

Article - Food/Feed Science and Technology

# Nanoemulsion Improves Babassu Palm Oil (*Orbignya phalerata*) Antioxidant Properties

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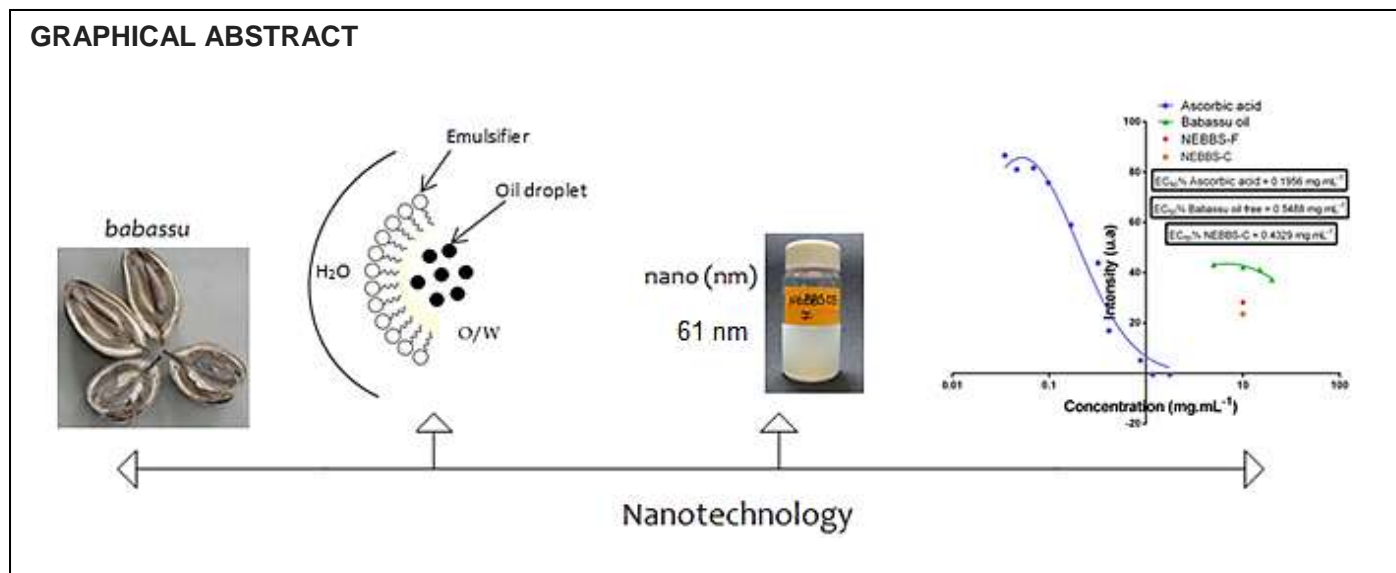
## HIGHLIGHTS

- Production of lipid nanoemulsions (<100 nm) of industrial interest with low energy demand.
- The antioxidant properties of babassu oil have been improved and the nanoemulsions are not cytotoxic.
- Babassu oil is a food and medicinal product.
- The nanoemulsion is strategic for the developed of new antioxidants phytotherapeutics.

**Abstract:** Background: Babassu oil is an extract from a Brazilian native coconut (*Orbignya phalerata* Martius) and is used both as a food and a medicinal product. Methods: we produced two babassu oil nanoemulsions and evaluated them regarding their nanoscopic stability, antioxidant activity and cytotoxicity. The nanoemulsions were characterized by Dynamic Light Scattering, and their stability was investigated for 120 days. The antioxidant activity was assessed by Spectroscopy Electron Paramagnetic Resonance, and the cytotoxicity was assessed by a colorimetric method (MTT) with the NIH/3T3 cell lineage. Results: the results showed nanoemulsions with average hydrodynamic diameter lower than 100 nm ( $p \leq 0.001$ ), and a polydispersity index of less than 0.3 ( $p \leq 0.001$ ), indicating monodisperse systems and good stability at room

temperature. The exposure of nanoemulsions at varying pH revealed that the isoelectric point was at 3.0, and the images obtained by Transmission Electron Microscopy showed spherical droplets with a size 27 nm. The antioxidant activity showed that the babassu nanoemulsions exposed to free radicals had a better response when compared to the oil free samples. The cell viability assays showed low toxicity of the formulation with viability over 92% ( $p \leq 0.05$ ). Conclusion: babassu oil nanoformulations showed low polydispersity and kinetic stability with effective antioxidant action. Therefore, they can be promising for application in the food industry or as antioxidant phytotherapeutics.

**Keywords:** babassu coconut oil; nanotechnology; development; antioxidant potential.



## INTRODUCTION

Babassu palm (*Orbignya phalerata* Martius) is widely present in Latin America. In Brazil, the plant is mainly present in northeastern and mid-western regions, where it has been exploited sustainably for decades [1-6]. The oil extract from its fruit is one of the most important products from the tree due to several biological activities [7-10], such as antioxidant properties [11-12]. Due to these properties, babassu oil is marketed as an important ingredient for different industries, especially for pharmaceutical and food companies in the north and northeast regions of Brazil [13-15]. Babassu oil is a natural source of fatty saturated acids [16-21], composed mainly of capric, caprylic, lauric, myristic, palmitic, oleic, stearic, and linoleic, which are sources of omega-9 [16-21]. These components are responsible for the biological properties of the oil [7-10], but its use in food and beverages has been limited due to its poor water solubility. To overcome this, different types of colloidal systems have been proposed to increase babassu oil dispersibility in aqueous solutions [22-25]. The idea is to entrap the hydrophobic oil components inside small, micro or nanostructured carriers, which are stable and dispersible in aqueous colloidal suspensions.

In addition to the dispersion properties provided by the colloidal systems, these technological solutions are also used to protect the oil components against oxidation. It is known that fatty acids present in natural oil sources are biomolecules that are highly susceptible to oxidation [26]. The idea is that steric separation between the dispersed oily core and the continuous aqueous phase, provided by the colloidal carrier's structure, protects the oil components against chemical attack and degradation.

Different types of colloidal carriers have been used to entrap natural oils and provide the desirable characteristics mentioned above [27]. Among the different technological approaches, our research group has invested in the development of a lipid nanoemulsion containing natural oils as the core matrix. Nanoemulsions, defined as a stable dispersion of lipid nanodroplets stabilized by surfactants in a continuous aqueous phase, were selected due to (1) the highly colloidal stability; (2) the possibility of industrial scale-up production; and (3) the fact that this nanocarrier can improve oral absorption for nutritional applications.

Thus, the objective of this study is to present the development of a lipid nanoemulsion produced with babassu oil using a spontaneous emulsification method. As main result, we highlight that the nanoemulsification process improved the babassu oil's antioxidant properties. In addition, the nanoemulsion

presented stability for at least four months (120 days); and presented slight cytotoxicity activity against mammalian culture cells, indicating that the formulation could be used for in vivo and market applications.

## MATERIAL AND METHODS

### Materials

PEG-35-castor oil and castor oil (Sigma-Aldrich, São Paulo-Brazil) were used as nanoemulsion surfactant and oil matrix respectively. The ingredients were selected based on the Hydrophilic-lipophilic balance (HLB), which was 13 and 14 respectively. Ultrapure water (18 M $\Omega$ .cm) Milli-Q (Millipore, Bedford, USA) was used as the solvent for nanoemulsion production.

Babassu oil was extracted from palms (*O. phalerata* Martius) located in Cocais areas in Maranhão state (Northeast of Brazil), from the following municipalities: Fortuna (FOR) (Latitude: 05°44'00" N and Longitude: 44°09'30" W) and Caxias (CAX) (Latitude: 04°51'32" N and Longitude: 43°21'22" W). These samples were provided by the Laboratory of Macromolecules and Natural Products at the State University of Maranhão.

### Formulation of nanoemulsions (NEBBS)

The babassu oil nanoemulsions (NEBBS) were produced using a spontaneous emulsification method [28]. The protocol was adjusted to physical parameters related to homogenization time, rotational speed and the dispersing solvent. The required amount of surfactant was dissolved in fixed babassu oil, which constituted the oily phase (dispersed phase).

The dispersion solvent used was ultrapure water (18 M $\Omega$ .cm) with sufficient quantity for 10 mL. For nanoemulsion preparation, the oil phase was weighed and homogenized by magnetic stirring for 10 minutes (37°C). After that, an aliquot (5 mL) of aqueous phase was added until the completed dispersion of the oil phase. The oil phase dispersion indicates the formation of the lipid nanodroplet. For the next experiments, two nanoformulations with babassu oil (BBS) were prepared: NEBBS-C (babassu oil from Caxias) and NEBBS-F (babassu oil from Fortuna). The use of two different oil sources aims to evaluate if the geographic localization could interfere with the nano emulsification process. As known, oils extracts composition can vary over the year seasons as well as and the location of their production. The oil phase composition was 1:4:2 (BBS oil: Cremophor: Castor Oil; w/w). After nanoemulsion preparation, the samples were kept protected from light at 4°C. The concentrations of the formulations are contained in the national patent application for Invention, Utility Model, Certificate of Addition of Invention and entry into the national phase of the PCT of the National Institute of Industrial Property - INPI under the Process number: BR 10 2016 017598 4 [29].

### Assay of characterization and stability

Nanoemulsion characterization and stability were evaluated by the (a) Hydrodynamic Diameter and Polydispersity Index, (b) Centrifugation Assays and Cooling/Heating tests, (c) Transmission Electron Microscopy (TEM), (d) Zeta Potential ( $\zeta$ ), pH and (e) Isoelectric Point (pI). These parameters (a, d) were analyzed at different time points (1, 7, 15, 30, 45, 60, 90 and 120 days) after nanoemulsion preparation. The (b) centrifugation test was carried out after 1, 7, 15, 30, 45 and 60 days and Cooling/Heating tests were carried out in 5 cycles.

### Hydrodynamic Diameter (nm) of the Droplets and Polydispersity Index – PDI

The lipid nanodroplets' Hydrodynamic Diameter (nm) was obtained by Dynamic Light Scattering technique (DLS) [30], using the Zetasizer Nano Series equipment (ZEN3690 model, Malvern Instruments, United Kingdom). For measurements, nanoemulsions (NEBBS) were diluted (1:20 v/v) in Milli-Q Ultrapure water (18 M $\Omega$ .cm). The operating conditions were: temperature (25 °C), fixed angle (90°) and equilibration time (60 seconds).

### Centrifugation Test

For the centrifugation test, an Ultra centrifuge (Mikro 220R model, Hettich Zentrifugen, Germany) was used according to the following protocol: 2 mL of NEBBS was placed in a plastic tube, and this was centrifuged at 94, 587, 1,150 x g for 15 minutes at 25°C. The centrifugation test was carried out after 1, 7, 15, 30, 45 and 60 days.

## Cooling and Heating Test

The experimental protocol was adapted from [31]. For the experiments, 2 mL of nanoemulsion (NEBBS) was transferred to eppendorfs tubes, cooled for 24 hours and heated for 24 hours, therefore completing a cooling/heating cycle. After the end of each cycle, in a total of five, the nanodroplet size was determined.

## Transmission Electron Microscopy (TEM)

For the electron microscopy, the Transmission Electron Microscope (JEM-1011 Electron Microscope model, JEOL Ltd., Japan) was used to visualize the nanodroplets' morphology. The nanoemulsion was diluted 1:10 (v/v) in ultrapure water (18 M $\Omega$ .cm) and 3  $\mu$ l was pipetted on to a copper screen covered with Formvar film. For negative staining, the samples were exposed to osmium tetroxide vapor, using a closed chamber, and dried for 24h at room temperature. For morphological analysis, images were taken at 100x magnification with 80 kv. The quantitative modal size of nanoemulsion analysis was performed using the software Image-Pro Plus 5.1 (Media Cybernetics, USA).

## Zeta Potential ( $\zeta$ )

Determination of the Zeta Potential ( $\zeta$ ) of NEBBS was carried out by electrophoretic light scattering technique [32] using the Zetasizer Nano Series equipment (ZEN3690 model, Malvern Instruments, United Kingdom). A laser was focused on the samples, and the Doppler shift in the light scattered by the particles was measured. The nanoemulsions (NEBBS) were diluted 1:20 (v/v) in Milli-Q Ultrapure water (18 M $\Omega$ .cm).

## pH

The pH of NEBBS was determined using a pH meter, accuracy  $\pm$  0.01, sensitivity 99% (NTPHM model, New Technology, Brazil) [33], previously calibrated with standard solutions at pH 4.0 and pH 7.0. The dilutions were performed at 1:10 (v/v) with three reading determinations at 25°C. The electrode was initially washed with 70% ethanol and Milli-Q ultrapure water (18 M $\Omega$ .cm).

## Isoelectric Point – pIE

To determine the isoelectric point (pIE) [34], the NEBBS were previously exposed to different pH conditions (pH values 3, 5, 7, 9 and 11) using HCl (0.1M) and NaOH (0.1M) solutions. The solutions were certified using a pH meter, resolution  $\pm$  0.01 (PHM NT model, New Technique, Brazil). The Zeta Potential  $\zeta$  of the nanoemulsions at different pH values were measured.

## *In vitro* antioxidant activity of free oil and NEBBS

The *in vitro* antioxidant activity test of NEBBS and BBS oil was conducted using the synthetic free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH•). DPPH extinction after antioxidant exposure, measured by EPR, is an experimental indication of the antioxidant activity. For the experiments, DPPH• (0.250 mM) in ethanol P.A., 100 mM sodium acetate buffer pH 5.5, 95% ethanol solution was added to babassu oil solutions in ethanol P.A at concentrations: 4.9920; 24.960; 49.920; 74.881 and 99.841 mM. Ascorbic acid (Vitamin C) in the range of 0.0024 to 0.1250 mM was used as a standard antioxidant control.

Electron Paramagnetic Resonance (EPR) Spectroscopy measurements were performed in an EPR spectrometer EMX plus (Bruker, Germany), by using X-band (9 GHz) and a high-resolution cavity (ER 4119HS, Bruker, Germany) with 1 G modulation field, 100 kHz modulation frequency, 20 mW microwave power, on an average of 4 scans, 100 G sweep width, 60 s sweep time and 3391 G center field. The analysis of the data was performed by double integration on Origin Pro 8.5.1 (Origin Lab Corporation, USA).

## Cell viability assay of nanoemulsions of babassu

### Cell Culture

For the *in vitro* test, murine embryonic fibroblast cell lines (NIH/3T3, ATCCR CRL-1658) were cultivated in sterile DMEM (Dulbecco Modified Eagle medium), sodium bicarbonate buffered and supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.5% (v/v) antibiotic (100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin) at pH 7.2. Cells were maintained in cell culture flasks in standard conditions (37°C, 80% humidified air and 5% CO<sub>2</sub>).

### Cell Viability Assay (MTT)

For the cell viability assay, cells were seeded in 96-well plates at a concentration of  $1 \times 10^3$  cells per well. After 24 hours, the initial culture medium was replaced with 200  $\mu\text{L}$  of fresh culture medium containing NEBBS-F in 07 different concentrations ( $\mu\text{g}/\text{mL}^{-1}$ ) (39.06; 78.12; 156.25; 312.50; 625.0; 1,250.0 and 2,500.0). The cells were exposed to the oil babassu nanoemulsion for 24, 72 and 48 hours, and the MTT assay was conducted according to our research group's standard protocol.

### Statistical analysis

The quantitative data for the stability characterizations and antioxidant activity were evaluated by parametric statistical tests according to the distribution of normality. The comparisons between groups and between time points were evaluated by Analysis of Variance 2-way ANOVA with Sidak's multiple comparisons test, and the level of significance adopted was  $p \leq 0.001$  (99.9%). Data related to cell viability assays composed a mean of three independent experiments in quadruplicates with standard deviation. Significance was considered to be of  $p \leq 0.05$  (95%), evaluated by the ANOVA test, with Bonferroni post-test and subsequent calculation of  $\text{IC}_{50}$ . All statistical treatments were conducted using Graphpad Prism 6 software (GraphPad Software, California).

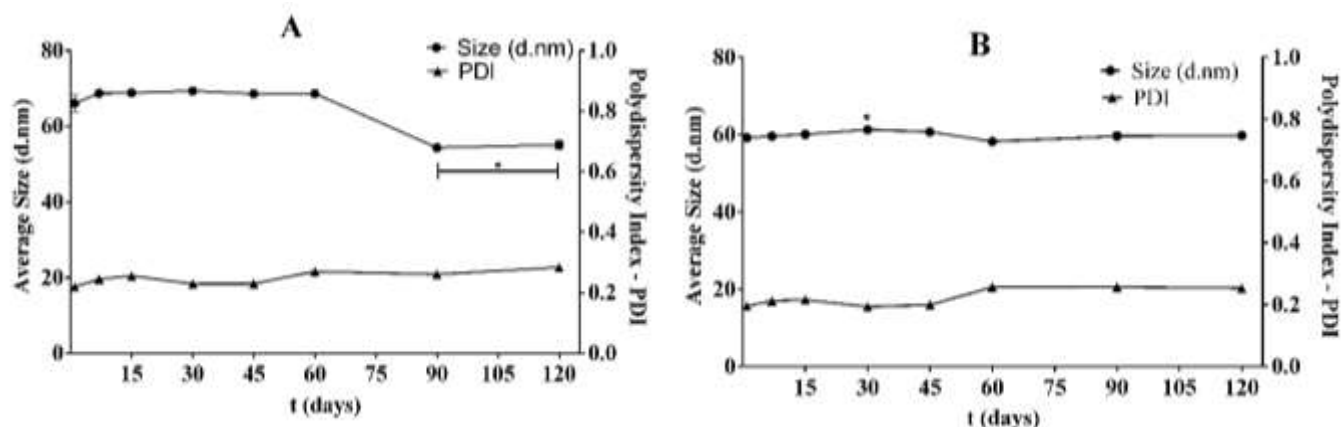
## RESULTS AND DISCUSSION

The stabilization of natural oils, such as babassu oil, in aqueous solutions inside the nanodroplets provided by the nanoemulsification process is one of the challenges that the food industry faces nowadays. Indeed, nanoemulsions containing natural oils are the subject of lively interest in this field, due to the chemical and physical protection provided by the nanocarrier. Furthermore, nanoemulsions are able to increase oral absorption of these ingredients when administered as nutritional products. In order to clarify and present the strategy and design used for the development of the babassu oil nanoemulsion, this result and discussion section is divided into three subsections: stability and characterization; *in vitro* antioxidant activity; and mammalian cell cytotoxicity.

### Colloidal characterization and stability

The stability of products is one of the most important features that should be evaluated during the development of nanoformulations. Due the emulsion thermodynamic instability, colloidal dispersion has an intrinsic trend to break this stability, creating bigger agglomerates, due to the coalescence of the dispersed particles. During instability, the nanodroplet grows over time, in a process classically known as Ostwald maturation, resulting in droplet coalescence [22-23,35-36]. One of the goals of the present report was to evaluate nanoscopic characteristics over 120 days, which strongly indicates the nanoemulsion's stability. In other reports in the literature, the evaluation of babassu nanoemulsions has usually been performed for periods shorter than one month [35-36].

As main results, the NEBBS-C and NEBBS-F nanoemulsions kept a average diameter of less than 70 nm, ( $69.3266 \text{ nm} \pm 0.8224$ ) Figure 1A and ( $61.2733 \text{ nm} \pm 0.6388$ ) Figure 1B, respectively. Average PDI less than 0.3 ( $0.2836 \pm 0.0023$  Figure 1A) and ( $0.2573 \pm 0.0066$  Figure 1B), indicating that samples had low polydispersity index values. The statistical analyses indicated that nanoemulsion size had a significant variation ( $p \leq 0.001$ ) at 90 and 120 days, in comparison to the initial values (day 0), for the NEBBS-C (Figure 1A) sample, and at 30 days for the NEBBS-F (Figure 1B) sample, in comparison to day 0. No significant difference was observed between the values found for the PDI, indicating that the samples had an acceptable colloidal stability.



**Figure 1.** Characterization of the hydrodynamic diameter and polydispersity index (PDI) of babassu nanoemulsion oil. (A) NEBBS-C; (B) NEBBS-F; t: hour. Values are expressed as mean  $\pm$  SD (n=3), ANOVA bidirectional analysis of variance with Sidak's multiple comparison test, significance adopted \* $p \leq 0.001$  (99.9%) when comparing times from 1 to 120 days. SD: standard deviation.

The second step for the nanoscopic stability assays was the investigation of the effects of physical stress on nanoemulsions' stability. As described in the literature [28,35-37], different types of physical conditions contribute to a breakdown in nanoemulsion stability. Among them, temperature variations are one of the most important variables that contribute to nanoemulsion instability. Due to this information, we performed the centrifugation and the cooling/heating tests to evaluate the nanoscopic stability of the samples.

For the centrifugation assays, both NEBBS-C and NEBBS-F nanoemulsions remained stable without signs of phase separation when subjected to a centrifugal field of 94, 587, 1,150 $\times$ g and analyzed over the 60 days period. In this kind of experiment, the nanodroplets' surface repulsion, provided by the surface surfactants, is tested in accelerated stressful conditions [37]. If the nanoemulsion remains stable, it is a strong indication that the droplet will remain dispersed even when submitted to other types of physical stress [38,39].

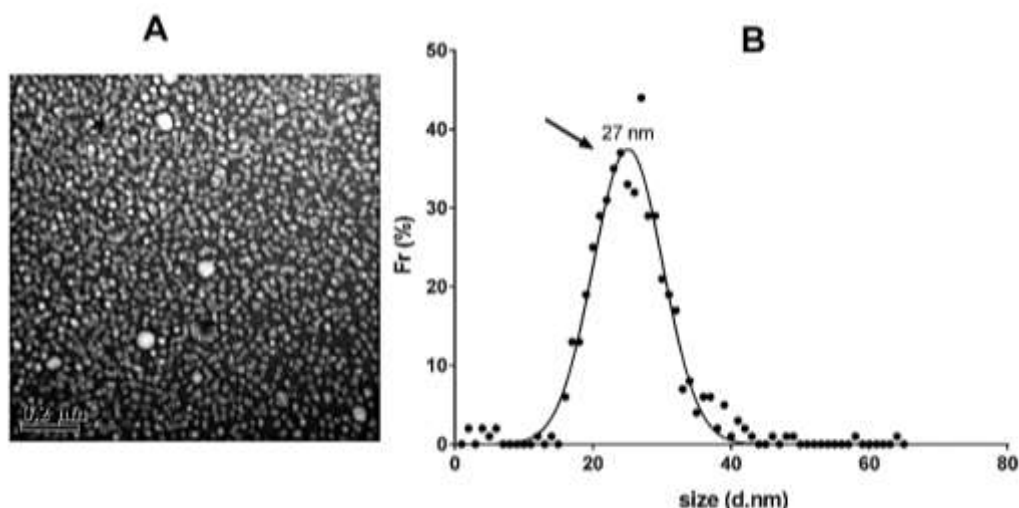
For the cooling/heating experiments, the Hydrodynamic Diameter (d.nm) and PDI of NEBBS-C showed significant size variation ( $p \leq 0.001$ ) for cycles number 01 and 04 with an increase in size and PDI during cycle number 04. For NEBBS-F, size variation ( $p \leq 0.001$ ) was observed only for cycle number 01 (Table 1). Despite the nanoscopic variations, no macroscopic alteration or phase separation was observed, indicating the stability of the nanoemulsion after this stress temperature experiment.

**Table 1.** Hydrodynamic Diameter (nm) and Polydispersity - PDI of oil babassu nanoemulsions subjected to cooling and heating cycles.

Cycles	NEBBS-C		NEBBS-F	
	Size (nm) $\pm$ SD	PDI $\pm$ SD	Size (nm) $\pm$ SD	PDI $\pm$ SD
1	151.120* $\pm$ 2.038	0.3513 $\pm$ 0.045	127.546* $\pm$ 0.877	0.275 $\pm$ 0.009
2	69.840 $\pm$ 0.104	0.305 $\pm$ 0.030	63.534 $\pm$ 0.273	0.267 $\pm$ 0.007
3	52.694 $\pm$ 0.531	0.379 $\pm$ 0.015	62.680 $\pm$ 0.334	0.277 $\pm$ 0.003
4	419.934* $\pm$ 78.422	0.737* $\pm$ 0.067	61.734 $\pm$ 0.850	0.298 $\pm$ 0.037
5	45.694 $\pm$ 0.559	0.282 $\pm$ 0.010	61.280 $\pm$ 0.600	0.357 $\pm$ 0.007

The values are expressed as mean  $\pm$  SD (n=3). Analysis of Variance 2-way ANOVA with Sidak's multiple comparisons test, significance adopted \* $p \leq 0.001$  (99.9%) when compared to the cycles. SD: Standard Deviation.

This was a very important result, because temperature variations are very marked in South America, the region where babassu oil is extracted [2-3,26-17]. In addition to the DLS size analyses, we also investigated the nanoemulsion size and morphology using Transmission Electron Microscopy (TEM). The nanoemulsion morphology and the size dispersion are presented in Figures 2A, 2B, respectively. As shown in Figure 2A, the lipid nanodroplet presents spherical morphology with a narrow size dispersion. Size quantification (Figure 2B) showed a normal Gaussian size dispersion with a size peak of 27 nm. The size obtained by TEM analysis is not exactly the same as that observed in the DLS experiments, but this kind of result is quite common in the literature [39,40].



**Figure 2.** Photomicrographs of the nanoemulsion droplets NEBBS-F obtained by (A) Transmission Electron Microscopy – TEM and (B) size distribution curve (d.nm) of the particles present in nanoformulation. Fr (%): Relative frequency in percentage. Significance adopted  $p \leq 0.001$  (99.9%).

As different techniques, TEM and DLS measure different nanodroplet parameters. TEM measures the electron beam transmittance through the samples, and the detectors are positioned in the same direction. Due to this microscope organization, and the electron beam wavelengths, the measurements are closer to reality. On the other hand, the DLS analyses measures the lipid nanodroplets' Brownian motion in aqueous media [39,40]. So, due to these different measurement's techniques, differences between size measurement values are common and reported in the literature [41].

As noted in the introduction section, one important property provided by nanoemulsions is the chemical protection of compounds entrapped inside the nanodroplets. For fatty acids, this condition is especially important, due to the possibility of lipid oxidation. Lipid oxidation in nanoemulsions causes the release of oxidative metabolites to the aqueous phase, alterations in the ionic tension and consequent colloidal suspension disruption [42]. This process can be monitored over time using different techniques, including measuring pH variation, and the consequent zeta potential alteration. In our experiments, we measured these both variables, aiming to correlate these chemical variables with the structural stability of the nanoemulsion.

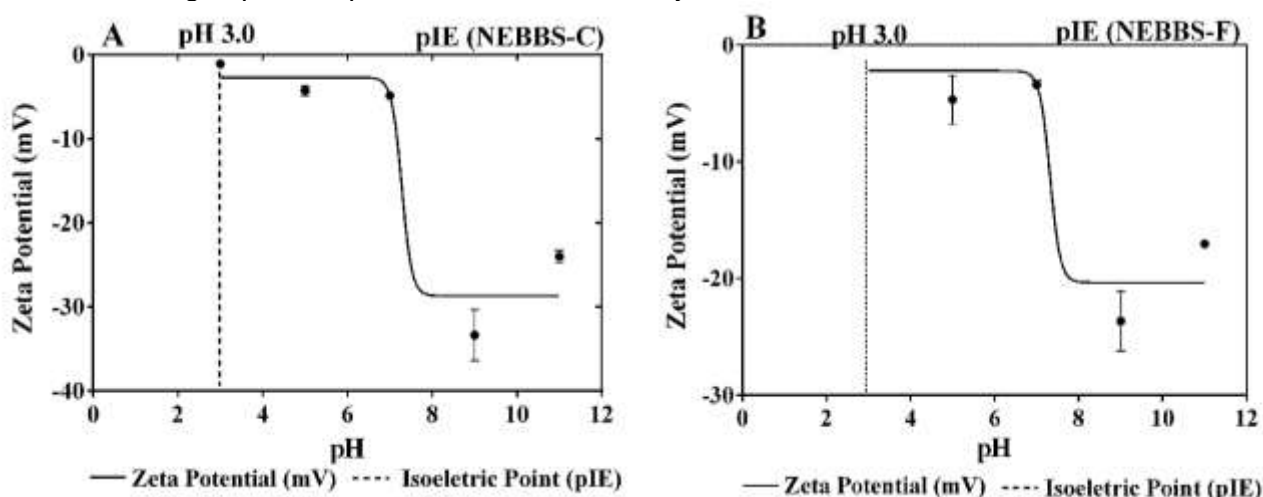
The Zeta Potential ( $\zeta$ ) and pH results are presented in Table 2. As shown, NEBBS-C and NEBBS-F presented negative Zeta Potential values, -6.51 mV and -5.05 mV respectively, at the first day after nanoemulsion preparation. This value varied over time; and was observed a statically significant difference for NEBBS-F at day 120. The pH values had a similar behavior, with results on the first day after preparation showing pH of  $6.34 \pm 0.58$  and  $6.39 \pm 0.36$  for NEBBS-C and NEBBS-F, respectively. And at the end of 120 days, the nanoemulsions showed neutral pH values of  $7.60 \pm 0.38$  (NEBBS-C) and  $7.26 \pm 0.20$  (NEBBS-F). As observed for the zeta potential measurements, pH measurements showed no significant change over time. These data are a good indicator of a nanoemulsion's chemical stability and might be correlated with the structural stability previously presented.

**Table 2.** Zeta Potential and pH of the nanoemulsions NEBBS-C and NEBBS-F as a function of time.

Zeta Potential (mV)		
T (days)	NEBBS-C	NEBBS-F
1	-6.51 ± 1.45	-5.05 ± 1.97
7	-4.94 ± 0.89	-3.02 ± 0.56
15	-0.18* ± 0.84	-5.20 ± 0.79
30	-10.35 ± 0.34	-3.77 ± 0.09
45	-11.56* ± 1.85	-2.77 ± 0,50
60	-8.43 ± 0.80	-7.69 ± 0.27
90	-4.60 ± 0.29	-6.66 ± 0.68
120	-8.35 ± 0.46	-8.05* ± 0.05
Potential Hydrogen (pH)		
T (days)	NEBBS-C	NEBBS-F
1	6,34 ± 0,58	6,39 ± 0,36
7	6,05 ± 0,03	6,60 ± 0,06
15	7,38 ± 0,02	6,94 ± 0,16
30	5,46 ± 0,30	7,62 ± 0,09
45	7,22 ± 0,43	6,82 ± 0,27
60	6,22 ± 0,19	6,58 ± 0,005
90	5,89 ± 0,09	6,25 ± 0,01
120	7,60 ± 0,38	7,26 ± 0,20

The values are expressed as mean±SD (n=3). Analysis of Variance 2-way ANOVA with Sidak's multiple comparisons test, significance adopted \*p≤0.001 (99.9%) when compared to the time. SD: Standard Deviation.

As is known, the zeta potential is the measurement of the superficial charges of colloidal structures, and it is established at the superficial interface of the dispersed colloidal structures, such as lipid nanoemulsions, and the dispersant media, such as continuous aqueous media. As an interface charge, both superficial and continuous media contribute to the zeta potential [32]. The event can easily be observed in the isoelectric point determination, when pH variations induce changes in the zeta potential measurements (Figure 3), the isoelectric point (pIE) is established at the pH value where the ZP value tends to go or is equal the zero. As observed, acidic pHs tend to increase the values of the zeta potential (ZP) in the positive direction of the Y line, while basic pHs reduce the ZP in the negative direction of the Y line. This phenomenon is explained by the number of charged positive protons available in the system.



**Figure 3.** Isoelectric Point (pIE) of babassu oil nanoemulsions and the influence of zeta potential. A: pIE of NEBBS-C; B: pIE of NEBBS-F. The values are expressed as mean ± SD (n=3), Analysis of Variance 2-way ANOVA with Sidak's multiple comparisons test, significance adopted p≤0.001 (99.9%). SD: Standard Deviation.

As an interfacial charge, the zeta potential contributes as a repulsive force to the stability of colloidal systems, such as nanoemulsions. Regarding stability, several articles report that the zeta potential needs to



be higher than 30 mV in the module to maintain the nanoemulsion's colloidal stability [32,37,39]. However, in our results, the measurements collected were around -5mV, a value lower than is reported in the literature [43]. This can be explained by the presence of portions of non-ionic surfactants on the surface of the nanoemulsion, which contribute to repulsion forces that maintain the stability of the colloidal suspension.

### ***In vitro* antioxidant activity assay**

The second group of results obtained were related to babassu oil's antioxidant activity. This property was evaluated by the reduction in free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH•), using Electronic Paramagnetic Resonance Spectroscopy (EPR) techniques. The DPPH• assay is a classical experimental approach to evaluate the antioxidant activity of different types of compounds [44,45]. In the traditional methodology, researchers measure DPPH• color modification, using spectrophotometric evaluations. Basically, DPPH• of violet colored turns yellow when antioxidant molecules are present, due to the reduction of this free radical that occurs through a Redox reaction. The antioxidant compound acts as a donor of a hydrogen (H) atom by reducing the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) to form the more stable diphenyl-picryl-hydrazine DPPH-H diamagnetic molecule.

Despite the good results obtained with these techniques, and the vast literature published using these optical evaluations, some drawbacks related to this methodology are reported when natural oils, such as babassu, are evaluated. The concerns are related to the presence of natural dyes in oil samples, which can interfere with the optical density, thus creating errors during the interpretation of results [45]. To overcome this, our research group is investing in the EPR technique to measure DPPH• upon exposure to antioxidant actives. Using this technique, the presence of natural chromophores does not interfere with the experiments.

It is well known in the literature that DPPH• radical has paramagnetic properties and generates a complex EPR spectrum with a typical signal of five bands with different intensities [46]. According to the literature [46], the intensity of the EPR signal can be adjusted to an exponential decay depending on the individual concentration of each sample, this result can be compatible with the action of more than one antioxidant substance in the sample with different kinetic rates, and can also be applied in unpurified extracts.

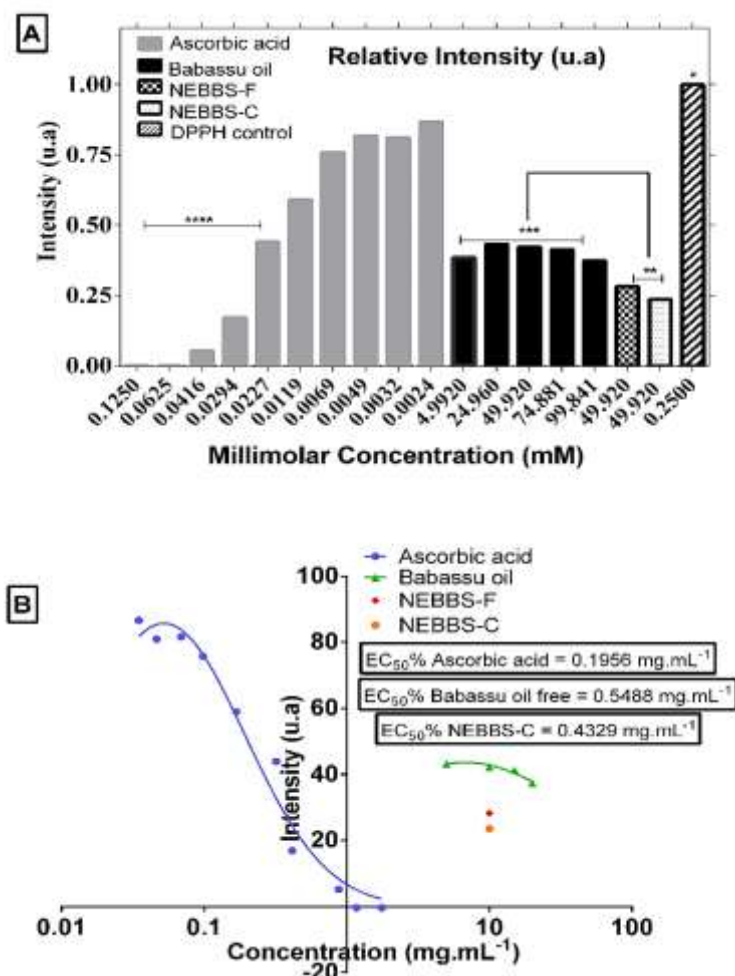
Due to the action of an antioxidant (AH), this radical is reduced to form diphenyl-picryl-hydrazine (DPPH<sub>2</sub>), which can be monitored by decreasing the intensity of the RPE signal. From the results obtained, the antioxidant activity is expressed as a percentage of reduction in DPPH• and the results are presented in 50% effective concentration (EC<sub>50</sub>). The greater the consumption of DPPH• by a sample, the greater its antioxidant activity [46, 47].

Figure 4 shows the antioxidant activity of the free babassu oil at different concentrations, as well as the NEBBS-C and NEBBS-F nanoemulsions. Furthermore, we used ascorbic acid as an internal standard antioxidant control. In Figure 4A we have the comparisons of millimolar concentrations of the samples ascorbic acid, free babassu oil, NEBBS-F and NEBBS-C in the presence of the free radical DPPH•. It was observed that the concentrations of ascorbic acid: 0.125 mM to 0.0227 mM significantly decreased ( $p \leq 0.05$ ) the relative intensity of DPPH•. Notably this phenomenon was also observed for the two babassu nanoemulsions (49.920 mM) which significantly ( $p \leq 0.05$ ) had a lower intensity of DPPH• free radicals compared to free babassu oil at the same concentration (49.920 mM). This result reveals that the condition of the nanoemulsified oil was important for present a better antioxidant response. Babassu nanoemulsion molarity was calculated based on the lauric acid concentration (200,3178 g/mol). this component was selected because it is one of the main fatty acids present in babassu oil.

As presented in Figure 4B, we used the EC<sub>50</sub> value to compare the antioxidants activities. The EC<sub>50</sub> is a value that represents the effective concentration (EC) in mg.mL<sup>-1</sup> of the antioxidant compound required to reduce the initial DPPH• concentration by 50%. Data are presented as a concentration response curve, and is clear to identify that as higher the antioxidant concentration, higher is the antioxidant capacity, represented by the decrease in the EPR relative signal. For the NBBS samples, it is possible to identify that with the same babassu oil concentration, we have a higher antioxidant capacity, proving that the antioxidant capacity is improved by the nanoemulsification process.

The results obtained demonstrate that the EC<sub>50</sub> of Ascorbic Acid was 0.1956 mg.mL<sup>-1</sup>, of free babassu oil 0.5488 mg.mL<sup>-1</sup> and babassu nanoemulsion 0.4329 mg.mL<sup>-1</sup>. The results show that the nanoemulsion improved the babassu oil's antioxidant capacity. Since a minimum effective concentration of babassu nanoemulsion was observed to reduce the initial DPPH concentration by 50% compared to free babassu oil. This improvement of babassu oil's antioxidant activity can be explained by the nanoemulsification process. Inside the nanodroplets, the oil is more dispersible, has a higher surface area in consequence of the droplet reduction, and the antioxidant compounds are more available to contact the DPPH• free radical.

It is important to state here that the nanoemulsification process will improve the dispersion of babassu oil and, for *in vivo* application, can greatly help to increase oral absorption, however the antioxidant activity is provided by the oil molecules. Our hypothesis is that the increase in oil dispersion, promoted by the nanoemulsion, will create large surface areas and the antioxidant activity can be improved, as demonstrated in our *in vitro* results.



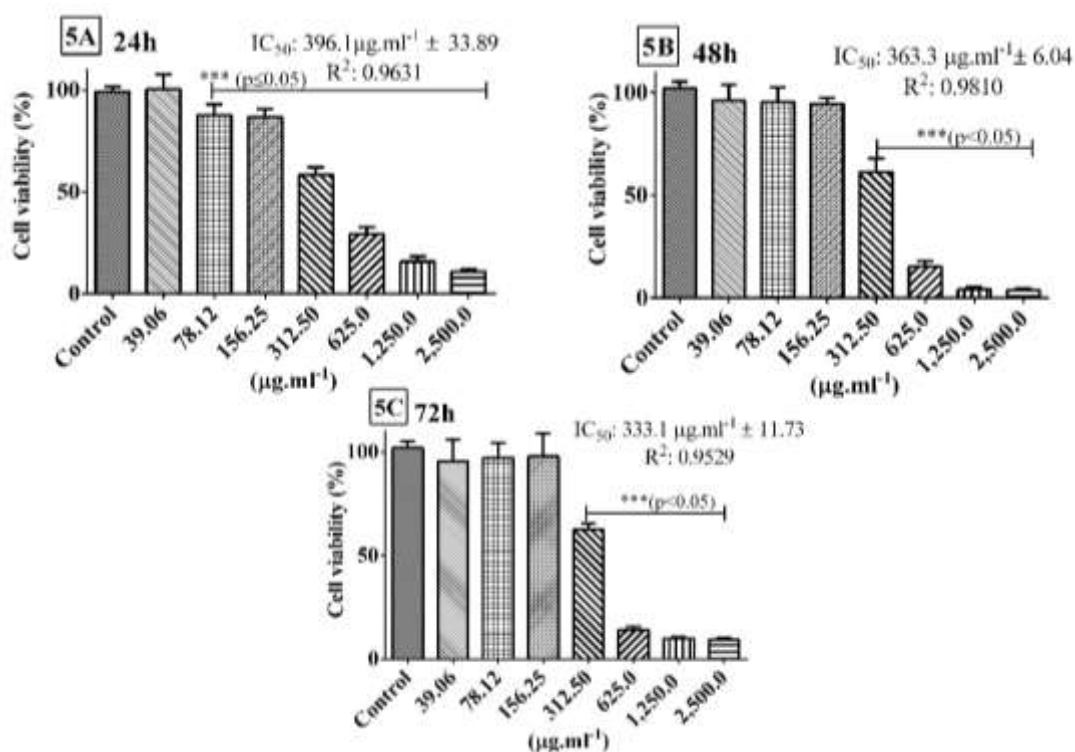
**Figure 4.** (A) Antioxidant effect monitored by Spectroscopy Electron Paramagnetic Resonance - EPR. (B) Integrated data obtained for ascorbic acid, babassu oil and nanoemulsions in the presence of DPPH•. Curve are represented in log concentration (mg.mL<sup>-1</sup>). \*p≤0.001 (99.9%) analysis of Variance 2-way ANOVA with Sidak's multiple comparisons test.

### Cell viability assessment

The last step in our investigation was to evaluate the nanoemulsion's cytotoxicity in mammalian cells. To achieve this objective, we exposed murine fibroblast cells (NIH-3T3) to different nanoemulsion concentrations. The NIH-3T3 cell line, a fibroblast culture cell, was selected because it is one of the most abundant cell types in the body and is one of the first cells that would come into contact with nanoemulsions in the absorption processes [48]. Moreover, this type of cell is one of the most used around the world, which facilitates its comparison with other published articles [49-51].

Cell viability results, measured by the MTT assay, are presented in Figure 5. The nanoemulsion's cytotoxicity presented time and concentration dependence. The IC<sub>50</sub> (%) values (concentration required to induce 50% of cell viability reduction) were calculated and were 396.1  $\mu\text{g.mL}^{-1} \pm 33.89$  (Figure 5A), 363.3  $\mu\text{g.mL}^{-1} \pm 6.04$  (Figure 5B) and 333.1  $\mu\text{g.mL}^{-1} \pm 11.73$  (Figure 5C) for 24, 48 and 72 hours of exposure, respectively. The evaluation of the babassu oil cytotoxicity was not evaluated in this experiment due to the high lipophilicity of these types of samples. In this situation, the free oil could form phase separation and the

oil molecules will not get in contact with the monolayer cells and some false negative biocompatibility results could be collected wrongly.



**Figure 5.** Cytotoxicity of NEBBS-F (39.06–2,500.0  $\mu\text{g.mL}^{-1}$ ) was analyzed using cells fibroblast (NIH/3T3) with MTT method. (A): 24 hours; (B): 48 hours; (C): 72 hours. Data are represented as mean  $\pm$  SD of four replicates from three independent experiments. The values are represented as the percentage cell viability where untreated cells were regarded as 100%. Data were analyzed using 2-way ANOVA with Bonferroni posthoc test considering  $*p \leq 0.05$ . SD: Standard Deviation.

Significant cell cytotoxicity was observed only at higher concentrations of babassu oil nanoemulsion above 78.12  $\mu\text{g.mL}^{-1}$  in 24h time ( $p \leq 0.05$ ) and above 312.50  $\mu\text{g.mL}^{-1}$  for 48 and 72h time ( $p \leq 0.05$ ). Furthermore, data showed that the presence of oil babassu nanoemulsion (NEBBS-F) did not promote significant cell death over time, since the percentage of cell viability remained proportionally ( $p \leq 0.05$ ) constant when comparing the times 24h, 48h and 72h at the lower concentrations. These results are in agreement with the cytotoxicity data of nanoemulsions based on natural oils extracts [49-51]. This is interesting, since even for a longer period of exposure the lower nanoemulsion concentrations did not affect the cell viability significantly, and future *in vivo* evaluations should be considered.

## CONCLUSION

In conclusion, this work reports the development of nanoemulsions produced with babassu oil (*Orbignya phalerata* Martius). In the tests used in this study, the babassu oil nanoemulsion presented low polydispersity and kinetic stability. The analysis of the *in vitro* antioxidant potential monitored by EPR corroborate the hypothesis that the antioxidant action of babassu oil can be improved by using a nanoemulsion. The cell viability assays in murine fibroblast cells showed low toxicity for the babassu oil nanoemulsion, with viability over 92% ( $p \leq 0.05$ ) in the studied times. These results regarding antioxidant and cytotoxic effects indicate that babassu oil nanoemulsions may have potential for future applications in nanostructured foods or for the development of new antioxidant phytotherapies.

## PATENTS

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**Conflicts of Interest:** The authors report no conflicts of interest.

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