

## PHARMACOLOGICAL SAFETY PROFILE AND HEMOCOMPATIBILITY OF LIPID NANOCARRIERS BASED ON TUCUMAN BUTTER<sup>1</sup>

### *PERFIL DE SEGURANÇA FARMACOLÓGICA E HEMOCOMPATIBILIDADE DE NANOCARREADORES LIPÍDICOS A BASE DE MANTEIGA DE TUCUMÃ<sup>1</sup>.*

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#### ABSTRACT

With the growing need to develop effective and sustainable therapeutic alternatives, research has been focusing on the association of products of natural origin with nanotechnology, which besides enhancing the action, acts to protect the constituents from degradation. A fruit highlighted in research today is *Astrocaryum vulgare* (Tucumã), as well as natural fixed oils extracted from seeds, such as grape seed oil and microalgae biomass. The present study aimed to evaluate the parameters of pharmacological safety and in vitro hemocompatibility of lipid nanocarriers (CLN) from the association of tucumã butter with two oils (grape seed and microalgae). Hemocompatibility rates were assessed by analyzing hemolytic activity, in addition to clotting time and clot retraction, whereas pharmacological safety analyzed parameters such as cell viability, DNA damage, dichloride and nitric oxide production. The nanocarriers produced through the association of butter with oils showed a satisfactory pharmacological safety and hemocompatibility profile.

**Keywords:** Nanotechnology, Nanosciences, *Chlorella homosphaera*, *Vitis vinifera*.

#### RESUMO

*Com a crescente necessidade em desenvolver alternativas terapêuticas eficazes e sustentáveis, as pesquisas têm apostado na associação de produtos de origem natural à nanotecnologia que além de potencializar a ação, atua protegendo os constituintes de degradação. Um fruto em destaque nas pesquisas atualmente é o *Astrocaryum vulgare* (Tucumã), assim como óleos fixos naturais extraídos de sementes, como o óleo de semente de uva e de biomassa microalgais. O presente estudo teve como objetivo avaliar os parâmetros de segurança farmacológica e hemocompatibilidade in vitro de nanocarreadores lipídicos (CLN) oriundos da associação de manteiga de tucumã com dois óleos (de semente de uva e microalga). As taxas de hemocompatibilidade foram avaliadas através da análise da atividade hemolítica, além do tempo de coagulação e retração de coágulo, já a segurança farmacológica analisou parâmetros como viabilidade celular, danos de DNA, produção de dicloro e óxido nítrico. Os nanocarreadores produzidos através da*

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associação da manteiga com os óleos apresentaram satisfatório perfil de segurança farmacológica e também de hemocompatibilidade.

**Palavras-chave:** Nanotecnologia, Nanociências, *Chlorella homosfaera*, *Vitis vinífera*.

## INTRODUCTION

Currently, studies combining natural bioactive agents such as natural oils and nanotechnology are being carried out as adjuvants in therapeutic processes, such as healing and antimicrobial action. Brazil, due to its great richness in medicinal plants, has been standing out in the use of *Astrocaryum vulgare* butter (Tucumã). Also, natural oils extracted from seeds, such as grape seed oil and fixed oils from microalgae biomass (such as *Chlorella homosfaera*) have become the target of major research (GHOSH; GABA, 2013; FERREIRA *et al.*, 2017).

Tucumã, which is used for food and beauty, is considered a rich source of bioactive substances, with high lipid content, large amounts of carotene, precursor to vitamin A, high levels of fiber and vitamin E. In addition to its high antioxidant capacity, it has some other biological activities such as anti-inflammatory and antimicrobial action against Gram-positive strains and, antifungal action demonstrated against *Candida albicans* (FERREIRA *et al.*, 2008; BONY *et al.*, 2012; SOUZA FILHO *et al.*, 2013; JOBIM *et al.*, 2014).

Grape seed oil is known for its great richness in vitamin E, vitamin C,  $\beta$ -carotene in addition to matter rich in tocopherols, steroids and various fatty acids, acting as a fat-soluble antioxidant in plasma and the tissue membrane, protecting cell integrity. Grape seed stands out for being a source of linoleic and oleic acid, containing traces of linolenic and palmitoleic. Linoleic acid and linolenic acid are essential for the proper functioning of various organs and are essential for maintaining cell membranes with normal functioning (CHOI; LEE, 2009; ANDRADE *et al.*, 2012; MEDINA *et al.*, 2015).

Microalgae are microscopic algae made up of a cell and chlorophyll and live in colonies and are usually found in fresh and salt water (RICHMOND, 2004). Currently, they have been the focus of many studies given their great applicability in the food, pharmaceutical, biomedicine and environmental industries, making cultivation more and more widespread worldwide. The great interest is because microalgae as an alternative for sustainable development because they originate a natural product and that their high proliferation in rivers and lakes often becomes an environmental problem. The cultivation of microalgae has been increasing and diversifying for some decades, due to the advance in the knowledge of cultivation techniques and commercial applications for the generated biomass. The history of microalgae cultivations clearly shows how the activity has been growing and diversifying around the world (LOURENÇO, 2006). Extraction is commonly used to obtain oil from plants, some of the known extraction methods are pressure, osmosis pressure, microwaves, supercritics and ultrasound (SZENTMIHALYI, 2002; SHAH *et al.*, 2005).

In addition to natural bioactives, research has bet on its association with one of the most advanced technologies, nanotechnology, which in addition to increasing the stability of assets, enhances its action and because they are biocompatible, biodegradable and have low or almost no toxicity (MERHNET ; MADER, 2001; HEURTAULT *et al.*, 2003; FATHIA *et al.*, 2012). Given this context, the present study aimed to prepare NLS, evaluate its hemocompatibility and pharmacological safety *in vitro*.

## MATERIALS AND METHODS

### ACQUISITION OF BUTTER AND OILS

The tucumã butter was provided by the company Amazon oil-Indústria Pará-Amazonas-Brazil (Amazon oil, 2019). *Vitis vinifera* grape seed oil was cordially supplied by the Franciscan University pharmacology laboratory, which was purchased commercially without requiring any type of purification. The microalgae oil *Chlorella homosphaera* was grown and extracted in the Nanotechnology laboratory of the Franciscan University.

The cultivation of microalgae was performed using modified BG11 medium (ACREMAN, 2017) and enriched with Vitamin B (GUILLARD; RYTHER, 1962; GUILLARD 1975). Thus, the culture was carried out with a volume of 300 mL of stock culture, in a 3L flask containing the medium, the culture being subjected to aeration (2 L min<sup>-1</sup>), kept under artificial light with a photoperiod consisting of a cycle 14:10 chiaro-scuro, provided by 40 W fluorescent lamps. The ambient temperature was maintained at around 24 ° C. The cultivation was followed for 14 days. For the extraction, the chosen method was the Soxhlet, a conventional method widely used to extract natural products from organic matrices. For this, a mass of 3 g of dry microalgae was used, in an oven at 60 ° C, for each extraction. Biomass was added to the extraction chamber, and a solvent mixture (250 mL of chloroform: methanol, 2: 1 v / v) was added to the distillation flask for lipid extraction (RAMOLA *et al.*, 2019). The solvent was refluxed for 8 h, passing through the phases of evaporation, condensation, and percolation of the solvent through biomass, the flask containing the mixture of solvent and extracted lipids was removed and the solvent was vaporized in a rotary evaporator to recover the lipids. The yield obtained was approximately 40%.

### PREPARATION AND CHARACTERIZATION OF LIPID NANOCARCHERS

The lipid nanocarriers based on tucumã butter were prepared using a method developed by pre - formulation tests, where the Ultra Turrax® equipment was used, using the high-speed homogenization technique. Two formulations were prepared: Tucumã Butter (*Astrocaryum vulgare*) with

grapeseed oil (*Vitis vinifera*) - (Nano 1) and Tucumã Butter (*Astrocaryum vulgare*) with microalgae oil (*Chlorella homosphaera*) - (Nano 2). The formulations were prepared at n=3 and maintained under different temperature and storage conditions (refrigerator at -4 °C, oven at 40 °C, room temperature with light exposure and darkroom temperature) for 60 days. The characterization of the nanoparticles was carried out through the analysis of parameters, such as pH, polydispersion index (PDI), size, and zeta potential. The diameter and PDI determinations of the nanoparticles were performed through dynamic light scattering; the zeta potential was measured by electrophoresis (Zetasizer® nano-Zs model ZEN 3600, Malvern), the determination of the pH of the nanoparticles was carried out in a pH-meter.

## EVALUATION OF HEMOCOMPATIBILITY PARAMETERS

### Blood collection

The samples were collected by venipuncture using vacuum tubes containing EDTA (ethylenediaminetetraacetic acid) for hemolysis and sodium citrate for coagulation and clot retraction (SOUSA FILHO *et al.*, 2019). Approved by the UFN Human Ethics Committee (CAAE 31211214.4.0000.5306).

### Hemolysis

The first parameter analyzed was through the hemolysis assay, as adapted from the literature (SOUSA FILHO *et al.*, 2019). The test was carried out in Eppendorf microtubes where blood and 1x PBS were added in the proportion of 1: 1 v / v, centrifuged for 15 minutes at 1000 rpm, the supernatant was discarded, and this procedure was performed three times. Later, in a new tube containing 1 mL of 1x PBS, 400 µL of washed red blood cells and 80 µL of treatment were added. As negative control cells (400 µL) and 1x PBS (1 mL), and as positive control cells (400 µL), 1x PBS (1 mL) and H<sub>2</sub>O<sub>2</sub> (80 µL) were used. The tubes were incubated at 37 ° C at 5% CO<sub>2</sub> for 1 hour, centrifuged for 5 minutes at 1000 rpm and 100 µL of the supernatant in a 96-well plate, which was read in the ELISA reader at 405 nm.

### Clotting time

Whole blood was collected and centrifuged 10 minutes at 2500 rpm. Then, 225 µL of plasma were separated into wells together with 25 µL of treatments and incubated at 37 ° C for 30 minutes. Subsequently, the reading was performed on the Quick Timer II (Drake) coagulometer properly cali-

brated, according to the manufacturer's recommendations for tests of PT hemostasis (Prothrombin time) and TTPA hemostasis (Partially activated thromboplastin time), used as a reference for normal APTT value, with an interval between 25 and 35 seconds. While the baseline values for TP, the interval between 12 and 15 seconds was used, as adapted from the literature (SOUSA FILHO *et al.*, 2019).

### **Clot retraction**

The percentage of clot retraction was analyzed through the volume of serum obtained, after the coagulation of a determined amount of blood. The initial clot contains all the elements of the blood. After its retraction, the serum is expelled from the fibrin mesh, which is retracted by the action of platelets, providing data on platelet activity. Thus, to perform the clot retraction analysis, after the clotting time was completed, an hour was set with the tube in the bath at 37 °C. After the end of the indicated time, using a 1 mL volumetric pipette, all the serum in the tube was aspirated. The aspirated volume of the total volume is considered the value of the clot retraction (LORENZI, 1999).

## **IN VITRO BIOCOMPATIBILITY ASSESSMENT**

### **Blood collection**

The samples were collected by venipuncture using vacuum tubes to obtain peripheral blood mononucleated cells (PBMCs), according to the experimental protocol approved by the UFN Human Ethics Committee (CAAE number: 31211214.4.0000.5306)

### **Cell culture and treatment**

The cells (PBMC) were separated by the procedure based on the difference in density gradient using the Ficoll Histopaque-1077VR reagent (Sigma-Aldrich). After adding the blood to the container with the reagent in volume 1: 1 v / v, the samples were centrifuged for 30 minutes at room temperature. PBMCs were distributed in 96-well plates containing RPMI 1640 cell medium (Sigma-Aldrich), 10% fetal bovine serum, and supplemented with 1% antibiotics. The cells were grown at a concentration of  $2 \times 10^5$  cells mL<sup>-1</sup> per well (BOTTON *et al.*, 2015). Then, the cells were exposed to treatments for 24 h. Afterward, its effect on cell modulation was evaluated using different colorimetric and fluorimetric assays. All treatments and trials were carried out in triplicate to ensure coherent statistical analysis. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) at 200 μmol L<sup>-1</sup> was used as a positive control for all tests.

### **MTT reduction test (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina)**

The method was carried out according to Mossman (1983). Upon completion of the incubation times, 20  $\mu\text{L}$  of the MTT solution (0.01 mol L<sup>-1</sup> and pH 7.4) was added in a concentration of 5 mg mL<sup>-1</sup> diluted in PBS (1X phosphate buffer). The plates that received the MTT solution were protected from light and kept at 37 ° C, in an oven with 5% CO<sub>2</sub>, which was homogenized in the shaker incubated for 4 hours. After 4 hours, the supernatant was removed from the wells, and the cells resuspended in 200  $\mu\text{L}$  of dimethyl sulfoxide (DMSO). The absorbance was determined in an ELISA reader at a wavelength of 560 nm. The determination of the cytotoxic potential was calculated in relation to the negative control (cells in culture medium).

### **Dichlorofluorescein diacetate test (DCFH-DA)**

To check the total free radical rate, the 2',7'-dichlorofluorescein diacetate reagent (DCFH-DA) was used, as adapted from the literature (ESPOSTI *et al.*, 2002), which can cross the cell membrane, being deacetylated by mitochondrial enzymes, giving rise to 2',7'-dichlorodihydrofluorescein, which reacts with reactive oxygen species (ROSs), mainly hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and produces 2',7'-dichlorofluorescein that emits fluorescence. Thus, fluorescence in the spectrofluorimeter apparatus was determined based on the lengths of where 488 nm excitation and 525 nm emission.

### **Fluorimetric DNA Quantification Assay by DNA-PicoGreen® reagent**

To complement the determination of cell viability and genotoxic capacity, the fluorimetric assay of quantification of free DNA in the medium was conducted using the DNA-PicoGreen® reagent, from the Invitrogen brand (Life Technologies), which is a fluorescent dye that is bound to double-stranded DNA. This procedure was performed in the culture medium where the cells were treated to determine the presence of double-stranded DNA in this medium due to possible cell disruption and cell death. The dye was added to the sample in a dark 96-well Elisa plate, with a 5-minute incubation and fluorescence reading in the Spectrofluorimeter at 480 nm excitation and 520 nm emission, according to Sagrillo *et al.* (2015).

## Nitric Oxide Test Protocol

The nitric oxide test detects the presence of organic nitrite in the sample. The nitrite is detected and analyzed by the formation of a pink color when the Griess reagent is added to the sample containing NO<sup>2-</sup>. The sulfanilamide of the Griess reagent is responsible for the formation of diazonium in the nitrite sample. When the azo compound (N-1-naphthylenediamino-bichlorohydrate) interacts with the diazonium salts, the pink color appears in the sample. The sample used is the cell culture supernatant, where 100 µL of the sample supernatant is pipetted into a 96-well plate. 100 µL of Griess reagent was added to the supernatant; left at room temperature for 15 minutes and read at 540 nm on the spectrophotometer (CHOI *et al.*, 2012).

## STATISTICAL ANALYSIS

The results were presented as a percentage of the untreated control group (negative control). The analyzes were performed using one-way bilateral analysis of variance (ANOVA) followed by Dunnett's post hoc test. Values with  $p < 0.05$  were considered statistically significant. The data were expressed as mean  $\pm$  standard deviation. The graphs were prepared using GraphPadPrism version 5.01 (GraphPad Software, La Jolla, CA, USA).

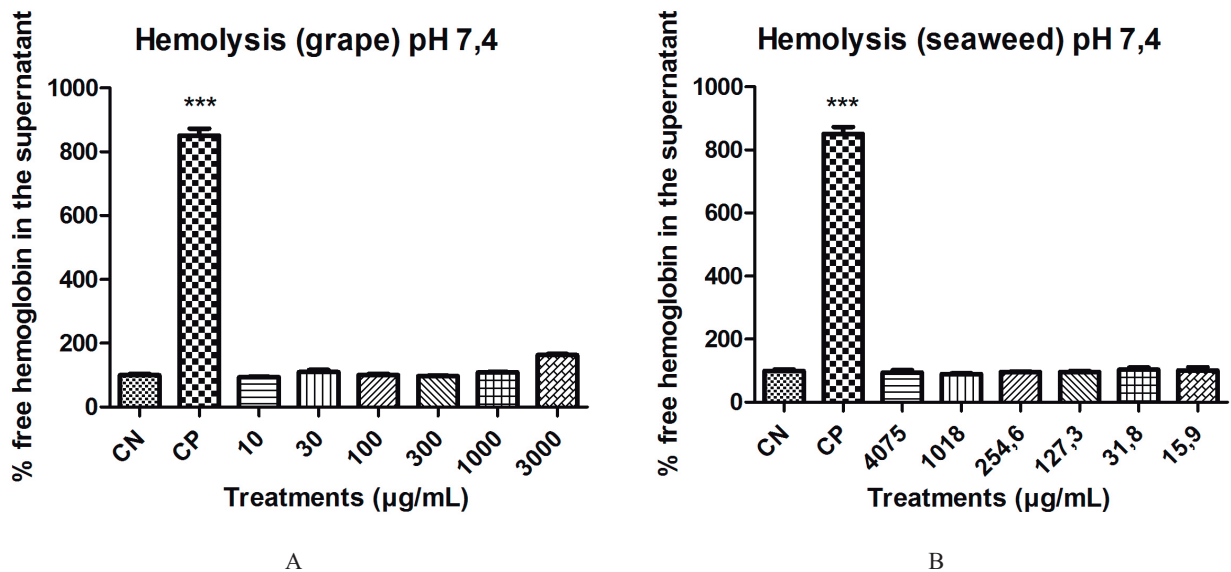
## RESULTS AND DISCUSSION

Through the characterization of the nanoparticles it was possible to observe that at time 0, that is, on the day of manufacture, formulation 1 consisting of Tucumã butter and grape seed oil showed a monodispersed distribution of particles with an average value of PDI 0.260 where according to the literature the ideal value is  $< 0.3$  (MACHADO *et al.*, 2019); the average CLN size of approximately 181.3 nm. The value for the zeta potential was -12.2 mV. Formulation 2, consisting of Tucumã Butter and microalgae oil, also showed a monodispersed distribution of particles showing an average value of PDI 0.200, the average size approximately 105.8 nm, and the results for the zeta potential was -9.00 mV. The pH analysis did not show significant changes for both formulations in the different storage conditions, remaining in a range between 5 and 6.

One of the most important hematological parameters to be analyzed in relation to a nanostructure is the hemolytic activity of the formulations because hemolysis is a process in which the rupture of the membrane of red cells occurs and can cause serious health problems, such as hemolytic anemia,

a disease characterized by the early destruction of red blood cells and can also cause an increase in bilirubin, which can cause the formation of gallstones. The assessment of hemolytic activity, in addition to indicative of in vitro toxicity, can also serve as a simple measure to estimate possible damage caused by the membrane formulation in vivo (DAL PIZZOL *et al.*, 2014; JOSHI *et al.*, 2018). The hemolytic activity of the CLN was carried out at pH 7.4, simulating the physiological pH. Figure 1 shows the hemolytic activity of the formulations at different treatment concentrations.

**Figure 1** - Results of the hemolysis test simulating the physiological pH for Nano 1 (a) and Nano 2 (b), negative control (CN) and positive control (CP).



The results were expressed as a percentage of positive control (100%). The data were expressed as mean  $\pm$  standard deviation (SD). The analyzes were performed by 1-way ANOVA, followed by Dunnett's post hoc test.

Values with  $p < 0.05$  were considered statistically significant.

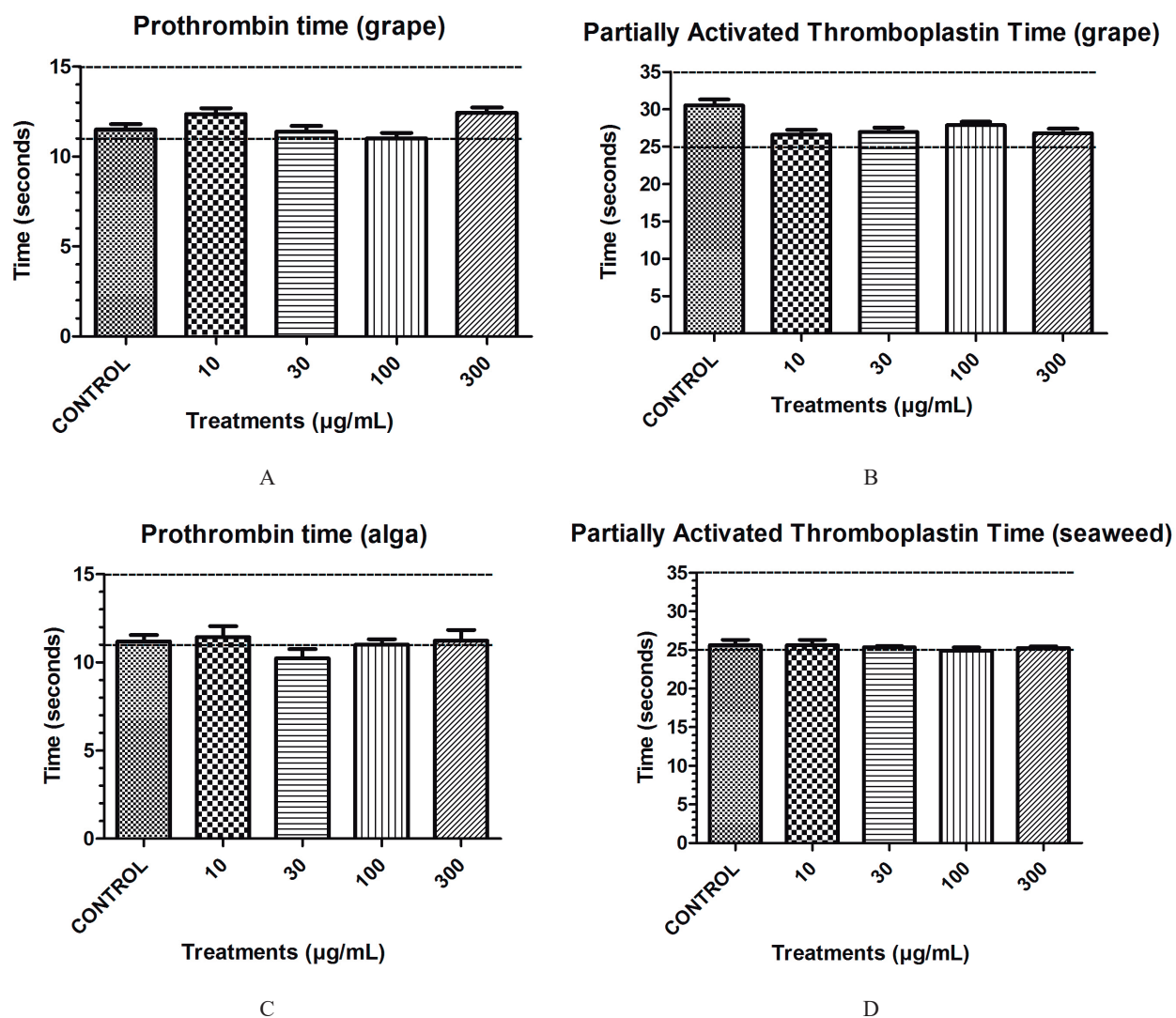
As shown in Figure 1, in none of the tested concentrations of the different formulations was the presence of hemoglobin in the supernatant detected, suggesting that the formulations do not have a hemolytic character and are safe in relation to the interaction with red blood cells. According to Kuznetsova *et al.* (2012), the low degree of hemolysis found is probably due to the repulsion between the nanoparticles and the erythrocytes as both have negative zeta potential, which causes repulsion between them.

In addition to hemolytic activity, Dobrovolskaia and Mcneil, (2013) assess that it is extremely important to evaluate formulations on the coagulation cascade since the activation of this process can lead to thrombosis or disseminated intravascular coagulation. Blood clotting is a complex sequence of chemical reactions that result in the formation of a fibrin clot, being an important part of hemostasis in which the damaged blood vessel wall is covered by a fibrin clot to stop bleeding and help repair the damaged tissue. Besides, clotting disorders can lead to an increased risk of bleeding, thrombosis or embolism, because when clotting is deficient, even a slight injury to a blood vessel can cause



severe blood loss; Likewise, when clotting is excessive, small blood vessels in critical locations can be blocked by clots. Blocked vessels in the brain can cause strokes and obstruction of vessels that carry blood to the heart can cause myocardial infarction (MOAKE, 2018). Figure 2 shows the clotting time for both formulations (Nano 1 and 2), where the Prothrombin Time (TP) and the Partially Activated Thromboplastin Time (TTP) are represented.

**Figure 2** - Representation of the results of clotting times for Nano 1 (a) (TP) and (b) (TTP) e and Nano 2 (c) (TP) and (d) (TTP).



The results were expressed as a percentage of positive control (100%). The data were expressed as mean ± standard deviation (SD). The analyzes were performed by 1-way ANOVA, followed by Dunnett's post hoc test.

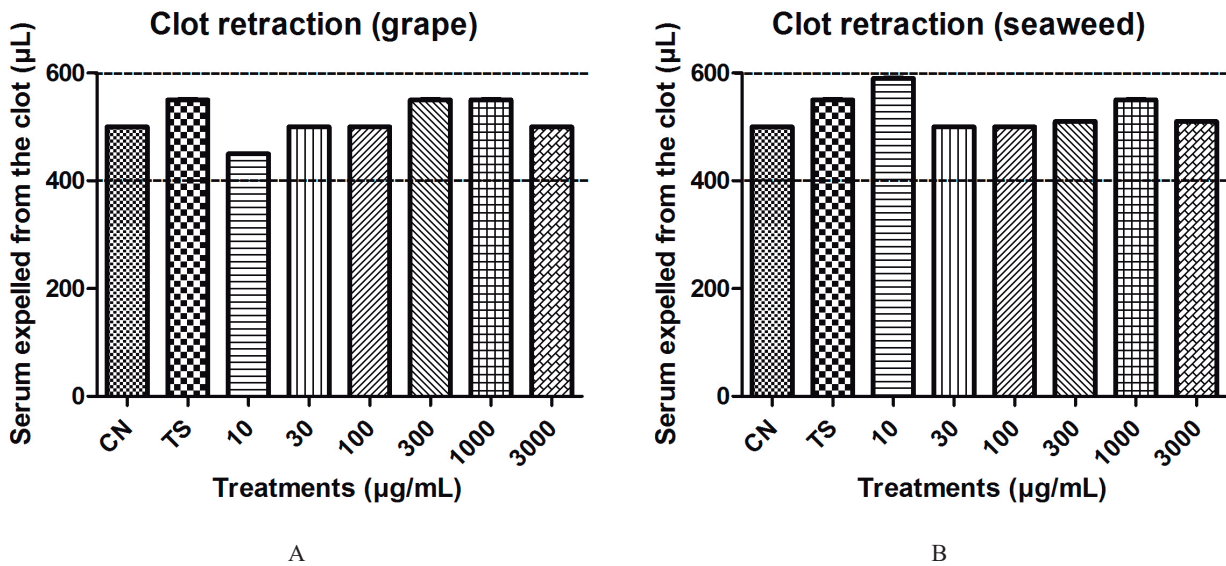
Values with  $p < 0.05$  were considered statistically significant.

In the present study, the evaluation of the effect of the formulations on the coagulation cascade demonstrated that none of the concentrations of the tested formulations altered the clotting time after incubation with human plasmas. Thus, both the Prothrombin Time and the Activated Partial Thromboplastin Time (figure 2), which evaluate, respectively, the extrinsic and intrinsic pathways of

coagulation were not altered. Correlated to hemolysis studies report that the release of the stroma of red blood cells during the hemolytic process can be the trigger for disseminated intravascular coagulation (MORAES *et al.*, 2013; SANTOS 2020).

After blood clotting, the formed clot retracts retaining the figured elements of the blood and releasing the liquid part, the serum. The clot retraction is due to the release of substances, by the platelets located at the intersection points of the fibrin network meshes. The volume of serum released is expressed as a percentage to the initial volume of blood that could clot. Clot retraction is the final stage of the clotting process and is directly related to the number and functionality of platelets, in normal situations the clot usually occupies 50 to 60% of the original volume leaving 40% or more for the serum and erythrocytes that escape the clot, so, according to the literature, a volume of serum below 40% is abnormal. The clot retraction is decreased when the fibrinogen level is decreased and platelets are less than 100,000 / mm<sup>3</sup> (WALLACH, 2003; ZANUSSO, 2013), which was not verified in our results, where both formulations presented percentages within the value of reference (Figure 3).

**Figure 3** - Representation of the results of clot retraction for Nano 1 (a) and Nano 2 (b), negative control (CN) and surfactant control (TS).



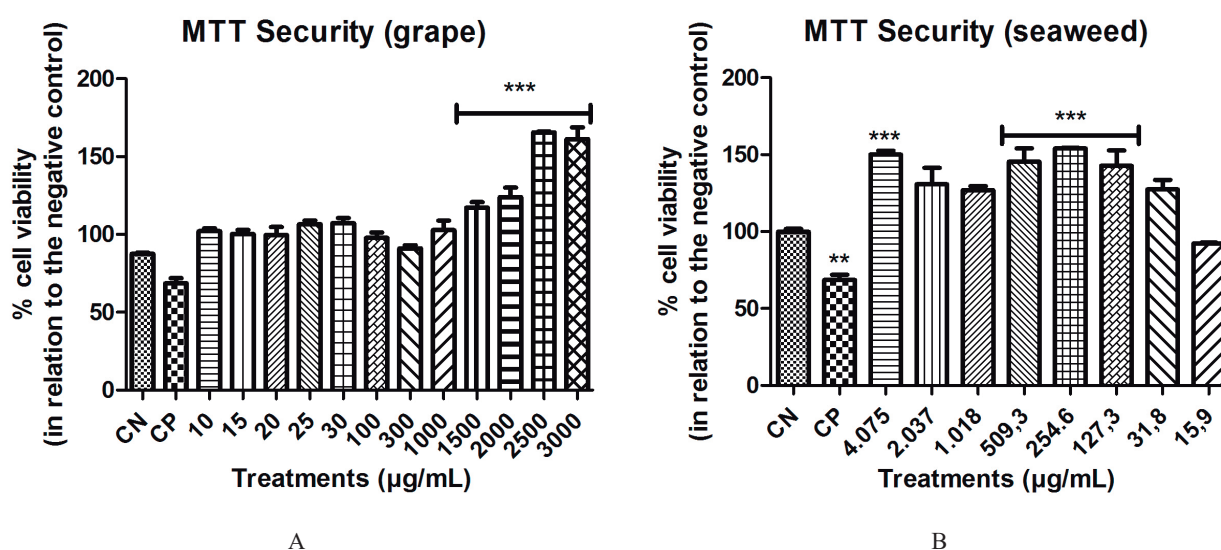
The results were expressed as a percentage of positive control (100%). The data were expressed as mean ± standard deviation (SD). The analyzes were performed by 1-way ANOVA, followed by Dunnett’s post hoc test. Values with  $p < 0.05$  were considered statistically significant.

The percentage of clot retraction is represented by the volume of serum obtained, after coagulation and clot retraction, of a determined amount of blood. The initial clot contains all the elements of the blood and after its retraction, the serum is expelled from the fibrin mesh, which is retracted by the action of the platelets. Such analysis provides data on platelet activity. Based on the results presented, we verified that the clot retraction time in all tested concentrations of the different formulations does not present a statistical difference from the negative control.

Taking into account that nanomaterials will have direct access to the cells of the human organism, it is necessary to carry out pharmacological safety tests to evaluate potential undesirable pharmacodynamic effects of the substance, because even knowing the content of its composition, little is known about its possible toxicity, biocompatibility interactions and adverse reactions of nanomaterials. Even because any constituent after assuming a nanostructured form can assume properties different from the original ones (DORA *et al.*, 2019).

The cell viability of lipid nanocarriers was evaluated from the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay. As a positive control of death, H<sub>2</sub>O<sub>2</sub> was used, as shown in Figure 4, with the negative control it significantly decreased cell viability. Both formulations were evaluated separately. In figure 4.A corresponding to the formulation with *Chlorella homosphaera* oil we can see that some concentrations tested (4.075, 509.3, 254.6, 127.3 µg / mL) demonstrated a significant increase in cell viability in 24h, the same occurred in figure 4.B corresponding to the formulation with grape seed oil in the highest concentrations.

**Figure 4** - Results of the MTT reduction test for formulations with microalgae oil (A) and grape seed oil (B), negative control (CN) and positive control (CP).

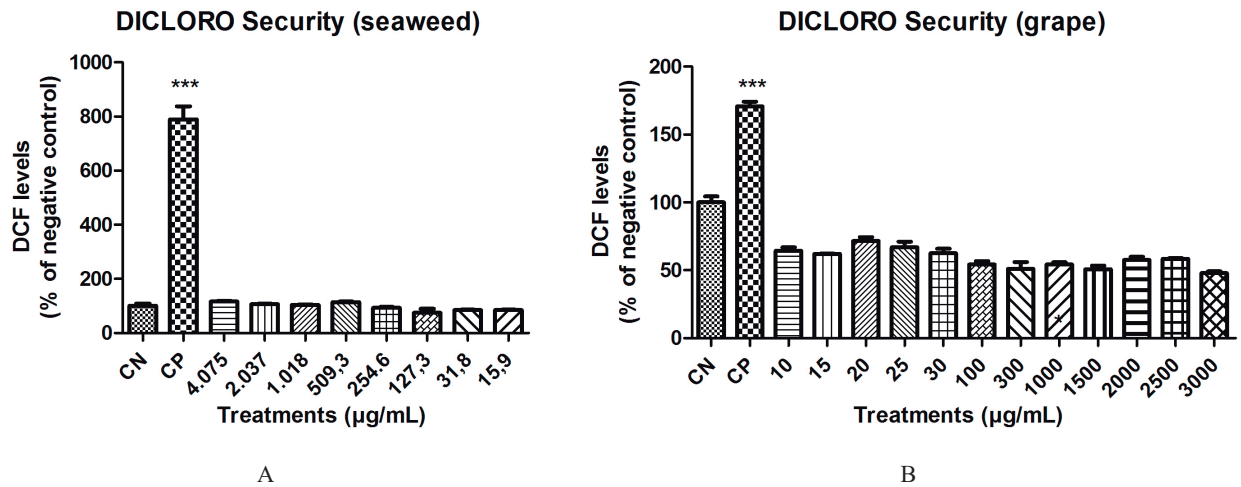


The results were expressed as a percentage of positive control (100%). The data were expressed as mean  $\pm$  standard deviation (SD). The analyzes were performed by 1-way ANOVA, followed by Dunnett's post hoc test.

Values with  $p < 0.05$  were considered statistically significant.

The total levels of reactive oxygen species were quantified using the dichlorofluorescein test where H<sub>2</sub>O<sub>2</sub> was used as a positive control. We can see in figure 5 that, as expected, the positive control presents a significant increase in DCF production, concerning the negative control. Both formulations were evaluated separately, in figure 5. A corresponding to the formulation with *Chlorella homosphaera* oil, figure 5.B the formulation containing grape seed oil. Analyzing the graphs, we can see that in all tested concentrations of both formulations they did not show DCF production concerning the negative control.

**Figure 5** - Results of the Dichlorofluorescein test for formulations with microalgae oil (A) and grape seed oil (B), negative control (CN) and positive control (CP).

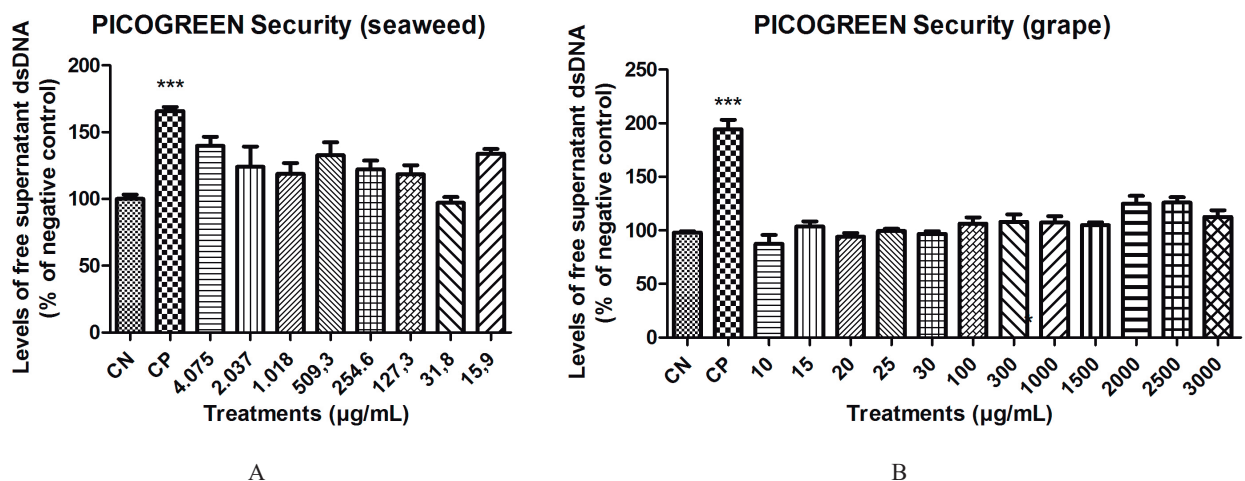


The results were expressed as a percentage of positive control (100%). The data were expressed as mean  $\pm$  standard deviation (SD). The analyzes were performed by 1-way ANOVA, followed by Dunnett's post hoc test.

Values with  $p < 0.05$  were considered statistically significant.

The Fluorimetric Assay for DNA Quantification by reagent was performed to detect double-stranded DNA damage in the supernatant after treatments. As shown in Figure 6, none of the two formulations showed a statistically significant difference with the negative control, suggesting then that they did not cause double-stranded damage to the cells' DNA (dsDNA).

**Figure 6** - Results of the PicoGreen test for formulations with microalgae oil (A) and grape seed oil (B), negative control (CN) and positive control (CP).



The results were expressed as a percentage of positive control (100%). The data were expressed as mean  $\pm$  standard deviation (SD). The analyzes were performed by 1-way ANOVA, followed by Dunnett's post hoc test.

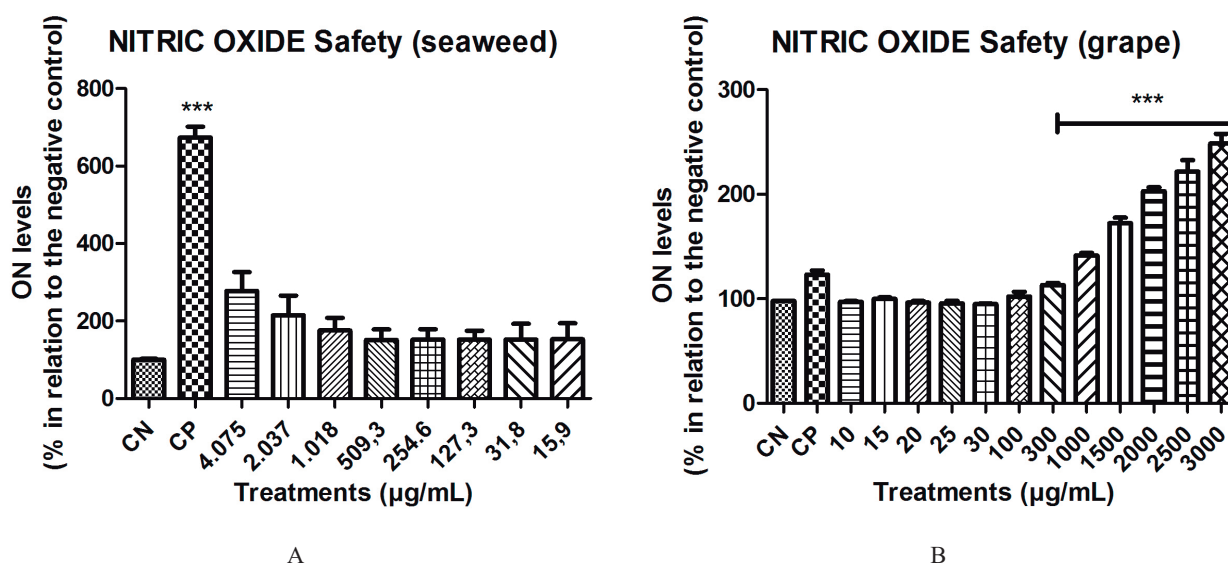
Values with  $p < 0.05$  were considered statistically significant.

Analyzing figures 4, 5, and 6 we can see that the two formulations studied showed a significant increase in cell viability, in addition to not increasing DCF production and not causing damage to

double-stranded DNA, such result can be attributed to the constituents of Butter, because according to Makpol *et al.* (2010),  $\alpha$ -tocopherol can protect cells against DNA damage and cell death.

To determine the nitrite production rates of the cells after treatment with the formulations, the nitric oxide test was performed, which is a free radical with an extremely short life in biological systems, where endogenous production by the synthesis of nitric oxide synthase is established as a performance of an important role in the mechanisms of vascular homeostasis, neurotransmission, and defense of the host (BRYAN; GRISHAM, 2007). According to Figure 7.A, for none of the concentrations, there was a statistically significant difference with the negative control, differently from what occurs in Figure 7.B where we observed that in the concentrations from 1000  $\mu\text{g} / \text{mL}$  to 3000  $\mu\text{g} / \text{mL}$  there was a significant increase in nitrite production.

**Figure 7** - Nitric Oxide test results for formulations with microalgae oil (A) and grape seed oil (B), negative control (CN) and positive control (CP).



The results were expressed as a percentage of positive control (100%). The data were expressed as mean  $\pm$  standard deviation (SD). The analyzes were performed by 1-way ANOVA, followed by Dunnett's post hoc test. Values with  $p < 0.05$  were considered statistically significant.

In figure 7 we can notice an increase in nitrite production in the highest concentrations of the formulation containing grape seed oil and, in the formulation, containing microalgae oil, there was no statistically significant increase. Nitric oxide is an endogenous molecule considered an important mediator of several biological actions such as vasodilation, neurotransmission, in addition to inhibiting adhesion and platelet aggregation. In addition, it is recognized as a modulator of the healing phases that control collagen deposition, angiogenesis, cell proliferation and even apoptosis (AMADEU *et al.*, 2008; CHAN *et al.*, 2015).

The increase in cell viability can be attributed to cytoprotective characteristics that the Butter of Tucumã has according to Makpol *et al.* (2010), where  $\beta$ -carotene is identified as the main constituent

of tucumã butter and oil, being able to prevent cell damage and decrease the level of DCF generation (BESTWICK and MILNE, 1999).

## CONCLUSION

Both the formulation containing grape seed oil and the one containing microalgae oil proved to be stable Lipid Nanocarriers for 60 days and in various storage locations as exposed to light, protected from light, in the refrigerator and greenhouse. Also, they presented an acceptable pH with the physiological one, enabling a possible application and they did not show visible signs of sedimentation, phase separation or color change.

Furthermore, the analyzes showed that the formulations do not have hemolytic activity against human erythrocytes and through the evaluation of the clotting time it was observed that the formulations do not promote changes in the coagulation cascade, being hemocompatible to a safe application and both formulations demonstrated pharmacological safety in the face of the cells tested.

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