

# Safety profile, antimicrobial and antibiofilm activities of a nanostructured lipid carrier containing oil and butter from *Astrocaryum vulgare*: *in vitro* studies

Aline Rossato<sup>a</sup>, Larissa da Silva Silveira<sup>a</sup>, Pâmella Scharamm Oliveira<sup>a</sup>, Thobias Toniolo de Souza<sup>b</sup>, Ana Paula Becker<sup>b</sup>, Roger Wagner<sup>c</sup>, Bruna Klein<sup>c</sup>, Walter Paixão de Souza Filho<sup>a</sup>, Roberto Christ Vianna dos Santos<sup>d</sup>, Diego de Souza<sup>a</sup>, Matheus Dellaméa Baldissera<sup>e</sup>, Michele Rorato Sagrillo<sup>a</sup>

<sup>a</sup>Graduate Program in Nanosciences, Universidade Franciscana, Santa Maria, RS, Brazil.

<sup>b</sup>Biomedicine Course, Universidade Franciscana, Santa Maria, RS, Brazil.

<sup>c</sup>Department of Technology and Food Science, Federal University of Santa Maria, Santa Maria, Rio Grande do Sul state, Brazil.

<sup>d</sup>Graduate Program of Pharmaceutical Sciences, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil.

<sup>e</sup>Graduate Program of Pharmacology, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil.

**Correspondence:** Dr. Michele Rorato Sagrillo, Program in Nanosciences, Universidade Franciscana, Santa Maria, RS, Brazil. E-mail: [sagrillorm18@gmail.com](mailto:sagrillorm18@gmail.com).

## Abstract

**Ethnopharmacological relevance:** *Tucumã (Astrocaryum vulgare)* is a fruit native to the Amazon region. Extracts from the peel and pulp are thought of as promising treatments for bacterial infections. The primary constituents of *Tucumã* oil and butter possess unsaturated carbon chains that are susceptible to oxidation by light or heat. The oils have high volatility and low aqueous solubility that limits their use without a vehicle. Nanotechnology refers to techniques to solve these problems. Nanostructured lipid carriers (NLC), for example, protect fixed oils degradation by heat or light, as well as from oxidation and evaporation, ensuring greater stability and function, thereby prolonging the useful life of the final product. **Study objectives:** The objective of this study was to evaluate the hemolytic, cytotoxic, antimicrobial and antibiofilm properties of an NLC containing *Tucumã* butter and oil so as to improve the solubility and photosensitivity of the compounds, generating better pharmacological efficacy. **Materials and methods:** The NLC was assessed for stability for 60 days. The cytotoxicity of nanoparticles in peripheral blood mononucleated cells was determined in culture using assays for cell viability, DNA damage, oxidative metabolism and damage to human erythrocytes. Antimicrobial activity was determined using the broth microdilution technique and antibiofilm activity according to standardized protocols. **Results:** The *Tucumã* NLC remained stable throughout the evaluated period, with pH between 5.22–5.35, monodisperse distribution ( $PDI < 0.3$ ) and average particle size of  $170.7 \pm 3$  nm. Cytotoxicity studies revealed that the NLC is safe and modulates inflammatory processes, demonstrated by increased cell viability and nitric oxide levels. There was low hemolytic activity of the NLC against human erythrocytes at most concentrations.

tested. **Conclusion:** Taken together, the data suggest that NLC containing Tucumã oil and butter showed antimicrobial and antibiofilm activity against organisms that cause morbidity and mortality in humans. They may be alternative solutions to public health problems related to bacterial infections.

**Keywords:** Microorganisms; Infections; Nanoparticles; Tucumã.

## 1. Introduction

Bacterial infections are one of the main causes of chronic diseases and mortality, causing economic losses for patients and public health systems. Each year in the United States, at least 2.8 million people are infected with antibiotic-resistant bacteria, with at least 35,000 deaths (CDC, 2019). Based on the incidence of antibiotic-resistant infections in 2014, each infection would cost \$ 1,383 to be properly treated, resulting in national health care costs for the adult population of approximately 2.8 billion per year (CDC, 2019). Antibiotics have been the preferred treatment method because antimicrobial agents kill microorganisms or stop their growth. Nevertheless, several studies have provided direct evidence that the widespread use of antibiotics leads to the emergence of multidrug-resistant bacterial strains (KNETSCH and KOOLE, 2011; PORSE et al., 2020), now considered a significant health problem worldwide (TARSILLO and PRIEFER, 2020).

Antibacterial agents based on herbal products have aroused growing interest because of their technological, economic, nutritional, and bactericidal activities (SUTILLI et al., 2018; JOSHI, 2018). These herbs include Tucumã (*Astrocaryum vulgare*) (HOVORKOVÁ et al., 2018), a fruit native to the Amazon region that is widely used as an anti-inflammatory and antioxidant (BONY et al., 2012). Recent studies have revealed its potential use as a hypoglycemic and antioxidant in a model of diabetes induced by alloxan (BALDISSERA et al., 2017). In the particular interest of the present study, Hovorková et al. (2018) studied the antibacterial activity of Tucumã against gram-positive and commensal pathogenic bacteria in the intestine, revealing that Tucumã is a promising herbal product for use as an antibacterial agent. Jobim et al. (2014) tested extracts of pulp and bark from Tucumã (*Astrocaryum aculeatum*) against 37 microorganisms and found significant antibacterial activity against three important gram-positive bacteria and antifungal activity against *C. albicans*. The antimicrobial mechanism of action of Tucumã appeared to involve redox imbalance that interrupts the growth of microorganisms and/or causes increased mortality. This effect has some specificity for each microorganism involving the role of different chemical compounds found in Tucumã extracts.

Even with good biological activity, oils in generation can undergo oxidation, with consequent loss of biological function. In this context, nanotechnology may provide an important alternative, because substances on the nanometer scale have different properties from those on the macrometric scale (FREIRE et al., 2018). In addition, nanoparticles (NPs) increase the retention of a drug in the particle, increasing the release time and controlling the release of the molecule. Nanostructured lipid carriers (NLC) protect fixed oils against degradation by heat and light, oxidation and evaporation, ensuring greater stability and function, consequently prolonging the useful life of the final product (PIRAN et al., 2017). We believe that nanotechnology can improve the antimicrobial activity of Tucumã oil and prevent its activity from being

reduced.

Most antimicrobial resistance mechanisms are irrelevant to NPs, because NPs are in direct contact with the bacterial cell wall, without the need to penetrate the cell. This shows that NPs are less likely to promote resistance in bacteria than are antibiotics. In addition, in view of the serious health concern associated with antimicrobial resistance, new approaches to inhibit microbial growth and the biofilms formed by them have been studied. The usefulness of nanomaterials for the efficient administration of antibacterials and the development of anti-biofilm agents is a promising strategy to overcome the resistance of microorganisms to antimicrobials (LOPES et al., 2019; WANG et al., 2017).

## **2. Materials and methods**

### **2.1 Acquisition of Tucumã oil and butter**

Tucumã oil and butter were purchased commercially from Amazon oil Industry - (Pará, Brazil).

#### **2.1.1 Characterization of Tucumã oil and butter**

The oil was previously characterized by Baldissera et al. (2017) using the gas chromatography method. The major components were oleic (368.7 mg/g) and palmitic acid (198.23 mg/g).

Tucumã butter was characterized using the method of Hartman and Lago (1973). A total of 20 mg of lipids from 1 mL of 0.4 M KOH methanolic solution were added in a test tube and vortexed for 1 min. The samples were kept in a water bath for 10 minutes at the boiling point and subsequently cooled to room temperature. Then, 3 mL of 1 M H<sub>2</sub>SO<sub>4</sub> methanolic solution were added and vortexed and kept in a water bath for 10 min. After cooling, 2 ml of hexane was added and centrifuged at 1050g for 5 min. Finally, the hexane with the fatty acid methyl esters (FAME) was subjected to chromatographic analysis. For FAME determination, a gas chromatograph model 3400CX was equipped with a flame ionization detector (Varian, Palo Alto, CA). A microliter of samples was injected into an injector without division, operated in split mode (20:1) at 250 °C. Hydrogen was used as a carrier gas at a constant pressure of 30 psi. The FAMES were separated using an HP-88 chromatography column (100 m × 0.25 mm × 0.20 µm thick film, Agilent, J & W, Folson, CA, USA). The initial oven temperature was programmed at 50 °C for 1 min and increased to 185 °C, at a rate of 15 °C/min. Then, increasing to 195 °C, at a rate of 0.5 °C/min, and finally up to 230 °C, increasing by 15 °C/min, and maintained for 5 min at isothermal. The detector temperature was kept constant at 250 °C. FAME compounds were identified by comparing the experimental retention time with those of the authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO). The results were presented as a percentage of each FA identified in the lipid fraction, considering the equivalent factor of the chain size from FAME to FID and the conversion factor of the ester in the respective acid, according to Visentainer and Franco (2006).

### **2.2 Preparation and characterization of the Nanostructured Lipid Carrier**

The NLC containing the Tucumã oil and butter was prepared using a method developed by pre-formulation tests using Ultra Turrax® equipment and characterized according to pH, polydispersion index (PDI), size and zeta potential. The diameter and PDI determinations of the nanoparticles were carried out through

dynamic light scattering; the zeta potential was measured using electrophoresis (Zetasizer® nano-Zs model ZEN 3600, Malvern), the determination of the pH of the nanoparticles was performed in pH-meter. The formulation was prepared at  $n = 3$  and maintained under various temperature and storage conditions (refrigerator at 28°C, oven at 40°C, room temperature with exposure to light and room temperature in a dark place) over 60 days.

## **2.3 Biocompatibility Parameters**

### **2.3.1 Hemolytic activity**

The hemolytic assay was determined according to Souza Filho et al. (2019) with modifications. Blood was added with 1x PBS solution (1:1 v/v) and centrifuged for 15 minutes at 168g. The supernatants were discarded, and this procedure was repeated three times. Subsequently, in microtubes containing 1mL of 1x PBS at different pHs (pH 7.2 simulating cases of sepsis due to metabolic acidosis; pH 7.4 simulating the normal pH of the organism and pH 7.5 simulating alkalemia), 50µL of red blood cells were added washed and 10µL were taken. As controls, we used the following: negative control (NC) (erythrocytes + 0.9% sodium chloride); positive control (PC) (erythrocytes + distilled water); surfactants (TS) (erythrocytes + mixture of surfactants, Tween® 60 + Spam 60®, in the same concentrations as treatments with NLC). The tubes were incubated at 37 °C at room temperature, under rotation, for 1 hour. After the microtubes were centrifuged for 15 minutes at 168g, 200µL of the supernatants were transferred to 96-well plates and were read in the ELISA reader at 540nm. The results were expressed as a percentage of the positive control.

### **2.3.2 Coagulation test**

This assay was performed according to Souza Filho et al. (2019). Whole blood was collected in citrate tubes and centrifuged for 10 minutes at 1050g. Then, 225 µL of plasma were separated into wells along with 25 µL of NLC treatments and incubated at 37 °C for 30 minutes. Two independent experiments were carried out in duplicate with different donors. Subsequently, a properly calibrated Quick Timer II (Drake) coagulometer was read, according to the manufacturer's recommendations for tests for TP hemostasis (Labtest - lot: 4008) and TTPa hemostasis (Labtest - lot: 4006). As a reference we used the interval between 25 and 35 seconds for the normal value of TTPa, while the baseline values of TP used the interval between 11 and 15 seconds, according to authors (SALVADOR-MORALES et al., 2009; ADAMSON et al., 1993).

## **2.4 Cytotoxicity evaluation**

### **2.4.1 Cell culture and treatments**

Peripheral blood mononuclear cells (PBMCs) derived from whole blood samples discarded from healthy adults were obtained from the Clinical Analysis Laboratory of the Franciscan University (LEAC-UFN) (experimental protocol approved by the UFN Ethics Committee on Human Beings) (CAAE número: 31211214.4.0000.5306) with absence of identification data.

Blood samples were processed for PBMC separation using procedure based on the difference in density gradient using the Ficoll Histopaque-1077VR reagent (Sigma-Aldrich). After the blood was disposed of in the reagent (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) containing 10% fetal

bovine serum and supplemented with 1% antibiotics. The cells were grown at  $2 \times 10^5$  cells  $\text{ml}^{-1}$  per well (BOTTON et al. 2015). Then, the cells were exposed to each compound tested for the efficacy protocol described in this research, in the same concentrations for 24 h, to evaluate its effect on cell modulation through different colorimetric and fluorometric assays. All treatments and trials were carried out in at least triplicate to ensure coherent statistical analysis; hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) at 200  $\mu\text{M}$  was used as a positive control for all tests.

#### 2.4.2 Cell viability measurements

After the treatment period, cell viability was assessed. The first assay performed was MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoline bromide) using the protocol according to Mossman (1983), when completing the incubation time of the cells with the treatment were 20  $\mu\text{L}$  of the MTT solution (0.01M and pH 7.4) was added at a concentration of 5mg/mL 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%  $\text{CO}_2$ , which was homogenized and incubated for 2 h. After incubation, the supernatants were removed from the wells and the cells resuspended in 150  $\mu\text{L}$  of dimethyl sulfoxide (DMSO). Absorbance was determined in an ELISA reader at 560nm.

The determination of cell viability was complemented by the quantification of free DNA in the medium using the DNA-PicoGreen® reagent (Invitrogen, Life Technologies). DNA-PicoGreen® reagent was incubated for 5 min together with the sample dark 96-well plates, and fluorescence reading was performed on the spectrofluorometer at 480nm (excitation) and 520nm (emission) (SAGRILLO et al., 2015).

#### 2.4.3 Quantification of total levels of reactive oxygen species

The total ROS levels were measured by fluorescence according to the technique described by Esposti (2002) using wavelengths of 488 nm (excitation) and 525 nm (emission). The results were expressed as% of the negative control.

#### 2.4.4 Determination of nitric oxide (NO) levels

NO levels were measured according to the technique described by Choi et al. (2012). For this, 50  $\mu\text{L}$  of cell culture supernatant and 50  $\mu\text{L}$  of Griess reagent were added to 96-well plates. The plates were maintained at room temperature for 15 min and the reading was performed spectrophotometrically at 540nm. The results were expressed in percentages in relation to the negative control.

#### 2.5 Evaluation of the antimicrobial activity of the Nanostructured Lipid Carrier

The sensitivity tests for determining the minimum inhibitory concentration (MIC) of the different microorganisms tested were determined using the broth microdilution method according to the protocol M07-A8 approved by the *Clinical and Laboratory Standards Institute* (CLSI).

The bacterial strains of *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus mutans*, *Enterococcus faecalis* and methicillin-resistant *Staphylococcus aureus* (MRSA) used in this study are clinical isolates from inmates at the Santa Maria University Hospital who were previously identified by gender-specific phenotypic



methods. These microorganisms are part of the collection of strains of the Microbiology Laboratory of the Franciscan University, Santa Maria - RS. The strains of *Aeromonas hydrophila* and *Pseudomonas aeruginosa* were provided by the Microbiology and Pathology Laboratory of the Federal University of Santa Maria - UFSM, Santa Maria - RS, Brazil.

MIC was determined to assess the NLC's antimicrobial activity against the microorganisms tested. In this sense, the bacterial samples were cultured on Mueller–Hinton agar (MH), and the colonies were inoculated in 5 mL of sterile saline, the absorbance was controlled until a transmittance of 0.5 was obtained on the MacFarland scale ( $1 \times 10^6$  to  $5 \times 10^6$  cells per mL), then a 1:10 dilution in MH broth was performed resulting in a concentration of  $10^4$  cells per mL.

After inoculum preparation, MIC was performed in 96-well polystyrene plates. The tests were performed in triplicate where, first, 100  $\mu$ L of MH broth were distributed in each well. A 100  $\mu$ L aliquot of the NLC solution containing Tucumã butter and oil was added to the first well and, after homogenization, transferred to the second, and so on until the twelfth well. Once the serial dilutions were made, the following dilutions were obtained: 25000, 12500, 6250, 3125, 1562.5, 781.25, 390.6, 195.3, 97.65, 48.8, 24.41, and 12.2  $\mu$ g/mL. Soon after, the microorganisms were inoculated, where 10  $\mu$ L of the standardized inoculum was then added to each well of the microdilution plate. After pipetting, the plates were incubated at 37 °C for 48 h. The plates were read by adding 20  $\mu$ L of a 1% solution of the 2,3,5-triphenyl tetrazolium chloride dye (Vetec®), in order to assist in the MIC development. As a negative control (-) three wells were used with the MH broth and, as a positive control (+), three wells were used with the MH broth and the bacterial inoculum and as a surfactant control (TS) three wells with a mixture of surfactants, Tween® 60 + Spam 60®, in the same concentrations as NLC treatments.

## **2.6 Interference of the Nanostructured Lipid Carrier in the formation of biofilm**

To assess the interference capacity of the NLC on the biofilm in formation of *K. pneumoniae*, *E.coli*, *P. aeruginosa*, *S.aureus*, *S. epidermidis*, *S.agalactiae*, methicillin-resistant *S.aureus* (MRSA) and *A.hydrophila*, one amount of each bacterial isolate was cultured in sterile petri dishes with MH Agar and incubated at 37°C for 24 h. Then the colonies were suspended in sterile 0.85% saline to prepare the inoculum. Cell density was adjusted in a spectrophotometer in order to obtain a transmittance equivalent to the 0.5 tube on the McFarland scale ( $1 \times 10^6$  a  $5 \times 10^6$  cells/mL) at a wavelength of 630nm.

In 96-well plates of sterile polystyrene, 90  $\mu$ L of brain–heart infusion (BHI) broth supplemented with 1% glucose was added and then 20  $\mu$ L of the inoculum of each microorganism in each well and 90  $\mu$ L of the NLC solution were dispensed to test the antibiofilm activity of the nanoparticles. For the negative control, only broth was added, and for the positive control, the culture medium plus the solution containing the microorganisms was added. For the surfactant control (TS), the medium and the mixture of surfactants, Tween® 60 + Spam, was added. 60®, in the same concentrations as NLC treatments. After pipetting, the plates were incubated at 37°C for 48 h. After this period, the samples were washed with 200  $\mu$ L of distilled water, three times. Then, the biofilm was fixed with 150  $\mu$ L of methane for 20min, after the plates were emptied and the wells were stained with 150  $\mu$ L of gentian violet for 15min and again washed with 200  $\mu$ L of distilled water. Subsequently, 150  $\mu$ L of 95% ethanol was added to dilute the crystals. Finally, the OD (optical density) was determined in a microplate reader, measured at 570nm (STEPANOVI et al. 2007).

## 2.7 Statistical analysis

The results of hemolysis and cytotoxicity were presented as a percentage of the untreated control group (negative control). The analyses 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).

The OD readings obtained in the biofilm formation assay were recorded as mean  $\pm$  standard deviation (SD) and bilateral one-way ANOVA followed by Dunnett's post hoc test. Values with  $p < 0.05$  were considered statistically significant. The graphs were prepared using GraphPadPrism version 5.01 (GraphPad Software, La Jolla, CA, USA).

## 3. Results

### 3.1 Characterization of Tucumã butter

The determination of the concentration of fatty acids is shown in Table 1. The analysis of Tucumã butter by gas chromatography identified 12 fatty acids, the majority of which was lauric acid (41.95 mg/g), myristic acid (28.08 mg/g) and oleic acid (10.07 mg/g).

Table 1. Determination of fatty acid composition in tucumã butter (*Astrocaryum vulgare*) by gas chromatography.

Fattyacid	mg of fatty acid/g of tucumã butter
Ácido capróico (C6:0)	0,07
Ácido caprílico (C8:0)	1,77
Ácido cáprico (C10:0)	1,89
Ácido undecanóico (C11:0)	0,07
Ácido láurico (C12:0)	41,94
Ácido isomerístico (C13:0)	0,10
Ácido mirístico (C14:0)	28,3
Ácido palmítico (C16:0)	9,03
Ácido esteárico (C18:0)	2,55
Ácido oléico (C18:1cis)	10,07
Ácido linoléico (C18:2cis)	2,64
Ácido $\gamma$ -linolênico (C18:3n6)	0,03

Construção do autor.

### 3.2 Preparation and characterization of the Nanostructured Lipid Carrier

The prepared formulation had a monodispersed distribution of particles showing PDI value of  $0.256 \pm 2$ , average size of  $170.7 \pm 3$  nm, zeta potential of  $-18.2 \pm 2$  and pH of 5.22–5.35. When evaluating the NLC formulations under various conditions of temperature and storage, there were no significant changes in the stability of the formulations over 60 days in any of the conditions of storage and temperature that they were

exposed to. The formulations stored in the refrigerator at  $-4^{\circ}\text{C}$ , showed a PDI value of  $0.252 \pm 2$ , average size of  $168.9 \pm 3\text{nm}$ , zeta potential of  $-18.8 \pm 2$  and pH of  $5.32\text{--}5.55$ . The formulations stored in an oven at  $40^{\circ}\text{C}$  showed a PDI value of  $0.255 \pm 2$ , an average size of  $165.9 \pm 3\text{nm}$ , zeta potential of  $-18.6 \pm 2$  and pH of  $5.42\text{--}5.53$ . When stored at room temperature in a dark environment, the formulations showed a PDI value of  $0.257 \pm 2$ , average size of  $171.3 \pm 3\text{nm}$ , zeta potential of  $-18.4 \pm 2$  and pH of  $5.37\text{--}5.43$ . In a clear environment, PDI value was  $0.254 \pm 2$ , average size was  $171.8 \pm 3\text{nm}$ , zeta potential was  $-18.0 \pm 2$  and pH was  $5.39\text{--}5.45$ . The formulations maintained in a clear environment and at room temperature underwent a color change.

### 3.3 Biocompatibility Parameters

#### 3.3.1 Hemolytic activity

Hemolytic activity was significantly higher in the treatment with  $12500 \mu\text{g/mL}$  NLC with pH 7.2 (acidosis) compared to the NC group, while it was significantly higher in the treatment with  $25000 \mu\text{g/mL}$  NLC with pH 7.4 (physiological) compared to the NC group. There was no significant difference between groups treated with NLC in relation to hemolytic activity at pH 7.5 (alkalosis) (Figure 1).

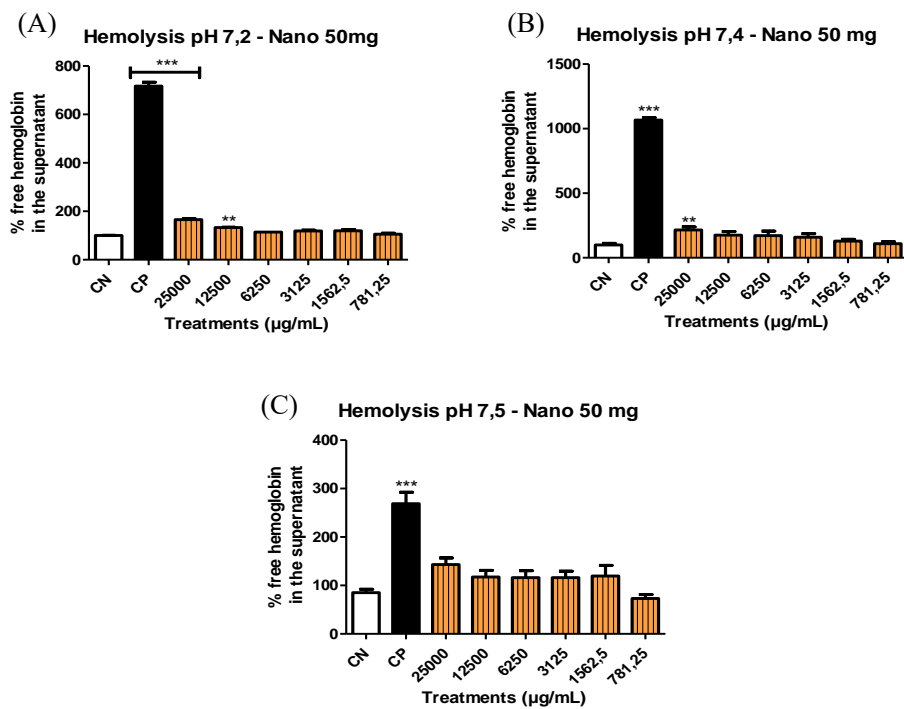


Figure 1. Results of hemolysis at different concentrations of the Nanostructured Lipid Carrier (NLC) at different pHs. (A) Results of hemolysis at pH 7.2 simulating cases of metabolic acidosis. (B) Hemolysis results at pH 7.4 simulating cases of physiological pH. (C) Hemolysis results at pH 7.5 simulating cases of metabolic alkalosis. Note: Results expressed as a percentage of positive control (100%). Values with  $p < 0.05$  were considered statistically significant.

#### 3.3.2 Coagulation test

The results of the coagulation tests are related to the type and concentration of treatments, as can be seen in Figure 2A (TP) and 2B (TTPa). Where for the TP test there were no results outside the biological range.



In the TTPa test at treatment concentrations of 781.25  $\mu\text{g/mL}$  and 12.2  $\mu\text{g/mL}$ . The results were below the biological range.

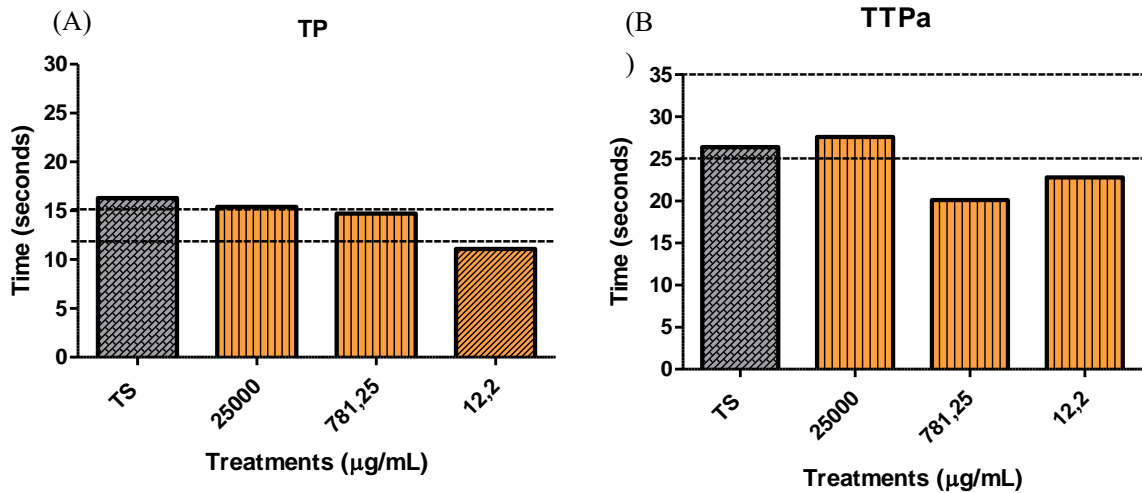


Figure 2. Results of coagulation tests at different concentrations of the Nanostructured Lipid Carrier (NLC). (A) prothrombin time (TP) results. (B) results from partially activated thromboplastin time (TTPa). The dotted limits correspond to the expected physiological times for healthy donors (between 12 and 15 seconds for PT and 25 and 35 seconds for TTPa). Values expressed as mean  $\pm$  standard deviation (SD).

### 3.4 Cytotoxicity evaluation

#### 3.4.1 Cell viability measurements

Cell viability was significantly higher in the treatment with 25000  $\mu\text{g/mL}$  NLC than in the NC group (Figure 3A). Figure 3B shows the results for detecting double-stranded DNA damage in the cell culture supernatant, where no DNA damage was observed in any of the tested concentrations.

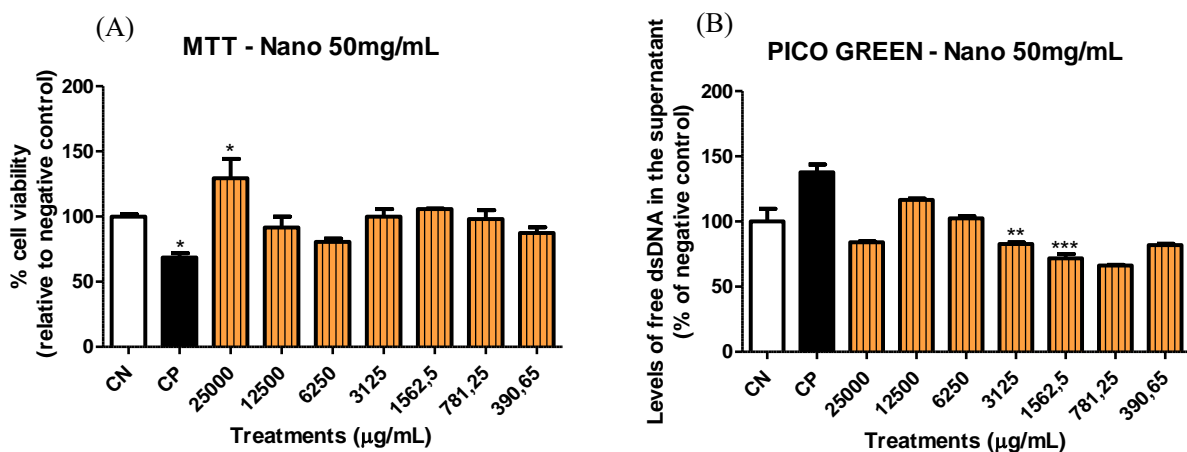


Figure 3. (A) MTT assay (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide) after 24h incubation. (B) Quantification quantity of free dsDNA after 24h of treatment. The data are presented as% of the untreated control group (CN). Values with  $p < 0.05$  were considered statistically significant.

### 3.4.2 Quantification of total levels of reactive oxygen species

Treatments with 6250 µg/mL and 3125 µg/mL NLC showed a significant increase in DCF production compared to the NC group (Figure 4).

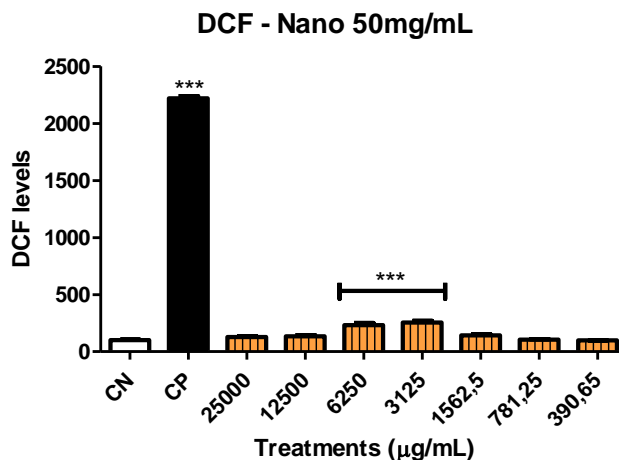


Figure 4. 2', 7'-dichlorofluorescein diacetate (DCF) assay with 24h incubation. The data are presented as% of the untreated control group (CN). Values with p <0.05 were considered statistically significant.

### 3.4.3 Determination of nitric oxide levels

ON levels were significantly higher in the treatment with 25000 µg/mL NLC than in the NC group (Figure 5).

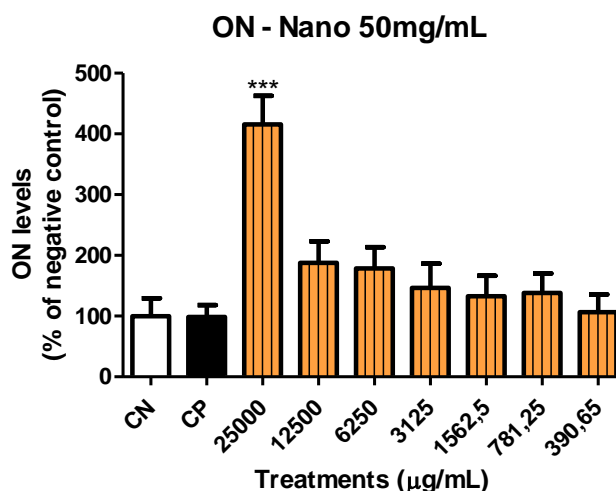


Figure 5. Nitric oxide (NO) assay with 24h incubation. The data are presented as% of the untreated control group (CN). Values with p <0.05 were considered statistically significant.

### 3.5 In vitro evaluation of the antimicrobial activity of the Nanostructured Lipid Carrier

The NLC containing Tucumã oil and butter showed antimicrobial activity in vitro against ten microorganisms of broad clinical importance to humans. The results also indicate that there was a greater antibacterial efficiency for gram-negative bacteria than for gram-positive bacteria (Table 2).

Table 2. Minimum Inhibitory Concentration (MIC) of the Nanostructured Lipid Carrier (CLN) containing Tucumã oil and butter *Astrocaryum vulgare*.

MICROORGANISMS	MIC( $\mu\text{g}/\text{mL}$ )
<i>Staphylococcus aureus</i>	12500
<i>Staphylococcus aureus</i> <i>resistente a Meticilina (MRSA)</i>	12500
<i>Staphylococcusepidermidis</i>	3125
<i>Streptococcus mutans</i>	---
<i>Streptococcus agalactiae</i>	6250
<i>Enterococcusfaecalis</i>	6250
<i>Klebsiellapneumoniae</i>	12500
<i>Escherichia coli</i>	12500
<i>Pseudomonasaeruginosa</i>	25000
<i>Aeromonashydrophila</i>	25000

Construção do autor.

### 3.6 Interference of the Nanostructured Lipid Carrier in the formation of biofilm

The biofilm formation for *A. hydrophila*, *E. coli*, *K. pneumoniae*, *S. agalactiae* and were significantly lower at concentrations of 25,000, 12500 and 6250  $\mu\text{g}/\text{mL}$  NLC compared to that of PC. By contrast, biofilm formation for *S. epidermidis* and *S. aureus* was significantly lower at concentrations of 25000 and 12500  $\mu\text{g}/\text{mL}$  NLC compared to PC (Figure 6).

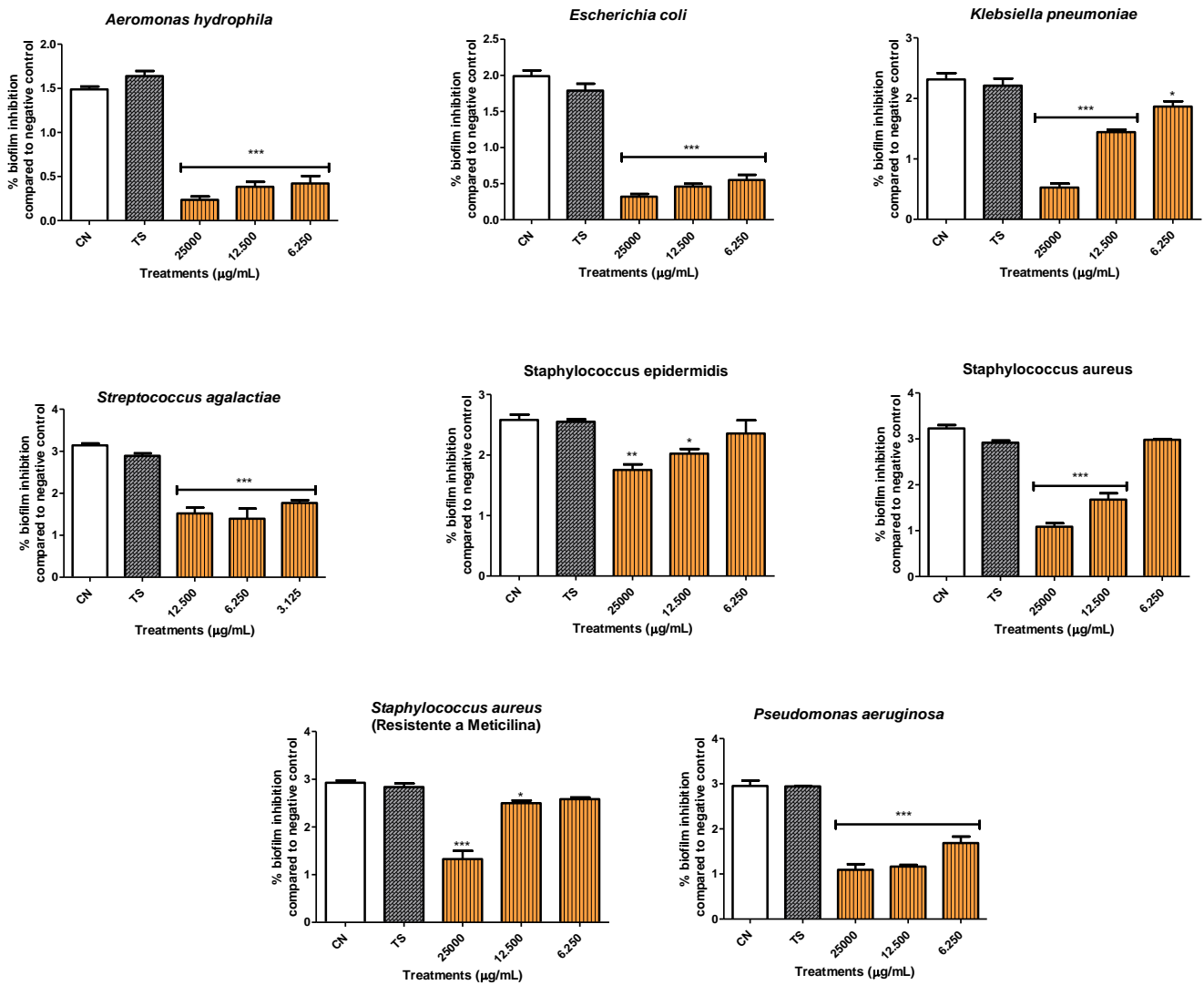


Figure 6. Effect of different concentrations of the Nanostructured Lipid Carrier (NLC) on the biofilm inhibition of different microorganisms. Significant difference between the tested concentration and the positive control (CP). Values with  $p < 0.05$  were considered statistically significant.

#### 4. Discussion

We observed for the first time that a nanoformulation of Tucumã oil in the form of NLC showed potent antimicrobial and anti-biofilm activities against various gram-positive and gram-negative bacteria that are human and animal pathogens. Our findings reveal that this formulation has morphological characteristics and an adequate safety profile, which makes this formulation an alternative to combat these pathogens. A complete characterization of NLC is important, considering the influence of such parameters in terms of biological effects, because the physical-chemical analysis provides important information about the stability of these types of nanoformulation over time (GIORDANI et al., 2014). Particle size is an important attribute of lipid nanocarriers, affecting stability, encapsulation efficiency, drug release profile, biodistribution, mucus adhesion, and cell uptake. A rapid increase in particle size, for example, indicates low stability of the colloidal system (BAHARI and HAMISHEHK, 2016). For a particle to be considered

nanometric, it is necessary that at least one of its dimensions is less than 1000 nm and that this changes its nature properties. Thus, the results found for the sizes of the produced nanoparticles agree with the criteria established in the literature, suggesting adequate stability and better therapeutic efficacy. The results of the characterization of the nanoparticles suggest adequate homogeneity: all formulations must be monodispersed (PDI <0.3) and diameter less than 300 nm. The numerical value of the PDI varies from 0.0 (for a perfectly uniform sample in relation to the particle size) to 1.0 (for a highly polydispersed sample with various particle size populations). Values of 0.2 and below are generally considered acceptable in practice for nanoparticles based on material polymers. In drug delivery applications using lipid-based carriers, such as liposomes and nanoliposomes formulations, a PDI of 0.3 and below is considered acceptable and indicates a homogeneous population of phospholipid vesicles (CHEN et al. 2011). In this study, the polydispersity index was low (<0.3) for all dispersions obtained, indicating the formation of monodispersed systems.

The stability studies of the Nanostructured Lipid Carrier (NLC) showed that it remained stable for the period of 60 days in all established temperature and storage conditions. There were no visible trends in sedimentation, phase separation or aggregation over time. The average particle size, PDI, zeta potential and pH are considered good indicators of stability of suspended nanoparticles (BERNARDI et al., 2011). Considering that the zeta potential reflects the electrostatic repulsion between the particles, and that the standard value of the zeta potential is equal to or greater than +/- 30mV, it is associated with stable solutions, it can be said that the prepared formulation was stable. In addition, high values of polydispersity index indicate heterogeneity in the diameter of the suspended particles; variations in polydispersity values as a function of time indicate the formation of particle populations with diameters that did not initially exist, which may be due to particle aggregation or breaking/degradation (MACHADO et al., 2019). The characterization and stability results obtained demonstrate that the NLC was successfully developed, remaining stable for a substantial period of time in all temperature and storage conditions to which it was exposed. In order for nanoformulations to be used efficiently in technological and biomedical applications, they must be able to remain stable for a long period of time, without losing their properties or structural changes.

The hemolysis assay is useful to determine cytotoxic activity and may be related to direct damage to the erythrocyte cell membrane (ALENCAR et al., 2015). For this reason, we performed this test in three conditions (physiological, acidosis, and alkalosis) to see if NLC were capable of causing damage to erythrocytes. We found that the highest concentrations of NLC (12500 µg/mL in the condition of acidosis; 25000 µg/mL in physiological conditions) caused hemolysis in the erythrocytes, suggesting a possible cytotoxic effect when applied in high concentrations. To the best of our knowledge, there are no reports on the hemolytic effects of NLC; however, that effect can be explained by one of its major compounds, i.e., oleic acid. Hoque et al. (2013) found that high concentrations of oleic acid caused hemolysis in human and goat erythrocytes. According to what was observed in the present study, where only the two highest concentrations of NLC have hemolytic effect, we believe that the high amount of oleic acid present in Tucumã oil and butter may be responsible for the hemolytic action of NLC. Ashokraja et al. (2017) suggested that the observed hemolysis properties of synthesized NPs can be essentially attributed to their size, surface chemistry, and physicochemical properties. Nevertheless, according to these authors, the

hemolytic process involves the denaturation of cells through the physical-chemical interaction between NPs and the cell surface. Red blood cells do not have cellular organelles and the structure of red blood cells is maintained by few membrane proteins, phospholipids and carbohydrates in their cell membrane. In such physiological conditions, red blood cell senescence occurs due to physical-chemical changes in its membrane. More molecules of the NLC are being released at pH 7.2 and 7.4 than the NLC at pH 7.5, as blood pH variation can also lead to hypertonic condition causing hemolysis. Likewise, the main cause of the higher hemolysis properties of the NLC may be due to the interactions of reactive species with the cell surface or due to changes in pH (ASHOKRAJA et al., 2017).

Nevertheless, nanoparticles can have longer systemic circulation times. This prolonged time of circulation in the blood stream increases the duration of contact with these blood components, including the coagulation system, potentially amplifying the activation of the coagulation cascade. The results of the coagulation tests showed normal TP activities, in all concentrations tested with TP values within the biological range, and abnormal TPPa values, below the biological range, in the two lowest concentrations tested, but without statistical significance. According to Zare-Zardini et al. (2018), one of the most important things about coagulation is the idea of the intrinsic and extrinsic arms of the coagulation cascade and the way they interact during coagulation in the body. For a potent application of any compound, an investigation of its effect on blood clotting is necessary. The results demonstrated that the NLC does not interfere in the extrinsic and intrinsic clotting pathways. However, as changes in the TTPa test were observed, we did not rule out interactions with intrinsic coagulation factors such as factors VIII, IX, XI, XII, and pre-kallikrein protein. For these reasons, different methods of analysis must be performed to confirm and better understand our findings.

To investigate the safety profile of this formulation, we performed MTT assays and measured changes in double-stranded DNA. In the MTT assay, no concentration was able to reduce the cellular viability of PBMCs after 24 h of culture, suggesting that this treatment does not have cytotoxic effects for mononuclear cells such as lymphocytes and monocytes. These results agree with those of Sagrillo et al. (2015), who reported that Tucumã fruit extracts did not have cytotoxic effects and reduced the toxic effects of human lymphocytes exposed to hydrogen peroxide. Furthermore, we did not detect the presence of double-stranded DNA damage in any of the tested concentrations.

To identify possible mechanisms of action associated with these few cytotoxic effects of the highest concentrations of NLC, we evaluated the production of ROS and nitric oxide, two important mediators of oxidative and inflammatory damage related to cytotoxic effects (Baldissera et al., 2017). According to these authors, the production of reactive species are among the main causes of DNA damage. These species react with various DNA components to produce DNA damage, modifying bases, inducing interconnection between chains and intra-chains, and promoting chromosomal abnormalities. In the present study, a significant increase in the production of ROS (6250 and 3125  $\mu\text{g}/\text{mL}$ ) and NO (25000 $\mu\text{g}/\text{mL}$ ) was observed compared to the negative control, suggesting that the production of ROS and NO may be involved in these cytotoxic effects found in red blood cells and PBMC exposed to NLC for 24 h. Although it has caused some negative effects, a recent study by Baldissera et al. (2017) found that Tucumã oil reduced oxidative damage in the brain of diabetic mice, which reveals its potent antioxidant action. On the other hand,  $\beta$ -carotene, another main constituent of Tucumã oil and butter prevented cell damage and decreased levels of



DCF (BESTWICK and MILNE, 1999). Another important constituent of both oil and Tucumã butter is tocopherol, an excellent inhibitor of lipid peroxidation, which acts as a hydrogen donor for the peroxy radical, interrupting the radical chain reaction (BARREIROS and DAVDI, 2006). It is important to note that the cytotoxic effects of NLC may be due to its constituents, because the literature reports the absence of toxic effects of Tucumã oil.

The results related to antimicrobial activity are important, because these microorganisms isolated in hospital environments can colonize and adhere to the surfaces of medical instruments and implants. Because they are already resistant to various drugs, the ability to adhere can effectively reduce antimicrobial options and can further worsen infections. The main reason why NPs are being considered as an alternative to antimicrobials is that they can effectively prevent microbial resistance to drugs in certain cases. The rampant use of antibiotics has led to the emergence of several risks to public health, including the emergence of superbugs that do not respond to any antibiotic and an existing epidemic against which the medication has no defense. The search for new effective antibacterial materials is significant for combating drug resistance, and NPs are being established as a promising approach to solving this problem (WANG et al., 2017). Rossato et al. (2019) showed that Tucumã-free oil showed antimicrobial activity against 5 microorganisms. *A. vulgare* oil is rich in lipids, carotenes, fibers and tocopherol, providing the fruit with high energy and nutritive content. Probably, the antimicrobial effect of Tucumã oil may be associated with its chemical composition, which includes several types of polyphenol molecules and fatty acids. Still, according to Rossato et al. (2019) polyphenols are secondary metabolites produced by higher plants, which play several essential roles in the physiology of plants that have potential healthy properties in the human body, mainly as antioxidants, anti-inflammatories, anti-allergens, and antimicrobials. This suggests that low uptake of free oil may limit its effectiveness, and that lipid encapsulation can help the active ingredients achieve their goals. Piran et al. (2017) demonstrated that NLC's increase the retention of a drug in the particle increased the release time and reduced the amount of drug needed to produce therapeutic action. According to these authors, this proficiency can be used to control the release of essential oils to improve the efficiency and reduce the quantity and toxicity of the essential oils used. By controlling the release of the drug molecule, the nanocarriers also protect essential oils against possible thermal or photoelectric degradation, oxidation or evaporation, which guarantees greater stability, flavor and function, consequently prolonging the useful life of the final product. According to Hu and Kwon (2011) most types of NP can overcome at least one of the common resistance mechanisms of microorganisms (including the interruption of bacterial membranes and the prevention of biofilm formation). These effects are the result of the bactericidal mode of NPs, which is based on their specific physicochemical properties. Unlike traditional antibiotics, NPs have dimensions <100 nm. The exceptionally small size results in properties such as greater interaction with the cells due to greater area-to-surface ratios and versatile and controllable application. According to Mühling et al. (2009), NPs cross the bacterial membrane and gather along the metabolic pathway, influencing the shape and function of the cell membrane. Then, NPs interact with the basic components of the bacterial cell, including DNA, lysosomes, ribosomes, and enzymes, leading to oxidative stress, disturbances of the electrolyte balance, inhibition of enzymes, heterogeneous changes, changes in the permeability of the cell membrane, deactivation of proteins, and changes in expression genes, all of which can be considered possible

mechanisms of action of nanoparticles against bacteria. According to Karimi et al. (2018) NLC systems allow the transfer of antibacterial extracts through the cell membrane layer. They act as carrier agents, bringing concentrations of compounds within the aqueous phase to microorganisms. According to these authors, there are "holes" in the cell membranes of the bacteria that act as component transfer areas, and smaller nanoparticles can be more easily inserted into the cavities and release their bioactive compounds, also contributing to the improvement of the antimicrobial activity of the nanoformulations. Finally, Mekarizadeh et al. (2017) found that lipid-based nanocarriers can protect essential oils against thermal or photoelectric degradation, increasing product stability and consequently, prolonging the life of the final product, in addition to not affecting the appearance, texture, or the taste of the product. The use of such delivery systems can increase the concentration of antimicrobials in areas in which microorganisms are preferentially located and can potentially increase the passive mechanisms of cell absorption that affect the stability of the lipid membrane and, as a result, increase antibacterial activity.

Comparing the MICs and the concentrations capable of inhibiting bacterial biofilm, we observed that the NLCs containing Tucumã oil and butter inhibited the formation of biofilm in concentrations lower than the MICs in most of the tested microorganisms. This suggests inhibition of biofilm formation of the microorganism in the sessile form (adhered to solid surfaces), not only in the inhibition of bacterial growth in the planktonic form (microorganisms in suspension and dispersed in the aqueous medium). Size of a NP can greatly affect its antibacterial activity. In addition to the rupture of bacterial membranes, the prevention of biofilm formation is an important mechanism because biofilms play fundamental roles in the development of bacterial resistance. The unique structure and composition of bacterial biofilms provides shelter or protection for embedded microorganisms, helping them to protect themselves from most antibiotics. In addition, bacterial biofilms are "a breeding ground" for frequent resistance mutations and the exchange and alteration of these mutations between bacterial cells. Studies have shown that many NPs can prevent or overcome biofilm formation (PEULEN and WILKINSON, 2011). Most bacteria exist in the form of biofilms, usually containing several species that interact with each other and with the environment. Biofilms are specifically microbial aggregates that depend on a solid surface and extracellular products, including extracellular polymeric substances (EPSs) (WANG et al., 2017). A study by Alalaiwe et al. (2019) using the biofilm, demonstrated that oxacillin-loaded nanoparticles penetrated the EPS, eradicating the MRSA biofilm more effectively than the individual treatment. According to these authors, extracellular DNA plays a fundamental role in the production of biofilm, acting as a chelator of cationic molecules. The interaction between EPS and nanoparticles with lipids can cause strong affinity and disintegration of the biofilm.

## **5. Conclusion**

The Tucumã nanostructured lipid carrier was successfully developed by the homogenization method using Ultra Turrax. It remained stable for a considerable period of time. Cytotoxicity studies demonstrated that the nanoparticle suspension is safe because no concentration reduced cellular viability of PBMCs or caused double-stranded DNA damage, in addition to having low NLC hemolytic activity against human erythrocytes in most concentrations tested and few toxic effects at high concentrations. The NLC-

containing Tucumã oil and butter showed antimicrobial and antibiofilm activity against important agents that cause human morbidity and mortality. Our findings suggest that this treatment, in nanostructured form, may be a potential alternative therapeutic agent for the elimination of bacteria and biofilms.

## 6. Acknowledgement

The research was not funded.

## 7. References

- A. Alalaiwe, P.W. Wang, L.P. Lu, P.Y. Chen, Y.J. Fang, and C.S. Yang. Synergistic Anti-MRSA Activity of Cationic Nanostructured Lipid Carriers in Combination With Oxacillin for Cutaneous Application, *Frontiers in Microbiology*. 2018; 9:1-14. <https://doi.org/10.3389/fmicb.2018.01493>
- B.D. Alencar, A.A. Melo, C.G. Silva, L.R. Lima, S.M.K. Pires-Cavalcante, F.R. Carneiro, S.A. Rabelo, V.O. Souza, F.S.H.R. Vieira, A.F. Viana, H.A. Sampaio, and S.S. Sampaio. Antioxidant, hemolytic, antimicrobial, and cytotoxic activities of the tropical Atlantic marine zoanthid *Palythoacaribaeorum*. *Annals of the Brazilian Academy of Sciences*. 2015; 87(2):1113-1123. <https://doi.org/10.1590/0001-3765201520140370>
- T.P. Amadeu, B.A. Seabra, M.G. de Oliveira, and A. Monte-Alto-Costa. Nitric oxide donor improves healing if applied on inflammatory and proliferative phase. *Journal of Surgical Research*. 2008; 149(1):84 – 93.
- C. Ashokraja, M. Sakar, and S. Balakumar. A perspective on the hemolytic activity of chemical and green-synthesized silver and silver oxide Nanoparticles. *Materials Research Express*. 2017; 4(10):1-25. <https://doi.org/10.1088/2053-1591/aa90f2>
- S.A.L. Bahari, and H. Hamishehkar. The Impact of Variables on Particle Size of Solid Lipid Nanoparticles and Nanostructured Lipid Carriers; A Comparative Literature Review. *Advanced Pharmaceutical Bulletin*. 2016; 6(2):143-151.
- D.M. Baldissera, F.C. Souza, H.T. Grando, F.L. Cossetin, R.M. Sagrillo, K. Nascimento, S.A. da Silva, K.A. Machado, M.B.I. da Cruz, M.L. Stefani, B. Klein, G.S. Monteiro, Antihyperglycemic, antioxidant activities of tucumã oil (*Astrocaryum vulgare*) in alloxan-induced diabetic mice, and identification of fatty acid profile by gas chromatograph: New natural source to treat hyperglycemia. *Chemico – Biological Interactions*. 2017; 270:51-58. <https://doi.org/10.1016/j.cbi.2017.04.001>
- S.B.I.A. Barreiros, and M.J. David. Estresse oxidativo: entre culturas de espécies reativas e defesa do organismo. *Química Nova*. 2006; 29(1):113 – 123.
- G. Barshtein, D. Arbell, and S. Yedgar. Hemolytic effect of polymeric nanoparticles: role of albumin. *IEEE Transactions on nanobioscience*. 2011; 10(4):259-261.
- S.D. BERNARDI, A.T. Pereira, R.N. Maciel, J. Bertoloto, S.G. Vieira, C.G. Oliveira, and A.P. Rocha-Filho. et al. Formation and stability of oil-in-water nanoemulsions containing rice bran oil: in vitro and in vivo assessments. *Journal of Nanobiotechnology*. 2011; 9(44):1-9. <https://doi.org/10.1186/1477-3155-9-44>
- S.C. Bestwick, and L. Milne. Effects of  $\beta$ -carotene on antioxidant enzyme activity, intracellular reactive

- oxygen and membrane integrity within post confluent Caco-2 intestinal cells. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 1999; 1474(1):47 – 55.
- E. Bony, F. Boudard, P. Brat, E. Dussossoy, K. Portet, P. Poucheret, J. Giaimis, and A. Michel. Awara (*Astrocaryum vulgare* M.) pulp oil: Chemical characterization, and anti-inflammatory properties in a mice model of endotoxic shock and a rat model of pulmonary inflammation. 2012; 83(1):33-43. <https://doi.org/10.1016/j.fitote.2011.09.007>
- G. Botton, C.F. Cadoná, A.K. Machado, F.V. Azzolin, I.B.M. da Cruz, M.R. Sagrillo, and J.R. Praetzel. Induction of cytotoxicity, oxidative stress, and genotoxicity by root filling pastes used in primary teeth. *International Endodontic Journal*. 2015; 49(8):737–745.
- S.N. Bryan, and B.M. Grisham. Methods to detect nitric oxide and its metabolites in biological samples. *Free Radical Biology and Medicine*. 2007; 43(5):645-657.
- A.P.C.P. Carlotti. Abordagem clínica dos distúrbios do equilíbrio ácido-base. *Medicina (Ribeirao Preto Online)*. *Revista USP*. 2012; 45(2).
- CDC - Centers for Disease Control and Prevention. <https://www.cdc.gov/drugresistance/index.html> (2019), Accessed 30th March 2020.
- M. Chen, X. Liu, and A. Fahr. Skin penetration and deposition of carboxyfluorescein and temoporfin from different lipid vesicular systems: In vitro study with finite and infinite dosage application. *International Journal of Pharmaceutics*. 2011; 408(1-2):223- 234. <https://doi.org/10.1016/j.ijpharm.2011.02.006>
- W.S. Choi, G.P. Shin, H.J. Lee, and D.G. Kim. The regulatory effect of veratric acid on NO production in LPS-stimulated RAW264.7 macrophage cells. *Cellular Immunology*. 2012; 280(2):164–170. <https://doi.org/10.1016/j.cellimm.2012.12.007>
- CLSI- CLINICAL LABORATORY AND STANDARDS INSTITUTE. Reference method for broth dilution antifungal susceptibility testing yeasts 3<sup>rd</sup> ed. Approved standard M27-A3. Clinical Laboratory and Standards Institute. 2008. Wayne, PA.
- M.D. Esposti. Measuring mitochondrial reactive oxygen species. *Methods*. 2002; 26(4):335–340.
- V. Fencl, A. Jabor, A. Kazda, and J. Figge. Diagnosis of metabolic acid-base disturbances in critically ill patients. *American Journal of Respiratory and Critical care Medicine*. 2000; 162(6):2246-2251.
- B.N. Freire, B. Naiana, L.C.S.R. Pires, H.P. Oliveira, and M.M. Costa. Atividade Antimicrobiana e Antibiofilme de Nanopartículas de Prata sobre Isolados de *Aeromonas spp.* obtidos de Organismos Aquáticos. *Pesquisa Veterinária Brasileira*. 2018; 38(2):244-249. <http://dx.doi.org/10.1590/1678-5150-pvb-4805>.
- P. Hovorkova, K. Lalouckova, and E. Skrivanová. Determination of in vitro antibacterial activity of plant oils containing medium-chain fatty acids against Gram-positive pathogenic and gut commensal bacteria. *Czech Journal of Animal Science*. 2018(3):119-125.
- A.J. Huh, and Y.J. Kwon. “Nanoantibiotics”: A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *Journal of Controlled Release*. 2011; 156(2):128-145.
- L.M. JOBIM, V.C.R. Santos; S.F.C. Alves, M.R. Oliveira, P.C. Mostardeiro, R.M. Sagrillo, C.O de Souza Filho, M.F.L Garcia, F.M. Manica-Cattani, E.E. Ribeiro, and M.B.I da Cruz. Antimicrobial activity of Amazon *Astrocaryum maculeatum* extracts and its association to oxidative metabolism. *Microbiological Research*. 2014; 169(4):314-323.

- K. R. Joshi. Role of natural products against Microorganisms. American Journal of Clinical Microbiology and Antimicrobial. 2018; 1(1).
- N. KARIMI, B. Ghanbarzadeh, H. Hamishehkar, B. Mehramuz, and H.S. Kafil. Antioxidant, Antimicrobial and Physicochemical Properties of Turmeric Extract-Loaded Nanostructured Lipid Carrier (CNL). [Colloid and Interface Science Communications](#). 2018; 22:18–24.
- M.L.W. Knetsch, and L.H. Koole. New strategies in the development of antimicrobial coatings: the example of increasing usage of silver and silver nanoparticles. *Polymers Basel*. 2011; 3:340–366.
- C. Li, R. Fu, C. Yu, Z. Li, H. Guan, D. Hu, D. Zhao, and L. Lu. Silver nanoparticle/chitosan oligosaccharide/poly(vinyl alcohol) nanofibers as wound dressings: a preclinical study. [International Journal of Nanomedicine](#). 2013; 8:4131-4145.
- L.S.Q. LOPES, A.R. Vaucher, L.J. Giongo, A. Gundel, and V.C.R. Santos. Characterisation and anti-biofilm activity of glycerol monolaurate nanocapsules against *Pseudomonas aeruginosa*. *Microbial Pathogenesis*. 2019; 130:178-185. <https://doi.org/10.1016/j.micpath.2019.03.007>
- P.T.G. Machado, B.M. Valeirinho, L. Mazzarino, P.C.L Machado Filho, M. Maraschin, A.L.R. Cerri, and S. Kuhnen. Development of propolis Nanoparticles for the treatment of bovine mastitis: in vitro studies on antimicrobial and cytotoxic activities. *Canadian Journal of Animal Science*. 2019; 99(4):713-723.
- S. Makpol, A. Zainuddin, A.N. Rahim, M.A.Y. Yusof, and W.Z.W. Ngah. Alpha-tocopherol modulates hydrogen peroxide-induced DNA damage and telomere shortening of human skin fibroblasts derived from differently aged individuals. *Planta Médica*. 2010; 76(9):869 – 875.
- M. Mekarizadeh, S.H. Kafil, S. Ghanbarzadeh, A. Alizadeh, and H. Hamishehkar. Improvement of citral antimicrobial activity by incorporation into nanostructured lipid carriers: a potential application in food stuffs as a natural preservative. *Research in Pharmaceutical Sciences*. 2017; 12(5):409-415.
- M. Moreno-Sastre, M. Pastor, A. Esquisabel, E. Sans, M. Vinas, A. Fleischer, E. Polomino, D. Bachiller, and L.J. Pedraz. Pulmonary delivery of tobramycin-loaded nanostructured lipid carriers for *Pseudomonas aeruginosa* infections associated with cystic fibrosis. *International Journal of Pharmaceutics*. 2016; 498(1-2):263-273.
- T. Mosmann. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 1983; 65(1-2):55–63.
- D. Mozaffarian, A. Ascherio, B.F. Hu, J.M. Stamfer, C.W. Willet, S.D. Siscovick, and B.E. Rimm. Interplay between different polyunsaturated fatty acids and risk of coronary heart disease in men. *Circulation*. 2005; 111(2):157-164.
- M. Mühlhling, A. Bradford, W.J. Readman, J.P. Somerfield, and D.R. Handy. An investigation into the effects of silver nanoparticles on antibiotic resistance of naturally occurring bacteria in an estuarine sediment. [Marine Environmental Research](#). 2009; 68(5):278-283.
- A.M. Partearroyo, A.M. Urbanja, and M.F. Goni. Effective detergent/lipid ratios in the solubilization of phosphatidylcholine vesicles by Triton X-100. *FEBS Letters*. 1992; 302(2):138-140. [https://doi.org/10.1016/0014-5793\(92\)80424-F](https://doi.org/10.1016/0014-5793(92)80424-F)
- T.O. Peulen, and K.J. Wilkinson. Diffusion of nanoparticles in a biofilm. *Environmental Science e Technology*. 2011; 45(8):3367-3373.



P. Piran, S.H Kafil, and H. Hamishehkar. Formulation of Menthol-Loaded Nanostructured Lipid Carriers to Enhance Its Antimicrobial Activity for Food Preservation. *Advanced Pharmaceutical Bulletin*. 2017; 7(2):261-268.

A. Porse, J.L. Jahn, H.M.M. Ellabaan, and A.O.M. Sommer. Dominant resistance and negative epistasis can limit the co-selection of de novo resistance mutations and antibioticresistance genes. *Nature Communications*. 2020; 11(1199).

M.D. Rizk, M.B. Witte, and A. Barbul. Nitric oxide and wound healing. *World Journal Surgery*. 2004; 28:301-306.

A. Rossato, S.L. Silveira, S.Q.L. Lopes, P.W. de Souza Filho, F.L. Schaffer, V.C.R. Santos, and R.M. Sagrillo. Evaluation in vitro of antimicrobial activity of tucumã oil (*Astrocaryum vulgare*). *Archives in Biosciences e Health*. 2019; 1(1):99-112. <https://doi.org/10.18593/abh.19701>

M.R. Sagrillo, M.F.L. Garcia, C.O. de Souza Filho, F.M.M.M. Duarte, E.E. Ribeiro, C.F. Cadoná, and M.B.I da Cruz. Tucumã fruit extracts (*Astrocaryum aculeatum Meyer*) decrease cytotoxic effects of hydrogen peroxide on human lymphocytes. *Food Chemistry*. 2015; 173:741–748. <https://doi.org/10.1016/j.foodchem.2014.10.067>

S.F. Siqueira, G.G. Rossi, K.A. Machado, S.F.C. Alves, C.V. Flores, D.V. Somavilla, A.V. Agertt, D.J. Siqueira, S.R. Dias, M.P. Copetti, R.M. Sagrillo, F.D. Voltar, and A.M.M. de Campos. Sulfamethoxazole derivatives complexed with metals: a new alternative against biofilms of rapidly growing mycobacteria. *Biofouling*. 2018; 34(8):893-911.

W.P.S. de Souza Filho, S.S. Homrich, M.P. Copetti, S.D. Peres, V.D. de Souza, C.R. Rieffel, K.A. Machado, F.A. Ourique, and R.M. Sagrillo. Effects of nanocapsules containing all-trans-retinoic acid under hemolytic and coagulation activity. *Archives in Biosciences & Health*. 2019; 1(1):125-138.

F.J. Sutilli, M.D. Gatlin, M.B. Heinzmann, and B. Baldisserotto. Plant essential oils as fish diet additives: benefits on fish health and stability in feed. *Reviews in Aquaculture*. 2018; 10(3):716-726. <https://doi.org/10.1111/raq.12197>

A. Schwentker, Y. Vodovotz, R. Weller, and R.T. Billiar. Nitric oxide and wound repair: Role of cytokines? *Nitric Oxide*. 2002; 7(1):1-10. [https://doi.org/10.1016/S1089-8603\(02\)00002-2](https://doi.org/10.1016/S1089-8603(02)00002-2)

S. Stepanovic, D. Vukovic, V. Hola, D.G. Bonaventura, S. Djukic, I. Cirkovic, and F. Ruzicka. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Journal of Pathology, Microbiology and Immunology*. 2007; 115(8):891-899. [https://doi.org/10.1111/j.1600-0463.2007.apm\\_630.x](https://doi.org/10.1111/j.1600-0463.2007.apm_630.x)

B. Tarsillo, and R. Prierer. Proteobiotics as a new antimicrobial therapy. *Microbial Pathogenesis*. 2020; 142. <https://doi.org/10.1016/j.micpath.2020.104093>

### Copyright Disclaimer

Copyright for this article is retained by the author(s), with first publication rights granted to the journal.

This is an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/4.0/>).