4096 pixels (FEI Company, Eindhoven, Netherlands).

2.5 Antioxidant activity

The liposomal antioxidant activity of G-Lip, W-Lip, and FG was evaluated by the DPPH method. This assay is based on the ability of an antioxidant substance to sequester the free radical DPPH and reduce it to 2,2-diphenyl-1-picrilhydrazyl with accompanying color change from violet to pale yellow according to the method described by Choi et al. [44].

Treatments were prepared at final concentrations of 1, 10, 25, and 50 µg.mL^{-1} using ethyl alcohol as the solvent and incubated under light protection for 30 min. Treatments were evaluated in relation to a control sample (white control) without DPPH and a negative control sample (only DPPH in solvent). The absorbances of the samples were determined using a UV/VIS spectrophotometer (UV-1650 PC; Shimadzu®, Kyoto, Japan) at a wavelength of 517 nm using absolute ethyl alcohol as the blank. The assay was performed in triplicate. The calculation of the ability to sequester DPPH molecule free radicals was determined by the following equation: % inhibition = 100 − [(sample absorbance − blank absorbance) × 100]/control absorbance.

2.6 Hemolysis assay

The hemolytic activity of G-Lip, W-Lip, and FG was evaluated by hemolysis assay using erythrocytes as a model for general cytotoxicity evaluation. For this purpose, rat erythrocytes were isolated from blood collected in EDTA tubes. The procedure was approved by the ethical committee of animal experimentation of University of Barcelona (UB), Spain. Red blood cells were isolated by centrifugation at 3000 rpm for 10 min and washed three times with an isotonic phosphate buffer solution (PBS; pH 7.4) [38].

In order to avoid false negative results, the ability of liposomes to capture hemoglobin was evaluated prior to assays. The objective of this procedure was to verify the interaction of liposomes with hemoglobin. The hemoglobin release technique was adopted, which consisted of adding 475 µL of water to 25 µL of blood. After

shaking and hemoglobin release, 500 µL of liposome formulation was added. This solution was kept under stirring for 1 h, followed by centrifugation at 10,000 rpm for 5 min (Heraeus Biofuge Pico®, Germany). The ability of the liposomes to capture hemoglobin was then observed.

For the hemolysis assay, 25-µL aliquots of erythrocytes were exposed to 500 µg.mL^{-1} FG, G-Lip, or W-Lip dissolved in PBS solution (pH 7.4) at a final volume of 1 mL [38]. Controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). Cells were maintained in different treatments for 1 and 24 h under agitation, followed by centrifugation at 10,000 rpm for 5 min. Supernatants were collected, and the hemoglobin released was detected by absorbance measured at 540 nm using a double-beam Shimadzu UV-VIS 160A® spectrophotometer (Kyoto, Japan). Percentages of hemolysis were determined by comparison with positive control samples totally hemolyzed with distilled water.

The same experimental protocol was performed for each component present in the formulation of liposomes (cholesterol, phospholipid, vitamin E, and polysorbate 80). Tests were performed considering concentrations of each component in the formulation, and PBS (pH 7.4) was used for solubilization. For lipophilic compounds, 1% ethanol was added to facilitate their solubilization.

2.7 Statistical analysis

All experiments were performed in triplicates. Results are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine statistical differences between data sets, followed by Dunnett's or Tukey's post-hoc test for multiple comparisons. All statistical analyses were performed using GraphPad Prism 7.0 software® (GraphPad Software, Inc., CA, USA). $p < 0.05$ value was considered statistically significant.

3. Results

3.1 Pre-formulation study

Formulations containing 10.0 mg.mL^{-1} of guarana powder had initial mean particle diameter of approximately 164 nm and 169 nm when produced by the RP and EI methods, respectively. The formulations remained stable for 14 days, after which an increase in mean particle diameter and PDI were observed, primarily when stored at RT (Table 1). Subsequently, organoleptic characteristics of appearance, color, and odor were also altered (Figure 1: I-1B, I-1C, II-1B, and II-1C).

Formulations with 5.0 mg.mL^{-1} of guarana powder maintained their characteristics for a period of 42 days, after which changes were observed, primarily related to mean diameter and PDI, specifically in formulations produced by evaporation method in RP and stored at RT. Of the organoleptic characteristics studied, only color was altered in this period for samples stored at RT and produced by the EI method (Figure 1: I-2B, I-2C, II-2B, and II-2C).

Better stability was obtained for formulations containing 1 mg.mL^{-1} of guarana powder. They remained stable without change in the mean particle diameter, PDI, and organoleptic characteristics when stored under RE for 70 days for both production methods. However, when produced by the RP method and stored at RT, analysis at 70 days showed a mean micron diameter (1534 nm ± 65.37), increase in PDI (0.493 ± 0.09), and change in coloration (Table 1 and Figure 1: I-3B, II-3B, and II-3C).

3.2 Physicochemical characterization and stability study

Based on pre-formulation results, subsequent experiments were performed using only G-Lip containing 1 mg.mL^{-1} of guarana powder produced by the RP and EI methods.

Initially, the mean diameter of particles was characterized using laser diffraction technique. The objective was to verify if all particles had dimensions of the nanoscale range. It was observed that all vesicles had dimensions in the nanoscale range, and the mean diameters were 240 nm ± 0.18 for liposomes produced by the

RP method and 201 nm ± 0.02 for liposomes produced by the EI method, similar to the diameter noted by dynamic light scattering (Figure 2). From this initial analysis, other physicochemical characteristics were evaluated, and results obtained are described in Figure 2.

The particle diameter value obtained by photon correlation spectroscopy during the experimental period was kept stable at approximately 174 nm with a low PDI of 0.2 when produced by RP. Likewise, by the EI method, particles with diameter of 115–124 nm were obtained with a low PDI of approximately 0.1, which was stable for 30 days (Figure 2A and 2B).

The initial zeta potential values of −8.50 mV ± 1.15 (RP) and −7.17 mV ± 0.28 (EI) were maintained without significant change ($p > 0.05$) during the 30 days of analysis (Figure 2C).

As shown in Figure 2D, initial pH values were approximately 7.32 ± 0.01 and 7.62 ± 0.09 when liposomes were produced by the RP method and the EI method, respectively. By RP, these values were significantly altered ($p < 0.05$) to 6.61 ± 0.20 during 30 days of analysis. However, by the EI method, the values remained stable throughout the analysis period.

It is important to mention that liposomes were produced with guarana powder. For this, firstly, the concentration of the major compounds (TEOB, TEOF, CAF, CAT, and EPICAT) present in guarana powder was evaluated. Results showed the concentration of 20.61 $\mu g.mL^{-1}$ methylxanthines [0.14 $\mu g.mL^{-1}$ (0.028%) of TEOB, 0.47 $\mu g.mL^{-1}$ (0.094%) of TEOF, and 20.00 $\mu g.mL^{-1}$ (4.0%) of CAF] and 26.00 $\mu g.mL^{-1}$ of polyphenols [13.00 $\mu g.mL^{-1}$ (2.6%) of CAT and 13.00 $\mu g.mL^{-1}$ (2.6%) of EPICAT]. After production of liposomes, these compounds were quantified simultaneously, and their stability was evaluated as described in Figure 3.

Both preparation methods (RP and EI) presented reductions in the content of active compounds, except for CAF, which remained stable ($p > 0.05$) for 30 days when stored RE (5 °C ± 2 °C) (Figure 3).

When liposomes were prepared by RP, the content of TEOF (30 days), CAT (30 days), and EPICAT (15 days) decreased. For CAF and TEOB, no reduction was observed during experiments.

When liposomes were prepared by the EI method, TEOB (30 days), TEOF (7 days), CAT (7 days), and EPICAT (7 days) decreased significantly ($p < 0.05$).

Among the compounds, EPICAT was the active substance that showed the highest concentration reduction. In 30 days of analysis, reductions of 43.76% were observed when liposomes were produced by the RP method, whereas 50.52% reduction was observed when liposomes were produced by the EI method.

In parallel to quantification of active compounds, EE% of these compounds was determined. It was not possible to determine EE% for TEOB and TEOF by the employed method because their concentration in guarana is very low, making it difficult to determine the nanoencapsulated content of these compounds. For other active substances (CAF, CAT, and EPICAT) different EE% were observed regardless of the liposome production method.

Interaction between drug and lipid vesicles decisively influenced its encapsulation in liposomes. Thus, higher encapsulation efficiencies for CAT [63.49 ± 1.90% (RP) and 47.80 ± 7.33% (EI)] and EPICAT [74.95 ± 5.08% (RP) and 71.36 ± 4.24% (EI)] were due to the higher lipophilic character of these substances, resulting in a greater interaction with the lipid lamellae and, consequently, higher EE% [45]. However, CAF presented EE% of 19.19 ± 4.53% when produced by the RP method and 2.35 ± 1.15% when produced by the EI method. These results were already expected due to the methods used to produce the liposomes.

Although the formulation produced by the EI method presented some physicochemical characteristics, such as a smaller particle diameter, narrower PDI, and stable pH during the stability period, which were more suitable, the RP method was chosen as the most convenient method of producing liposomes containing 1 $mg.mL^{-1}$ of guarana powder. This choice was based primarily on the quantification and stability of active compounds and EE% of these compounds, primarily CAF.

From this, remaining experiments were conducted only with liposomes containing 1 $mg.mL^{-1}$ guarana powder produced by the RP method.

It is also worth mentioning that by the RP method, W-Lip were also produced and characterized. These formulations had the following physicochemical characteristics: particle diameter of 172 nm ± 0.02 (laser diffraction) and 165 nm ± 2.39 (dynamic light scattering), PDI of 0.27 ± 0.02, zeta potential of −9.16 ± 0.57 mV, and pH of 7.33 ± 0.02. It should be noted that presence of guarana did not alter the physicochemical characteristics of liposomes, maintaining their desirable characteristics.

Similarly, photomicrographs obtained from Cryo-TEM for W-Lip and G-Lip evidenced the presence of spherical particles with an average diameter around 200 nm (Figure 4), indicating low polydispersity of the system. These results corroborated with the medium and low dispersion diameters obtained by laser diffraction and dynamic light scattering techniques, respectively, as shown in Figure 2.

Formation of multilamellar (MLV) and unilamellar (LUV) liposomes systems was also observed, with a higher amount of unilamellar systems, as indicated in Figure 4.

3.3 Antioxidant activity

Antioxidant activity of guarana and liposomes (W-Lip and G-Lip) are shown in Figure 5.

Regardless of the concentration tested, W-Lip showed no ability to sequester free radicals from the DPPH molecule. However, when DPPH was added to G-Lip and FG, antioxidant activity was observed at concentrations of 10, 25, and 50 $\mu g.mL^{-1}$ of guarana. The free radical reduction for G-Lip was 10.90%, 27.76%, and 29.84% at concentrations of 10, 25, and 50 $\mu g.mL^{-1}$, respectively. Similarly, for FG, 10.05%, 28.04%, and 44.13% reductions of DPPH radicals were observed at concentrations of 10, 25, and 50 $\mu g.mL^{-1}$, respectively.

3.4 Hemolysis

The hemolytic activity of guarana and liposomes (W-Lip and G-Lip) was determined after 1 and 24 h of incubation at a concentration of 500 $\mu g.mL^{-1}$ (Figure 6).

The results obtained evidenced the non-hemolytic effects after 1 h of incubation for the different treatments (FG, W-Lip, and G-Lip). However, after 24 h of incubation, hemolysis was observed for W-Lip (hemolysis 100%). These results clearly showed that membrane lysis is caused by the liposomal structure (W-Lip) after 24 h of exposure and indicates the protective effect of guarana.

Hemolytic activity of constituents used in liposome preparation showed that polysorbate 80 (Tween 80®) was the only hemolytic component with values of approximately 33% and 100% after 1 and 24 h, respectively. Moreover, a low hemolytic process (around 7%) was observed for vitamin E after 1 h of contact with erythrocytes. Other compounds (phospholipid and cholesterol) did not present hemolysis for 24 h of incubation with erythrocytes.

4. Discussion

In this study, we incorporated guarana powder into liposomal dispersions, which presented adequate physicochemical and stability characteristics. Moreover, these liposomal dispersions did not cause hemolysis in contact with erythrocytes, indicating promising application in pharmaceutical, food, and cosmetic industries.

From among different nanostructured systems, liposomes were selected for their biocompatibility, biodegradability [46], and primarily versatility in encapsulating amphiphilic compounds [47] like guarana. Soya phosphatidylcholine was the phospholipid of choice in our study as the use of natural phospholipids for liposome preparation generally presents a lower cost when compared with formulations prepared with synthetic phospholipids. These natural compounds generally exhibit high stability and biocompatibility, allowing production of lipid vesicles that are more resistant to oxidation processes [48]. Cholesterol was added to this phospholipid to improve the resistance of lipid membranes, thus increasing hydrophilic substance retention by reducing the rotational freedom of phospholipid hydrocarbon chains and inducing the formation of a less permeable structure [49]. Vitamin E, which acts as an antioxidant agent that prevents the oxidation of these substances and improves stability of liposomal dispersion, was also added [42,48].

Among various methods of liposome preparation, RP and EI methods were selected considering the laboratory conditions and possible industrial-scale production of these liposomes. The RP method afforded a higher rate of encapsulation of hydrophilic compounds, whereas the EI method was easy to perform [40,42,48].

To the best of our knowledge, considering this was the first time that guarana powder was associated with liposomes, pre-formulation studies were extremely important to select the ideal powder concentration to maintain the stability of the system. Through this initial study, the concentration of 1 $mg.mL^{-1}$ of guarana powder showed better physicochemical characteristics, such as mean particle diameter, PDI, and organoleptic characteristics.

PDI was used to determine homogeneity in particle size distribution in the dispersions. PDI values of ≤ 0.3 are considered monodisperse systems [50]. In our study, when prepared by the EI method, PDI was significantly lower ($p < 0.05$) than that in the RP method (Figure 2B). As already described in literature, these results were already expected as the EI method allows obtaining small unilamellar liposomal vesicles associated with relatively narrow vesicle distribution [51], whereas liposomes produced by the RP method predominantly produces large unilamellar liposomes [40,42,51]. In this sense, data obtained in this research are in agreement with those reported in literature for liposomes produced by RP and EI methods [41-43].

The slightly negative zeta potential obtained in our results was already expected (Figure 2C), which is justified by the presence of phosphoric acid in phospholipids, such as phosphatidylcholine, used in this experiment [52]. Negative potential (−6.8 to −7.7 mV) for liposomes produced by the supercritical fluid method using Lipoid S100® and cholesterol has been also observed by Karn et al. [53].

Results of zeta potential are generally used to predict the stability of colloidal systems in general; the higher the zeta potential in module, the greater is the stability of these systems as particles tend to repel each other, thus avoiding aggregation and loss of stability [54-55]. The not-so-high zeta potential obtained in our study can be justified by steric stabilization. In this case, stabilization occurred by changing the shear plane to a much greater distance from the surface of the particles, which led to a reduction in the measured zeta potential. This shear change is often related to the absorbent layers of polymers and/or molecules [56].

The pH values were found to be around 7.4, as expected (Figure 2D), as PBS with pH 7.4 was used for liposome production.

Stability analysis results (Figure 3) showed reductions in active compound content, particularly for catechins (CAT and EPICAT). This can be justified by the chemical structure of these compounds, which have an acid character, allowing degradation in neutral and/or basic medium. Data from literature show that neutral and basic conditions facilitate catechin degradation process, insofar under these conditions, catechins can undergo degradation as well as oxidation and polymerization [57-59]. Feng [60] reported that catechins present in green tea are generally unstable under neutral and alkaline conditions.

In general, when liposomes were prepared by the RP method, the content of the active substances was maintained for a longer time than when prepared by the EI method. By the EI method, significant reductions ($p < 0.05$) in TEOF, CAT, and EPICAT content were observed in 7 days of stability. However, by the RP method, stability of these compounds was maintained for up to 15 days (EPICAT) and 30 days (TEOF and CAT) (Figure 3).

Data from literature show that the behavior and stability of liposomal vesicles can be controlled by the method of obtaining these particles. Costa and Moraes [61] obtained greater physicochemical stability in 5-

fluorouracil liposomes prepared by the RP method when compared with dry lipid film hydration methods, followed by extrusion and the EI method.

CAT and EPICAT presented higher EE% when compared with CAF. These results are justified by higher lipophilicity of these compounds when compared with CAF. In general, lipophilic active compounds are incorporated into lipid bilayers, allowing greater incorporation and consequently higher EE% when compared with hydrophilic compounds that are generally dispersed in the aqueous phase [62]. Thus, low EE% values found for CAF [19.19 ± 4.53 % (RP) and 2.35 ± 1.15 % (EI)] were already expected as this compound presents higher hydrophilicity, consequently presenting a lower EE% when produced from soybean phosphatidylcholine [48,63].

In addition, as already described in literature and evidenced in our study when compared with other methods of liposome production, the RP method showed higher rates of encapsulation were obtained for hydrophilic compounds [36,54].

The antioxidant activity of guarana (Figure 5) was observed from the concentration of 10 $\mu g.mL^{-1}$ and was maintained when this active substance was associated with liposomes. A dose-dependent activity was also evidenced for both FG and G-Lip.

Numerous studies have already described the potential antioxidant effect of guarana [7,10,12-13,64-65]. Phenolic compounds, primarily found in guarana seeds, guarantees this activity. Similarly, their oxidation properties allow these compounds to act as a reducing agent by donating hydrogen and electrons. Another contributing factor is the chemical structure formed by at least one aromatic ring, which is attached to one or more hydroxyls, thus, playing an important role in neutralizing or sequestering free radicals and chelating transition metals, allowing to act in the initiation and propagation steps of the oxidative process [66].

Erythrocyte membrane resistance has been used by many authors as a tool in the evaluation of toxicity [67-72]. The hemolytic assay is based on the measurement of hemoglobin release from suspended red blood cells in solutions with gradual reduction in its concentrations in addition to detection of cells showing osmotic lysis. The released hemoglobin is positively correlated to the percentage of lysed cells [39].

Previously, for evaluating the hemolytic activity of guarana and liposomes, hemoglobin uptake by this liposomal system was studied. The results showed no interaction of liposomes with hemoglobin; therefore, the hemolysis experiments were conducted safely.

The results of hemolytic evaluation clearly showed that cellular damage was caused by liposomal structure (W-Lip) in contact with blood cells for 24 h, but this was prevented by the presence of guarana, which is an important anti-hemolytic compound (Figure 6).

The hemolytic effect observed for W-Lip can be attributed to a possible cytotoxic effect caused by polysorbate 80, which has previously been reported in literature. Tatsuishi et al. [73] observed that polysorbate 80 has the ability to increase cytotoxicity of hydrogen peroxide *in vitro*, thus, increasing cellular susceptibility to oxidative stress.

In addition, protection against cellular damage observed for G-Lip (Figure 6) can be justified by the potent antioxidant capacity found in our study (Figure 5) and also described in literature for guarana [7,10,12-13,64]. Flavonoids and polyphenols in erythrocyte membranes have a beneficial effect on membrane stability and protect the membrane from degradative processes like hemolysis [74-76]. It is believed that one of the factors contributing to membrane stabilization is related to Van der Waals excitation within the lipid bilayer by flavonoids [75].

In a study with aqueous extract of *Thymus satureioides*, a plant rich in polyphenols and flavonoids, Ranchoun et al. [77] observed that this extract neutralizes the free radicals released by 2,2-azobis-amidinopropane (AAPH) radical and can revert hemolysis induced by this radical. Justification for this erythrocyte protection ability is linked to the high content of polyphenols and flavonoids present in this extract, which shows antioxidant activity by protecting the erythrocyte membrane from injury by avoiding and/or increasing the half-life of the hemolytic process.

Baccarin et al. [78] suggested three possible mechanisms that lead to erythrocyte protection by products containing polyphenols. The first mechanism is related to partition coefficient or to the degree of lipophilicity of phenolic compounds that determines its interaction with biomembranes, which is a determining factor in its antioxidant capacity and/or ability to interact with free radicals. Second, the chemical structure of phenolic compounds determines their ability to react with free radicals. This ability is directly related to the number of hydroxyl groups that have the ability to donate hydrogens, forming a stable product, thus preventing the

continuation of oxidative process. Moreover, a third mechanism may be related to the synergism of different phenolic compounds that may contribute to oxidation inhibition.

Likewise, Bouhlali et al. [79] evaluated the antioxidant and antihemolytic activity of six varieties of *Phoenix dactylifera L*., and, similar to previous studies, attributed the stabilization of erythrocyte membrane to the presence of flavonoids and polyphenols. There was also a high correlation between antioxidant and antihemolytic activity.

5. Conclusions

Liposomes containing 1 $mg.mL^{-1}$ of guarana powder were successfully produced by the RP method. These structures presented adequate nanometric and physicochemical characteristics with the type of system developed, enabling future applications as nanomedicine. Hemolytic evaluation showed that W-Lip induces membrane damage caused by the presence of the surfactant polysorbate 80. Results obtained in this study allowed us to conclude that the presence of guarana in liposomes (G-Lip) affords protection against hemolytic process, possibly due to its flavonoid and polyphenol composition, which exert a potent antioxidant activity. This formulation demonstrates as a potential nanomedicine to be explored further. In this sense, more tests are being performed to prove the efficiency and safety of guarana-containing liposomes.

Conflicts of Interest: The authors declare no conflicts of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001 and CNPq/JST project (Process: 490760/2013-9) for financial support.

References

1. Brasil. Ministério do Meio Ambiente. Biodiversidade – Flora. Available: < http://www.mma.gov.br/mma-em-numeros/biodiversidade-flora>. Last accessed on 02 de dezembro de 2018.
2. Brasil. Ministério do Meio Ambiente. Plano Amazônia Sustentável. Diretrizes para o desenvolvimento sustentável da Amazônia brasileira, Brasília, 2008.
3. F.C. Schimpl, J.F. Da Silva, J.F.C. Gonçalves, P. Mazzafera, Guarana: Revisiting a higtly caffeinated plant from the Amazon, J. Ethnopharmacol. 150 (2013) 14-31. doi: 10.1016/j.jep.2013.08.023.
4. D.L. Woods, Guarana: Paullinia cupana, P. sorbilis; also known as Brazilian cocoa and 'zoom'. Journal of Primary Health Care. 4 (2012) 163-164.
5. N. Dalonso, C.L.De.O. Petkowicz, Guarana powder polysaccharides: Characterisation and evaluation of the antioxidant activity of a pectic fraction, Food Chem. 134 (2012) 1804-1812. doi: 10.1016/j.foodchem.2012.03.088.
6. T. Klein, R. Longhini, J.C.De.M. Palazzo, Development of an analytical method using reversed-phase HPLC-PDA for a semipurified extract of Paullinia cupana var. sorbilis (guaraná), Talanta. 88 (2012) 502-506. doi: 10.1016/j.talanta.2011.11.023.
7. L. Yonekura, C.A. Martins, G.R. Sampaio, M.P. Monteiro, L.A.M. César, B.M. Mioto, C.S. Mori, T.M.N. Mendes, M.L. Ribeiro, D.P. Arçari, A.F.Da.S. Torres, Bioavailability of catechins from guaraná (Paullinia cupana) and its effect on antioxidant enzymes and other oxidative stress markers in healthy human subjects. Food Funct. 7 (2016) 2970-2980. doi: 10.1039/c6fo00513f.
8. K.N. Machado, A.A. De Freitas, L.H. Cunha, A.A.G. Faraco, R.M. De Pádua, F.C. Braga, C.D. Vianna-Soares, R.O. Castilho, A rapid simultaneous determination of methylxantines and proanthocya_nidins in

Brazilian guaraná (*Paullinia* cupana Kunth.), FoodChem. 239 (2018) 180-188. doi: 10.1016/j.foodchem.2017.06.089.

9. T.M. Antonelli-Ushirobira, E.N. Kameshima, M. Gabriel, E.A. Audi, L.C. Marques, J.C.P. Mello, Acute and subchronic toxicological evaluation of the semipurified extract of seeds of guaraná (Paullinia cupana) in rodents, Food Chem. Toxicol. 48 (2010) 1817-1820. doi: 10.1016/j.fct.2010.04.013.
10. L.S. Bittencourt, D.C. Machado, M.M. Machado, G.F.F. Dos Santos, T.D. Algarve, D.R. Marinowic, E.E. Ribeiro, F.A.A. Soares, F. Barbisan, M.L. Athayde, I.B.M. Cruz, The protective effects of guaraná extract (Paullinia cupana) on fibroblast NIH-3T3 cells exposed to sodium nitroprussid. Food Chem.Toxicol. 53 (2013) 119-125. doi: 10.1016/j.fct.2012.11.041.
11. E. Matsuura, J.S.R. Godoy, P.S. Bonfim-Mendonça, J.C.P. De Mello, T.I.E. Svidzinski, A. Gasparetto, S.M. Maciel, In vitro effect of Paullinia cupana (guaraná) on hydrophobicity, biofilm formation, and adhesion of Candida albicans to polystyrene, composites, and buccal epithelial cells, Arch Oral Bio. 60 (2015) 471-478. doi: 10.1016/j.archoralbio.2014.05.026.
12. A. Basile, L. Ferrara, M.D. Pezzo, G. Melo, S. Sorbo, P. Bassi, D. Montesano, Antibacterial and antioxidant activities of ethanol extract from Paullinia cupana. Mart. J. Ethnopharmacol. 102 (2005) 32–36. doi: 10.1016/j.jep.2005.05.038.
13. R.L. Portella, R.P. Barcelos, E.J.D. Da Rosa, E.E. Ribeiro, I.B.M. Cruz, L. Suleiman, F.A.A. Soares, Guaraná (*Paullinia cupana Kunth*) effects on LDL oxidation in elderly people: an in vitro and in vivo study, Lipids Health Dis. 12 (2013) 1-9. doi: 10.1186/1476-511X-12-12.
14. C. Costa Krewer, L. Suleiman, M.M.M.F. Duarte, E.E. Ribeiro, C.P. Mostardeiro, M.A.M. Montano, M.I.U.M. Da Rocha, T.D. Algarve, G. Bresciani, I.B.M. Da Cruz, Guaraná, a supplement rich in caffeine and catechin, modulates cytokines: evidence from human in vitro and in vivo protocols, Eur Food Res Technol. 239 (2014) 49-57. doi.org/10.1007/s00217-014-2182-3.
15. A.K. Machado, F.C. Cadoná, V.F. Azzolin, E.B. Dornelles, F. Barbisan, E.E. Ribeiro, M.F.M. Cattani, M.M.M.F. Duarte, J.R.P. Saldanha, I.B.M. Da Cruz, Guaraná (*Paullinia cupana)* improves the proliferation and oxidative metabolism of senescent adipocyte stem cells derived from human lipoaspirates, Food Res Int. 67 (2015) 426–433. doi.org/10.1016/j.foodres.2014.11.056.
16. H. Kober, E. Tatsch, V.D. Torbitz, L.P. Cargnin, M.B. Sangoi, G.V. Bochi, A.R. Da Silva, F. Barbisan, E.E. Ribeiro, I.B.M. Da Cruz, R.N. Moresco, Genoprotective and hepatoprotective effects of Guarana (*Paullinia cupana Mart*. *var*. *sorbilis*) on CCl4-induced liver damage in rats, Drog Chem Toxicol. 39 (2016) 48-52. doi: 10.3109/01480545.2015.1020546.
17. L. Suleiman, F. Barbisan, E.E. Ribeiro, R.N. Moresco, G. Bochi, M.M.M.F. Duarte, M.F. Manica-Cattani, T.C. Unfer, V. Azzolin, J. Griner, I.B.M. Da Cruz, Guaraná Supplementation Modulates Tryglicerides and Some Metabolic Blood Biomarkers in Overweight Subjects, Annals of Obesity & Disorders, 1 (2016) 1-5.
18. M.M. Celestino, A.C. Gomes, P.B. Botelho, A. Gambero, L.M. Mesquita, W. Vilegas, M.L. Ribeiro, J.F. Mota, South American herbal extracts reduce food intake through modulation of gastrointestinal hormones in overweight and obese women, J Funct Foods. 35 (2017) 555–563. doi.org/10.1016/j.jff.2017.06.015.
19. J.B. Ruchel, J.F. Rezer, M.L. Thorstemberg, C.B. Dos Santos, F.L. Cabral, S.T. Lopes, C.B. Da Silva, A.K. Machado, I.B.M. Da Cruz, M.R. Schetinger, J.F. Gonçalves, D.B. Leal, Hypercholesterolemia and Ecto-enzymes of Purinergic System: Effects of *Paullinia cupana*, Phytother Res. 30 (2016) 49-57. doi:10.1002/ptr.5499.
20. N.D.S. Lima, C.R.E.P. Caria, A. Gambero, M.L. Ribeiro, The effect of Guarana *(Paullinia cupana)* on metabolic and inflammatory parameters in adult male mice programmed by maternal obesity. Eur J Nutr. (2018), doi:10.1007/s00394-018-1686-1.
21. L. Pomportes, J. Brisswalter, L. Casini, A. Hays, K. Davranche, Cognitive Performance Enhancement Induced by Caffeine, Carbohydrate and Guarana Mouth Rinsing during Submaximal Exercise. Nutrients. 9 (2017) 589. doi:10.3390/nu9060589.
22. D.J. White, D.A. Camfield, S. Maggini, A. Pipingas, R. Silberstein, C. Stough, A. Scholey, The effect of a single dose of multivitamin and mineral combinations with and without guaraná on functional brain activity during a continuous performance task, Nutr Neurosci. 20 (2017) 20, 8-22. doi: 10.1179/1476830514Y.0000000157.
23. R.C. Veasey, C.F. Haskell-Ramsay, D.O. Kennedy, K. Wishart, S. Maggini, C.J. Fuchs, E.J. Stevenson, The Effects of Supplementation with a Vitamin and Mineral Complex with Guaraná Prior to Fasted

Exercise on Affect, Exertion, Cognitive Performance, and Substrate Metabolism: A Randomized Controlled, Trial. Nutrients, 7 (2015) 6109-27. doi:10.3390/nu7085272.

24. J.B. Ruchel, J.B.S. Braun, S.A. Adefegha, A.G. Manzoni, F.H. Abdalla, J.S. Oliveira, K. Trelles, C. Signor, S.T.A. Lopes, C.B. Da Silva, L.G. Castilho, M.A. Rubin, D.B.R. Leal, Guarana (Paullinia cupana) meliorates memory impairment and modulates acetylcholinesterase activity in Poloxamer-407-induced hyperlipidemia in rat brain, Physiol Behav. 168 (2017) 11–19. doi:10.1016/j.physbeh.2016.10.003.
25. E. Hertz, F.C. Cadoná, A.K. Machado, V. Azzolin, S. Holmrich, C. Assmann, P. Ledur, E.E. Ribeiro, O.C. De Souza Filho, M.F. Mânica-Cattani, I.B.M. Da Cruz, Effect of Paullinia cupana on MCF-7 breast cancer cell response to chemotherapeutic drugs, Mol Clin Oncol. 3 (2015) 37-43. doi:10.3892/mco.2014.438.
26. F.C. Cadoná, A.K. Machado, V.F. Azzolin, F. Barbisan, E.B. Dornelles, W. Glanzner, P.B. Gonçalves, C.E. Assmann, E.E. Ribeiro, I.B.M. Da Cruz, Guaraná a Caffeine-Rich Food Increases Oxaliplatin Sensitivity of Colorectal HT-29 Cells by Apoptosis Pathway Modulation, Anticancer Agents Med Chem. 16 (2016) 1055-1065. doi:10.2174/1871520616666151217121138.
27. F.C. Cadoná, J.L. Rosa, T. Schneider, M. Cubillos-Rojas, S. Sanchez-Tena, V.F. Azzolin, C. Assmann, A.K. Machado, E.E. Ribeiro, I.B.M. Da Cruz, Guaraná, a Highly Caffeinated Food, Presents in vitro Antitumor Activity in Colorectal and Breast Cancer Cell Lines by Inhibiting AKT/mTOR/S6K and MAPKs Pathways, Nutr Cancer. 69 (2017) 800-810. doi:10.1080/01635581.2017.1324994.
28. E.R. Flores, M.Dal.A.R. Berto, F.C. Cadoná, A.K. Machado, G.F.F. Dos Santos, I.B.M. Da Cruz, F.B. Morrone, T. Graziotin, C.G. Bicca, Effect of guaraná extract (Paullinia cupana), an amazonian fruit richest in caffeine on human bladder cancer cell line, Revista Amazonense de Geriatria e gerontologia. 8 (2017) 88.
29. C.G. Palma, A.T. Lera, T. Lerner, M.M. De Oliveira, T.M. De Borta, R.P. Barbosa, G.M. Brito, C.A. Guazzelli, F.J. Cruz, A. Del Giglio, Guarana (Paullinia cupana) Improves Anorexia in Patients with Advanced Cancer, J Diet Suppl. 13 (2016) 221-231. doi:10.3109/19390211.2015.1008612.
30. W.G. Silva, P. Rovellini, P. Fusari, S. Venturini, Guaraná - Paullinia cupana, (H.B.K): Estudo da oxidação das formas em pó e em bastões defumados Guaraná, Rev Ciênc Agrovet. (edição on-line) Lages. 14 (2015) 235-241. On-line version, ISSN 2238-1171.
31. Z. Fang, B. Bhandari, Encapsulation of poliphenols: Review, Trends Food Sci Technol. 21 (2010) 510-523. doi.org/10.1016/j.tifs.2010.08.003.
32. P.V. Gadkari, M. Balaraman, Catechins: Sources, extraction and encapsulation: A review, Food Bioprod Proces. 93 (2015) 122-138. doi.org/10.1016/j.fbp.2013.12.004.
33. G. Zorzi, E. Carvalho, G.V. Poser, H.F. Teixeira, On the use of nanotechnology-based strategies for association of complexes matrices from plant extracts, Rev. Bras. Farmacogn. 25 (2015) 426-436. doi.org/10.1016/j.bjp.2015.07.015.
34. A. Grillone, T. Li, M. Battaglini, A. Scarpellini, M. Prato, S. Takeoka, G. Ciofani, Preparation, Characterization, and Preliminary In Vitro Testing of Nanoceria-Loaded Liposomes, Nanomaterials. 7 (2017) 276. doi:10.3390/nano7090276.
35. K.A. Terrón-Mejía, E. Martínez-Benavidez, I. Higuera-Ciapara, C. Virués, J. Hernández, Z. Domínguez, W. Argüelles-Monal, F.M. Goycoolea, R. López-Rendón, A. Gama Goicochea, Mesoscopic Modeling of the Encapsulation of Capsaicin by Lecithin/Chitosan Liposomal Nanoparticles, Nanomaterials. *8* (2018) 425. doi.org/10.3390/nano8060425.
36. M.T.F. Bara, P.A.M. Ribeiro, M.C.B. Arantes, L.L.S.S. Amorim, J.R. De Paula, Determinação do teor de princípios ativos em matérias-primas vegetais, Rev. Bras. farmacogn. 16 (2006) 211-215. doi:10.1590/S0102-695X2006000200014.
37. M. Pagano, C. Faggio, The use of erythrocyte fragility to assess xenobiotic 477 cytotoxicity. cell biochemistry and function, Cell Biochem Funct. (2015) doi: 478 10.1002/cbf.3135.
38. D.R. Nogueira, L.E. Scheeren, M.P. Vinardell, M. Mitjans, M.R. Infante, C.M.B. Rolim, Nanoparticles incorporating pH-responsive surfactants as a viable approach to improve the intracellular drug delivery, Mater. Sci. Eng. C. 57 (2015) 100–106. doi.org/10.1016/j.msec.2015.07.036.
39. M.R. Farag, M. Alagawany, Erythrocytes as a biological model for screening of xenobiotics toxicity, Chem Biol Interact. 5 (2018) 73-83. doi.org/10.1016/j.cbi.2017.11.007.
40. O. Mertins, M. Sebben, A. Pohlmann, N. Da Silveira, Production of soybean phosphatidylcholine chitosan nanovesicles by reverse phase evaporation: a step by step study, Chem Phys Lipids. 138 (2005) 29-37. doi.org/10.1016/j.chemphyslip.2005.07.004.

41. C.B. Oliveira, L.A. Rigo, L.D. Rosa, L.T. Gressler, C.E.P. Zimmermann, A.F. Ourique, A.S. Silva, L.C. Miletti, R.C.R. Beck, S.G. Monteiro, Liposomes produced by reverse phase evaporation: in vitro and in vivo efficacy of diminazene aceturate against Trypanosoma evansi, Parasitology. 141 (2014) 761-769. doi: 10.1017/S0031182013002114.
42. O.R. Justo, Â.M. Moraes, Analysis of process parameters on the characteristics of liposomes prepared by ethanol injection with a view to process scale-up: Effect of temperature and batch volume, Chem. Eng. Res. Des. 89 (2011) 785-792. doi.org/10.1016/j.cherd.2010.09.018.
43. A.F. Ourique, P. Do S. Chaves, G.D. Souto, A.R. Pohlmann, S.S. Guterres, R.C.R. Beck, Redispersible liposomal-N-acetylcysteine powder for pulmonary administration: Development, in vitro characterization and antioxidant activity, Eur J Pharm Sci. 65 (2014) 174–182. doi: 10.1016/j.ejps.2014.09.017.
44. C.W. Choi, S.C. Kim, S.S. Hwang, B.K. Choi, H.J. Ahn, Y.M. Lee, S.H. Park, S.K. Kim, Antioxidant activity and free radical scavenging capacity between korean medicinal plants and flavonoids by assay-guided comparison. Plant Science, 163(6) (2002) 1161-1168. https://doi.org/10.1016/S0168-9452(02)00332-1.
45. F. Frezard, D.A. Schettini, O.G.F. Rocha, C. Demicheli, Lipossomas: Propriedades físicas-químicas e farmacológicas, aplicações na quimioterapia à base de antimônio, Quim Nova. 28 (2005) 511-518. doi.org/10.1590/S0100-40422005000300025.
46. S.A. Saraf, Applications of novel drug delivery system for herbal formulations, Fitoterapia. 81 (2010) 680-689. doi.org/10.1016/j.fitote.2010.05.001.
47. R.M. Daudt, J. Emanuelli, I.C. Külkamp-Guerreiro, A.R. Pohlmann, S.S. Guterres, A nanotecnologia como estratégia para o desenvolvimento de cosméticos, Ciênc. Cult. 65 (2013) 28-31. On-line version, ISSN 2317-6660.
48. C.M. Batista, C.M.B. De Carvalho, N.S.S. Magalhães, Lipossomas e suas aplicações terapêuticas: Estado da arte, Rev. Bras. Cienc. Farm. 43 (2007) 167-179. doi.org/10.1590/S1516-93322007000200003.
49. D. Lombardo, P. Calandra, D. Barreca, S. Magazù, M.A. Kiselev, Soft Interaction in Liposome Nanocarriers for Therapeutic Drug Delivery, Nanomaterials. 6 (2016) 125. doi.org/10.3390/nano6070125.
50. A. Khare, I. Singh, P. Pawar, K. Grover, Design and Evaluation of Voriconazole Loaded Solid Lipid Nanoparticles for Ophthalmic Application, J Drug Deliv. 2016 (2016) 11. doi:10.1155/2016/6590361.
51. P. Stano, S. Bufali, C. Pisano, F. Bucci, M. Barbarino, M. Santaniello, P. Carminati, P.L. Luisi, Novel camptothecin analogue (Gimatecan)-containing liposomes prepared by the ethanol injection method, J Liposome Res. 14 (2004) 87–109. doi:10.1081/LPR-120039794.
52. B. Heurtault, P. Saulnier, B. Pech, J.E. Proust, J.P. Benoit, Physico-chemical stability of colloidal lipid particles, Biomaterials. 24 (2003) 4283–4300. doi.org/10.1016/S0142-9612(03)00331-4.
53. P.R. Karn, H.J. Parkl, S.J. Hwangl, Characterization and stability studies of a novel liposomal cyclosporin A prepared using the supercritical fluid method: comparison with the modified conventional, Int J Nanomedicine. 8 (2013) 365–377. doi: 10.2147/IJN.S39025.
54. A. Laouini, C. Jaafar-Maalej, I. Limayem-Blouza, S. Sfar, C. Charcosset, H. Fessi, Preparation, Characterization and Applications of Liposomes: State of the Art, J Colloid Sci. Biotechnol. 1 (2012) 147–168. doi:10.1166/jcsb.2012.1020.
55. L.M. Assis, A.R. Machado, A.S. Motta, J.A.V. Costa, L.A. Souza-Soares, Development and Characterization of Nanovesicles Containing Phenolic Compounds of Microalgae Spirulina Strain LEB-18 and Chlorella pyrenoidosa, AMPC. 4 (2014) 6-12. doi:10.4236/ampc.2014.41002.
56. S. Honary, F. Zahir, Effect of Zeta Potential on the Properties of Nano-Drug Delivery Systems - A Review (Part 2), Trop J Pharm Res. 12 (2013) 265-273. doi.org/10.4314/tjpr.v12i2.20
57. Q.Y. Zhu, A. Zhang, D. Tsang, Y. Huang, Z.Y. Chen, Stability of Green Tea Catechins, J Agric Food Chem. 45 (1997) 4624-4628. doi:10.1021/jf9706080
58. K. Yoshino, M. Suziki, K. Sasaki, T. Miyase, M. Sano, Formation of antioxidants from (-)-epigallocatechin gallate in mild alkaline fluids, such as authentic intestinal juice and mouse plasma, J Nutr Biochem. 10 (1999) 223-229.
59. Y. Lun Su, L.K. Leung, Y. Huang, Z.Y. Chen, Stability of tea theaflavins and catechins, Food Chem. 83 (2003) 189-195. doi.org/10.1016/S0308-8146(03)00062-1.
60. F. Wan Yong, Metabolism of Green Tea Catechins: An Overview, Curr Drug Metab. 7 (2006) 755-809. doi.org/10.2174/138920006778520552.

61. C.A.M. Costa, A.M. Moraes, Encapsulation of 5-fluorouracil in liposomes for topical administration, Acta Sci-Technol. 25 (2003) 53-61.
62. S.G.M. Ong, L.C. Ming, K.S. Lee, K.H. Yuen, Influence of the Encapsulation Efficiency and Size of Liposome on the Oral Bioavailability of Griseofulvin-Loaded Liposomes, Pharmaceutics. 8 (2016) 25. doi:10.3390/pharmaceutics8030025.
63. A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S.W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi, K. Nejati-Koshki, Liposome: classification, preparation, and applications, Nanoscale Res Lett. 8 (2013) 102. doi:10.1186/1556-276X-8-102.
64. T. Klein, R. Longhini, M.L. Bruschi, J.C.P. De Mello, Microparticles containing guaraná extract obtained by spray-drying technique: development and characterization, Rev. bras. farmacog. 25 (2015) 292–300. doi.org/10.1016/j.bjp.2015.03.010.
65. L.S. Bittencourt, R.C. Bortolin, E.A. Kolling, C.E. Schnorr, A. Zanotto-Filho, D.P. Gelain, J.C.F. Moreira, Antioxidant Profile Characterization of a Commercial *Paullinia cupana* (Guarana) Extracts. J. Nat. Prod. Resour. 2(1) (2016) 47–52.
66. S.E. Soares. Ácidos fenólicos como antioxidantes. Revista de Nutrição de Campinas. 15(1) (2002) 71-81.
67. D.R. Nogueira, M. Mitjans, M.R. Infante, M.P. Vinardell, The role of counterions in the membrane-disruptive properties of pH-sensitive lysine-based surfactants, Acta Biomaterialia. 7 (2011) 2846-2856. doi:10.1016/j.actbio.2011.03.017.
68. D.R. Nogueira, M. Mitjans, M.A. Busquets, L. Pérez, M.P. Vinardell, Phospholipid Bilayer-Perturbing Properties Underlying Lysis Induced by pH-Sensitive Cationic Lysine-Based Surfactants in Biomembranes, Langmuir. 28 (2012) 11687−11698. dx.doi.org/10.1021/la300626y.
69. D.R. Nogueira, M.D.C. Morán, M. Mitjans, L. Pérez, D. Ramos, J. De Lapuente, M.P. Vinardell, Lysine-based surfactants in nanovesicle formulations: the role of cationic charge position and hydrophobicity in in vitro cytotoxicity and intracellular delivery, Nanotoxicology. 8 (2014). doi: 10.3109/17435390.2013.793779.
70. D.R. Nogueira, L.E. Scheeren, L.B. Macedo, A.I.P. Marcolino, M.P. Vinardell, M. Mitjans, M.R. Infante, A.A. Farooqi, C.M.B. Rolim, Inclusion of a pH‐responsive amino acid‐based amphiphile in methotrexate‐loaded chitosan nanoparticles as a delivery strategy in cancer therapy, Amino Acids. 48 (2015) 157-168. doi 10.1007/s00726-015-2075-1.
71. D.R. Nogueira-Librelotto, L.E. Scheeren, M.P. Vinardell, M. Mitjans, C.M.B. Rolim, Chitosan-tripolyphosphate nanoparticles functionalized with a pH-responsive amphiphile improved the in vitro antineoplastic effects of doxorubicin. Colloids and Surfaces B: Biointerfaces, 147 (2016) 326-335. http://dx.doi.org/doi:10.1016/j.colsurfb.2016.08.014
72. C. Fornagueraa. G. Calderóa, M. Mitjans, M.P. Vinardell, C. Solansa, C. Vauthier, Interaction of PLGA nanoparticles with blood components: protein adsorption, coagulation, activation of the complement system and hemolysis studies, Nanoescale. 14 (2015) 6045-58. doi: 10.1039/c5nr00733j.
73. T. Tatsuishi, Y. Oyama, K. Iwase, J.Y. Yamaguchi, M. Kobayashi, Y. Nishimura, A. Kanada, S. Hirama, Polysorbate 80 increases the susceptibility to oxidative stress in rat thymocytes, Toxicology. 207 (2005) 1-14, doi:10.1016/j.tox.2004.07.020.
74. M. Sharma, K. Kishore, S.K. Gupta, S. Joshi, D.S. Arya, Cardioprotective potential of Ocimum sanctum in isoproterenol induced myocardial infarction in rats, Mol Cell Biochem. 225 (2001) 75-83. doi.org/10.1023/A:1012220908636.
75. M.V. De Freitas, R.M. Netto, J.C.H. Da Costa, T.M. De Souza, J.O. Costa, C.B. Firmino, N. Penha-Silva, Influence of aqueous crude extracts of medicinal plants on the osmotic stability of human erythrocytes, Toxicol In Vitro. 22 (2008) 219-224. doi:10.1016/j.tiv.2007.07.010.
76. J.P. Kehrer, J.D. Robertson, C.V. Smith, Free radicals and reactive oxygen species. Critical reviews in Toxicology, 1 (2015) 277-307. doi: 10.1016/j.cbi.2014.10.016.
77. M. Ramchoun, K. Sellam, H. Harnafi, C. Alem, M. Benlyas, F. Khallouki, S. Amrani, Investigation of antioxidant and antihemolytic properties of Thymus satureioides collected from Tafilalet Region, south-east of Morocco, Asian Pac J Trop Biomed. 5 (2015) 93-100. doi.org/10.1016/S2221-1691(15)30151-9.
78. T. Baccarin, M. Mitjans, E. Lemos-Senna, M.P. Vinardell, Protection against Oxidative Damage in Human Erythrocytes and Preliminary Photosafety Assessment of Punica granatum Seed Oil Nanoemulsions Entrapping Polyphenol-rich Ethyl Acetate Fraction, Toxicol In Vitro. 30 (2015) 421-428. doi:10.1016/j.tiv.2015.09.020.

79. E.D.T. Bouhlali, M. Bammou, K. Sellam, M. Benlyas, C. Alem, Y. Filali-Zegzouti, Evaluation of antioxidant, antihemolytic and antibacterial potential of six Moroccan date fruit (Phoenix dactylifera L.) varieties, JKSUS. 28 (2016) 136–142. doi.org/10.1016/j.jksus.2016.01.002.

Figure captions

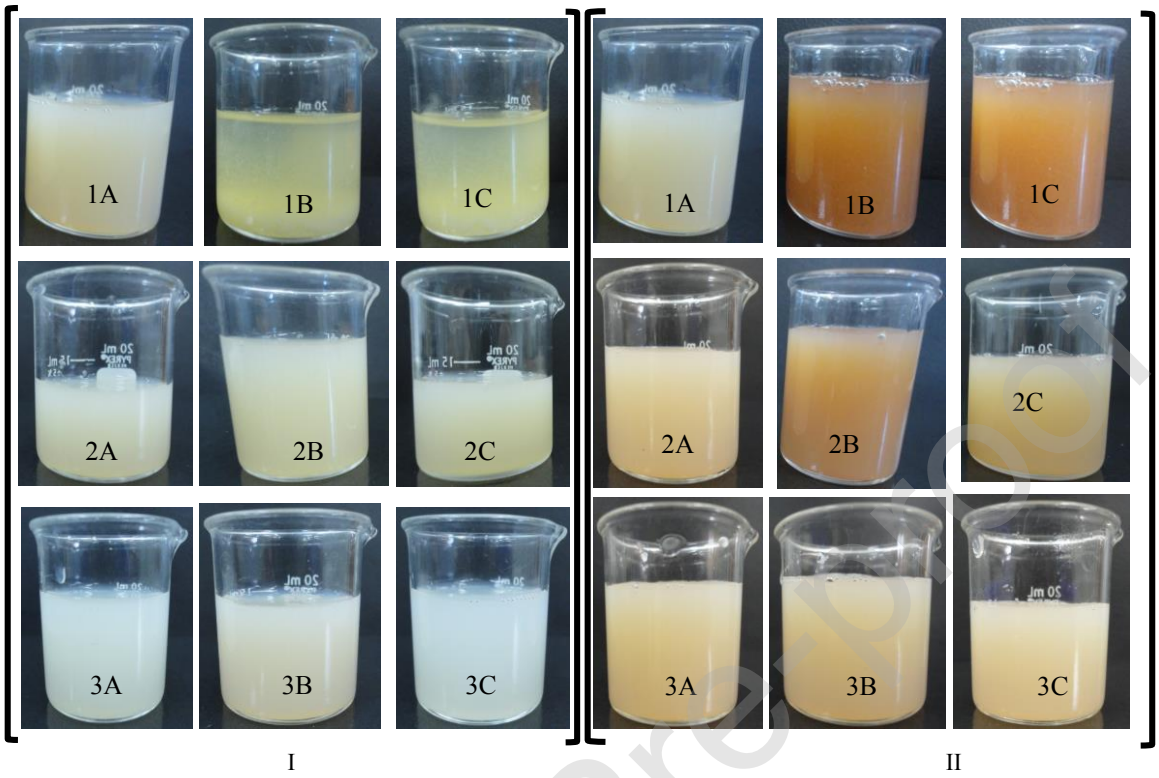


Figure 1 – Organoleptic characteristics of formulations prepared by RP (I) and EI (II) methods. (1) 10.0 mg.mL^{-1}; (2) 5.0 mg.mL^{-1}; (3) 1.0 mg.mL^{-1}. Initial (A), final at RT (B), and final at RE (C).

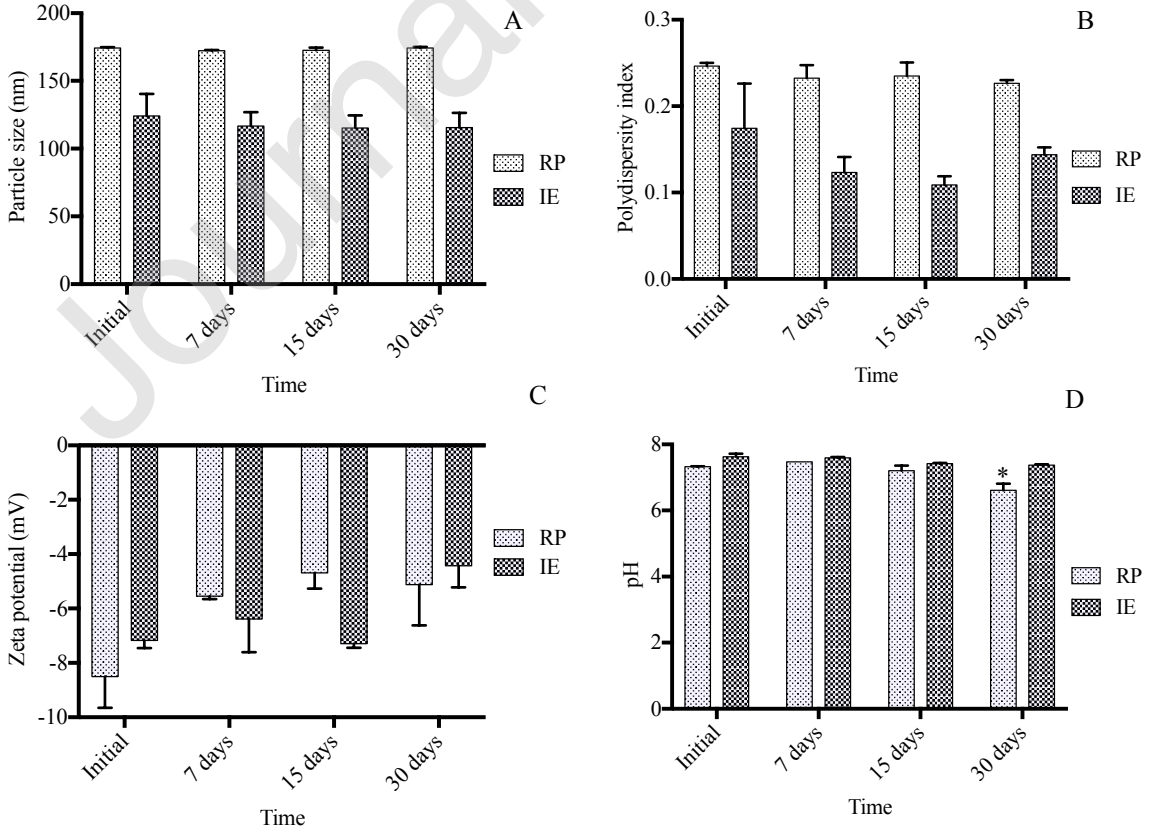


Figure 2 – Physicochemical parameters of liposome formulations containing 1 mg.mL^{-1} guarana powder stored at RE (n = 2). A: Particle size (nm); B: PDI; C: Zeta potential (mV); and D: pH. RP, liposomes containing 1 mg.mL^{-1} of guarana powder produced by the RP method; EI, liposomes containing 1 mg.mL^{-1} of guarana powder produced by the EI method. * value presents significant difference p <0.05 in relation to the initial value.

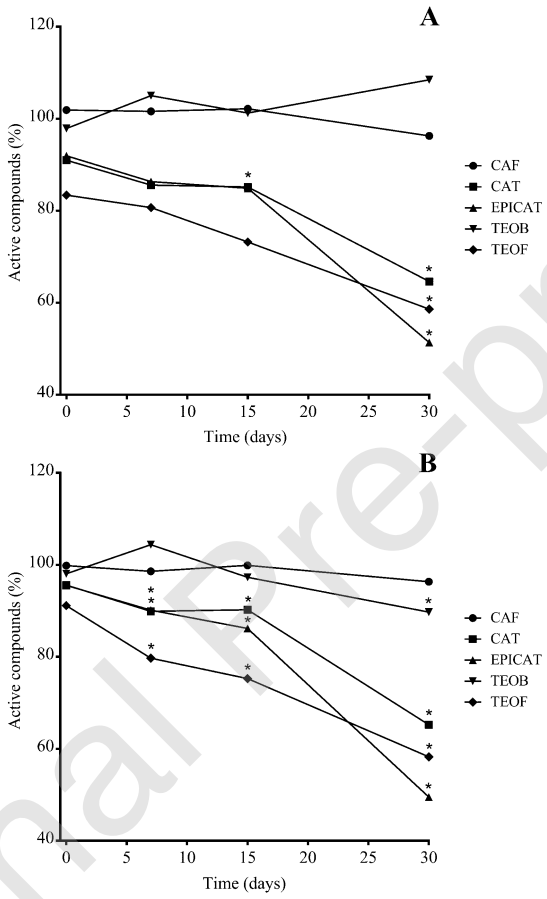


Figure 3 - Total content of active ingredients present in liposomes containing 1 mg.mL^{-1} guarana powder stored under RE (n = 2). A, liposomes produced by the RP method; B, liposomes produced by EI method. TEOB (theobromine), TEOF (theophylline), CAF (caffeine), CAT (catechin), and EPICAT (epicatechin) content. * Value presents significant difference p <0.05 in relation to the initial value.

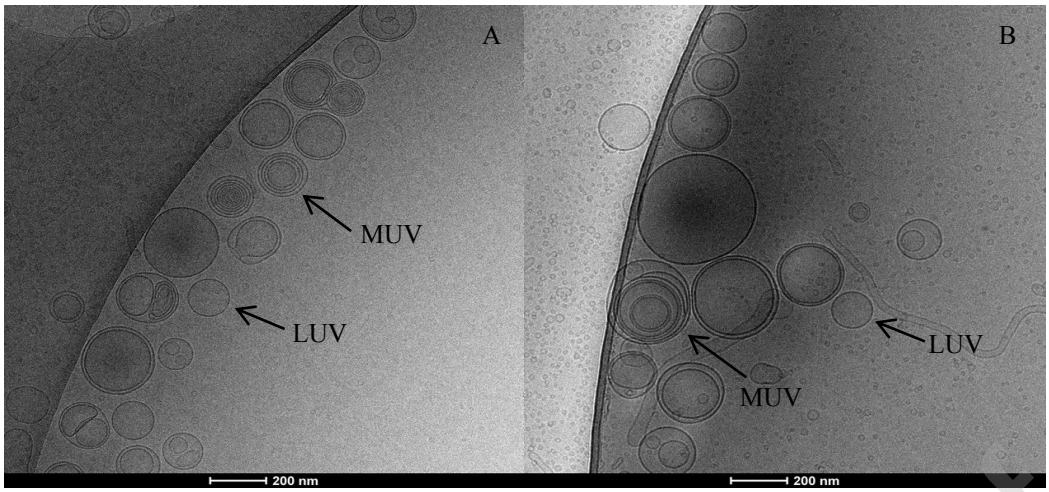


Figure 4 – Photomicrographs obtained by Cryo-TEM from liposome dispersions. A, white liposomes (W-Lip); B, liposomes containing 1 mg.mL^{-1} of guarana powder (G-Lip). LUV, large unilamellar vesicles; MLV, multilamellar vesicles.

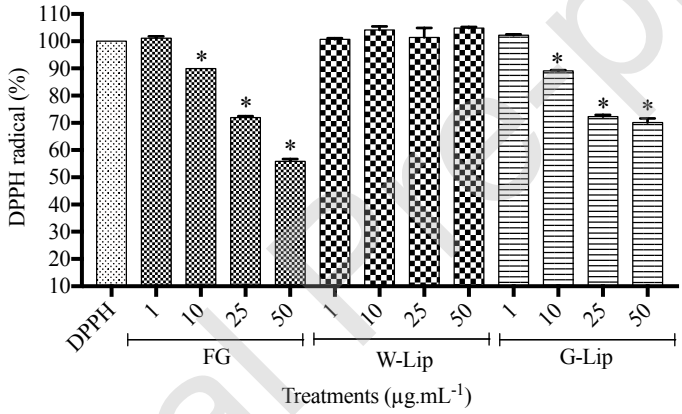


Figure 5 – Antioxidant activity for different treatments. FG (free guarana), W-Lip (white liposomes), G-Lip (liposomes containing guarana). *Statistically significant difference ($p < 0.05$) when compared with positive control with 100% DPPH.

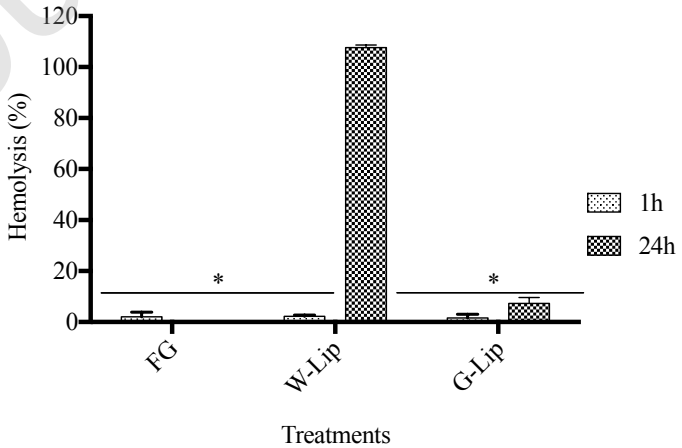


Figure 6 – Hemolytic activity for different treatments. FG (free guarana at 500 μg.mL^{-1} concentration), W-Lip (white liposomes), G-Lip (liposomes at 500 μg.mL^{-1} concentration). *Statistically significant difference ($p < 0.05$) when compared with positive control with 100% hemolysis.

Table

Table 1 – Mean particle diameter and PDI of liposomes produced by RP and EI methods.

Condition		RP		EI	
		Particle diameter (nm) ± DP	**PDI ± DP**	**Particle diameter (nm) ± DP**	**PDI ± DP**
10.0 mg.mL^{-1}	Initial	164 ± 6.32	0.426 ± 0.02	169 ± 0.23	0.238 ± 0.02
	RT -14 days	653 ± 184.69	0.696 ± 0.12	596 ± 266.32	0.706 ± 0.21
	RE -14 days	218 ± 12.86	0.487 ± 0.02	208 ± 23.55	0.456 ± 0.07
5.0 mg.mL^{-1}	Initial	140 ± 8.42	0.343 ± 0.05	115 ± 1.53	0.168 ± 0.01
	RT - 42 days	241 ± 27.84	0.710 ± 0.09	175 ± 2.08	0.477 ± 0.01
	RE - 42 days	181 ± 5.76	0.373 ± 0.04	146 ± 0.40	0.387 ± 0.05
1.0 mg.mL^{-1}	Initial	163 ± 8.63	0.313 ± 0.01	132 ± 0.57	0.181 ± 0.01
	RT - 70 days	1534 ± 65.37	0.493 ± 0.09	155 ± 2.76	0.242 ± 0.01
	RE - 70 days	168 ± 5.15	0.372 ± 0.04	141 ± 2.97	0.181 ± 0.01

RT (room temperature, 25 °C ± 2 °C), RE (under refrigeration, 5 °C ± 2 °C)