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MICROALGAE OIL AND VITAMIN E: FROM NANOSTRUCTURING TO SAFETY PROFILE[1]

ÓLEO DE MICROALGA E VITAMINA E: DA NANOESTRUTURAÇÃO AO PERFIL DE SEGURANÇA

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ABSTRACT

In the last few years, nanotechnology seeks to expand its nanocarrier systems, to increasingly ensure that the incorporated assets maintain their properties and benefits with greater efficiency and safety. Thus, nanoencapsulation has been widely used, mainly in the cosmetic industry, which aims to combine new technologies in its products that guarantee good efficacy of the active principles used in conjunction with renewable and sustainable natural bioactive. In this context, the objective of this study was to nanoencapsulate the oil extracted from the microalgae *Chlorella homosphaera* with the addition of the vitamin E, to guarantee the properties and stability of this oil and the vitamin, facing encapsulation. Then, evaluate the biocompatibility of the nanocapsules for a possible topical use formulation. Nanocapsules containing microalgae oil and vitamin E were produced by the nanoprecipitation method and then physically chemically characterized, after cytogenotoxicity and hemocompatibility tests with the nanoformulations were performed to assess the safety profile of the formulations. The nanocapsules showed satisfactory results, such as average particle diameter, polydispersion index, zeta potential, and pH with values within the expected limits for the formulation produced and did not show cytotoxicity in the tests performed. Concluding that nanoencapsulation is a possible alternative for using oil from the microalgal origin with vitamin E for cosmetic formulations and topical use.

Keywords: *Nanotechnology, Nanocapsule, Natural Bioactives, Biocompatibility.*

RESUMO

Nos últimos anos a nanotecnologia busca expandir seus sistemas de nanocarreadores, para garantir cada vez mais que os ativos incorporados mantenham suas propriedades e benefícios com maior eficácia e segurança. Deste modo, a nanoencapsulação vem sendo muito utilizada, principalmente na indústria cosmética, que visa aliar em seus produtos novas tecnologias, que garantam uma boa eficácia dos princípios ativos utilizados em conjunto com bioativos naturais renováveis e sustentáveis. Neste contexto, objetivou-se neste estudo nanoencapsular o óleo extraído da microalga Chlorella homosphaera com adição de vitamina E, para garantir as propriedades e estabilidade deste óleo e a vitamina, frente a encapsulação. Logo, avaliar a biocompatibilidade das nanocápsulas para uma possível formulação de uso tópico. Foram produzidas nanocápsulas contendo o óleo da microalga mais adição de vitamina E pelo método de nanoprecipitação e em seguida, caracterizadas físico-quimicamente, após, testes de cito-genotoxicidade e hemocompatibilidade com as nanoformulações foram efetuados, para avaliar o perfil de segurança das formulações. As nanocápsulas

1 Master's work.

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apresentaram resultados satisfatórios, como diâmetro médio das partículas, índice de polidispersão, potencial zeta e pH com valores dentro dos limites esperados para a formulação produzida e não apresentaram citotoxicidade nos testes realizados. Concluindo que a nanoencapsulação é uma possível alternativa para utilização de óleo de origem microalgal com vitamina E para utilização em formulações cosméticas de uso tópico.

Palavras-chave: *Nanotecnologia, Nanocápsulas, Bioativos Naturais, Biocompatibilidade.*

INTRODUCTION

Currently, there is a growing demand in the field of nanotechnology for the search and production of modern nanocarriers to improve the benefits of the actives incorporated in these devices. As a more appropriate alternative, nanoencapsulation offers controlled rates of release of the actives in specific action sites, expanding its specificity, improving the efficacy of the active, and reducing possible toxicity, while delaying the chemical and enzymatic effects that degrade it and allowing administration by other non-invasive routes (JORNADA *et al*., 2012; FRANK *et al*., 2015).

Nanocapsules are vesicular or reservoir systems in which the oil/water is essentially confined to a cavity surrounded by a small polymeric membrane, so after being injected intravenously or subcutaneously, the nanocapsules can be targeted to specific cells and locations in the body (DINESHKUMAR *et al*., 2013). Therefore, they stand out for the physical-chemical properties that make them important release systems, such as small size, large surface area, and several surface charge characteristics (SCHAFFAZICK *et al*., 2003; MAINARDES *et al*., 2009), it can also improve the stability of the active ingredients (OURIQUE *et al*., 2008) and can still be biocompatible with tissues and cells when synthesized from biocompatible or biodegradable materials (GUINEBRETIÈRE *et al*., 2002).

In this context, the cosmetic area aroused great interest in nanocapsules, as an option to obtain better results from their products (SCHMALTZ *et al*., 2005). Thus, the substances that show the most interest in encapsulation are lipids, proteins, vitamins, and natural pigments, such as β-carotene (PETERS *et al*., 2011).

Microalgae can produce more lipids than any other conventional culture, in addition, numerous other species can produce large amounts of essential fatty acids (EFA), especially ω 3 and ω 6, which are the two most abundant, as well as acid α linolenic (ALA, C18:3 ω 3) and linoleic acid (Al, C18:2 ω 6) (HO *et al*., 2014; BELLOU *et al*., 2016). According to the scientific literature, there are many *Chlorella* species with potential for oil production, with lipid accumulation in the range of 2% to 63% on a dry basis (MATA *et al*., 2010).

It is important to consider that the use of oils is usually limited by their solubility and by the need to disperse them in a way that guarantees their effective action (JIANG *et al*., 2009). Essential oils are unstable, therefore, easily degraded in the presence of oxygen, light, heat, moisture, and

metals, making them susceptible to degradation during the processing and storage of a product (GONÇALVES *et al*., 2017; HAC-WYDRO *et al*., 2017).

Considering the instability and high volatility of oils, nanoencapsulation emerges as an alternative to minimize losses, as it involves them by a physical barrier, providing protection against their oxidation or volatilization, ensuring the efficiency of the oil in the matrix to which it will be added (NGUEFACK *et al*., 2009; GONÇALVES *et al*., 2017).

Currently, there is a growing demand for the use of natural vegetable raw materials, due to an appeal of valuing Brazilian biodiversity and sustainability, the extraction of oil from microalgae becomes something renewable, but the use of this oil requires allied biotechnology to guarantee its properties beneficial. In this context, the alternative to nanoencapsulate the oil of the microalgae *Chlorella homosphaera* with the addition of vitamin E as something innovative, to guarantee the properties and stability and, before nanoencapsulation, evaluate the biocompatibility of the produced nanocapsules.

MATERIALS AND METHODS

FROM THE CULTIVATION OF MICROALGAE TO THE EXTRACTION OF *Chlorella homosphaera* OIL

The cultivation of microalgae of the genus *Chlorella homosphaera* was carried out using a standard BG 11 medium, containing macronutrients and micronutrients that are essential for the growth of microalgae (RIPPKA *et al*., 1979). Then, the biomass of *Chlorella homosphaera* was obtained by drying approximately 20 liters of algae cultivation in a greenhouse, for about 5 days at a temperature of 60 ˚C, with daily monitoring. Afterward, the Soxhlet method was used to extract the oil from *Chlorella homosphaera* (RAMOLA *et al*., 2019), the technique uses the Soxhlet equipment and 200 mL of solvents, chloroform: methanol in a 2:1 v/v ratio, standard solvent for lipid extraction, then heated with the aid of a heating blanket to approximately 70 ˚C. The extraction process took about 6 hours, then the flask with the solvents was taken to a rotary evaporator for a few minutes for total evaporation of the solvents.

Characterization of the oil extracted from *Chlorella homosphaera*

The characterization of the oil extracted from *C. homosphaera* was by the gas chromatography method, using a gas chromatograph with a mass spectrometer, model GCMS QP2010 Plus brand Shimadzu, this technique consists of the separation and analysis of mixtures by the interaction of its components between a phase stationary and a mobile phase (COLLINS *et al*., 2006). The parameters and conditions of the gas chromatograph were: ZB WAX capillary column (30m x 0.25mm di x 0.25 μm), helium carrier gas, column flow 10 mL min^{-1}, injection volume 1.0 μL, total process time 49.5 minutes.

Production of nanocapsules containing *Chlorella homosphaera* oil and vitamin E

The nanocapsule formulations were prepared using the preformed polymer nanoprecipitation technique, adapted from Venturini *et al.* (2011). Two phases were prepared separately, one organic and the other aqueous, kept under magnetic stirring for 30 minutes at a temperature of 40 ˚C until total solubilization of the components. Then, the organic phase was poured into the aqueous using a funnel, for the immediate formation of the nanocapsules with *C. homosphaera* oil (NC1). The suspension was kept under magnetic stirring for another 10 minutes, after which it was placed in a rotary evaporator at 80 rpm for about 2 hours for total evaporation of the solvent and excess water, up to a final volume of 10 mL of formulation The same protocol for the production of the other nanocapsules was followed, being NC 2 with oil and vitamin E (0.08 g of each component), and NC 3 with only vitamin E (0.16 g).

Table 1 - Composition of nanocapsule formulations containing *Chlorella homosphaera* oil for a final volume of 10 mL.

Phase	Component	Quantity
Organic	PCL polymer	0,1 g
	Span® 60	0,038 g
	Chlorella oil	0,16 g
	Acetone	27 mL
Aqueous	Tween® 40	0,077 g
	Milli-Q Water	53 mL

Source: author's construction.

Physical chemical characterization of nanocapsules

The nanocapsules were characterized by mean particle diameter, polydispersion index, zeta potential, and pH of the formulations. The dynamic light scattering technique was used to perform this analysis, using the Zetasizer® equipment. The samples were diluted 500 times in ultrapure water (Milli Q®) previously filtered on a 0.45 μm membrane and the reading was performed in triplicate, to obtain the results of the size and polydispersion index of the nanocapsules. To determine the zeta potential, the electrophoresis technique was performed using the same Zetasizer® equipment, expressing the results in millivolts (mV). The samples were diluted 500 times in a 10 mmol/L NaCl solution previously filtered on a 0.45 μm membrane and the reading was performed in triplicate. The pH of the formulations was determined by a potentiometer (DM 22, Digimed®) previously calibrated with a buffer solution pH 4.0 and 7.0.

IN VITRO EVALUATION OF CYTOGENOTOXICITY AND HEMOCOMPATIBILITY

In vitro cytogenotoxicity and hemocompatibility tests were performed on peripheral blood mononuclear cells (PBMCs) and with erythrocytes, under approval by the Franciscan University (UFN) Ethics Committee in Human Beings, under CAAE number: 44940821.3.0000.5306.

For isolation of PBMC and cell culture, blood samples were properly homogenized slowly and then centrifuged for 30 minutes at 1000 rpm with Histopaque solution. Subsequently, a leukocyte pellet was formed that was withdrawn into another tube, and culture medium was added for another centrifugation at 1000 rpm for 10 minutes. The supernatant was discarded and resuspended in a complete culture medium, as described below.

The cells were properly cultivated in a culture containing RPMI 1640 (Invitrogen®), 10% fetal bovine serum (Invitrogen®), inactivated at 56 ˚C for 1 hour, 100 $U.mL^{-1}$ of penicillin (Invitrogen®) and 100 $U.mL^{-1}$ of streptomycin (Invitrogen®), at 37 ˚C in a humid atmosphere containing 5% CO_2. Then, the cells were seeded in 96-well plates, with a concentration of 2 x 10^5 cells/mL, and treated with different concentrations of microalgae oil and nanoencapsulated vitamin E. As internal controls for the cytogenotoxicity tests were performed, a negative and positive control, composed of culture and cells, and culture-containing cells added with 100 $mmol.L^{-1}$ of hydrogen peroxide, respectively, were prepared. For the hemocompatibility tests, a saline solution (0.9%) was used as a negative control.

Cell viability (MTT), dichlorodihydrofluorescein (DCFH), nitric oxide (ON), and DNA double-stranded damage (PicoGreen®) assays were performed, both evaluating cytogenotoxicity, following the protocols according to Sagrillo *et al.* (2015), Choi *et al.* (2012), Esposti (2002), Ha *et al.* (2011), respectively. To assess the hemocompatibility of the nanocapsules, the prothrombin time (PT), activated partial thromboplastin time (APTT), clot retraction, and hemolysis tests were performed, following the protocols according to Vaucher *et al.* (2010).

STATISTICAL ANALYSIS

The results obtained were statistically analyzed using the GraphPad Prism program, version 5.0. Data were expressed as mean ± standard deviation (SD). All experiments were carried out in triplicate. Treatments were compared using a one-way analysis of variance followed by Tukey's or Dunnett's post hoc test when necessary, tests with $p<0.05$ were considered significant.

RESULTS AND DISCUSSIONS

YIELD OF OIL EXTRACTED FROM *Chlorella homosphaera*

The extraction by Soxhlet method, using chloroform solvents: methanol is widely described in the literature as the most widely used mixture of solvents in the extraction of microalgae lipids, since it can remove neutral and polar lipids, with excellent oil extraction yields (GRIMA *et al.*, 1994; SOARES, 2010; D'OCA *et al.*, 2011). From an experimental extraction, the yield of 1.5 g of oil corresponding to 10% of the dry biomass value was observed, this value was calculated using equation (1).

The oil yield was also observed in the other extractions performed and corroborated with the literature describing a yield of 12 to 14% using this same extraction method (GREENWELL *et al.*, 2010; RAMOLA *et al.*, 2019).

$$C_{lip} = \frac{Pf\ X100}{Pa} \quad (1)$$

(Where: C_{lip} = lipid concentration (% w/w); P_f = final weight (g); P_a = dry sample weight (g).

Analysis of the characterization of *Chlorella homosphaera* extracted oil by GC MS

Table 2 shows the components found in the oil extracted from *Chlorella homosphaera*, as well as the retention time, area, and percentage of each compound.

Table 2 - Compounds found in *Chlorella homosphaera* oil by GC MS.

Retention time	Area	Identification of the compound	Percentage relative to the total area
21.557	2538021	C14:0	0,13
22.880	203645	saturated fatty acid	0,01
23.217	112115	saturated fatty acid	0,01
24.020	521221	C15:0	0,03
25.735	427373	Aldehyde	0,02
26.025	226914	saturated fatty acid	0,01
26.415	21703005	C16:0	1,15
26.766	341958	C16:1	0,02
26.923	14688785	C16:1	0,78
27.629	1178253	saturated fatty acid	0,06
28.201	1450595	C16:2	0,08
28.384	1061611	saturated fatty acid	0,06
28.678	2301917	saturated fatty acid	0,12
28.904	1798682	C16:3	0,09
29.110	830049	unsaturated fatty acid	0,04
29.787	1144109	saturated fatty acid	0,06
30.129	1097592	hydrocarbon	0,06
30.913	19996595	C18:0	1,06
31.268	46470603	C18:1	2,45
31.408	14033870	C18:1	0,74
31.679	842163	C18:1	0,04
32.180	6402447	C18:2	0,34
33.496	6711703	C18:3	0,35
34.108	8892575	unsaturated fatty acid	0,47
35.130	24572805	C20:0	1,30
35.475	123102248	C20:1	6,50
35.606	14804208	saturated fatty acid	0,78
36.325	10588211	C20:2	0,56
36.824	5408581	C20:3	0,29
37.236	31049047	C20:4	1,64
37.573	8084336	unsaturated fatty acid	0,43
38.088	28702035	unsaturated fatty acid	1,51

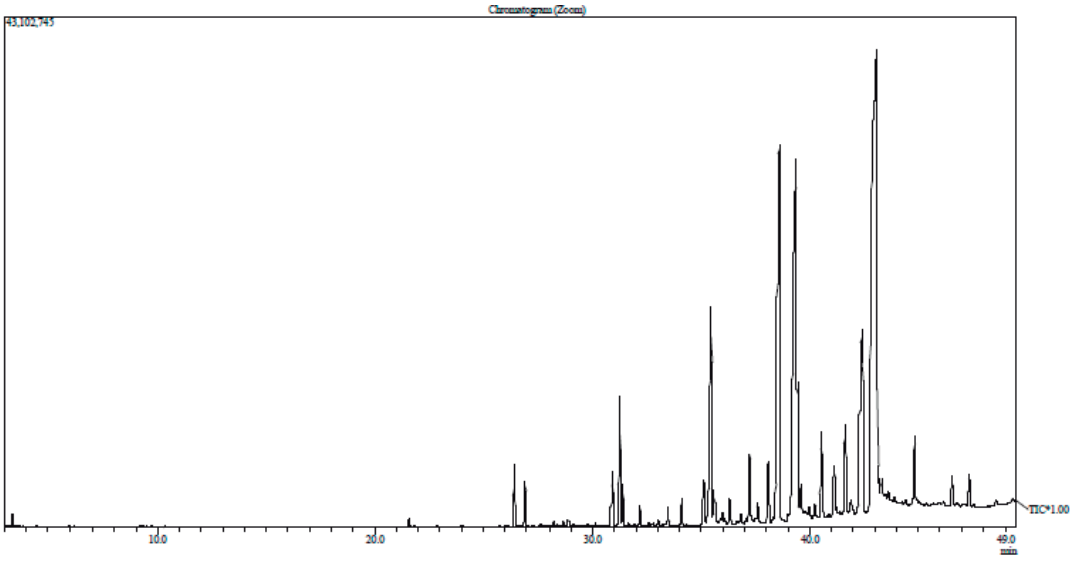
38.589	222081047	C20:5	11,72
39.380	333845516	unsaturated fatty acid	17,62
39.569	17833576	C22:1	0,94
40.518	36297166	unsaturated fatty acid	1,92
41.617	52327916	unsaturated fatty acid	2,76
42.399	150254699	unsaturated fatty acid	7,93
43.060	605477937	C22:6	31,96
44.827	37051015	hydrocarbon	1,96
46.538	20773310	unsaturated fatty acid	1,10
47.312	17341835	unsaturated fatty acid	0,92

Source: Data obtained from the oil extracted from microalgae,
after performing GC MS at the University of Santa Cruz do Sul UNISC.

Figure 1 shows the chromatographic characterization of the microalgae *C. homosphaera* oil. In the chromatogram we observed that the acids: palmitic (16:0), oleic (C18:1), achidic (C20:0), eicoseinoic (C20:1), EPA and DHA, were the fatty acids in greater quantity, with the proper retention times: 26.41; 31.26; 35.13; 35.47; 38.58 and 43.06 minutes and with a percentage relative to the total area of 1.15; 2,45; 1,40; 6,50; 11.72 and 31.96%, respectively. In this characterization by gas chromatography, the oil extracted from *C. homosphaera* demonstrated the same components of the oil extracted in studies conducted by D'oca *et al.* (2011).

It was observed in chromatographic analysis that unsaturated fatty acids are found in greater quantity than saturated fatty acids, data also found in studies conducted by Bjerk (2012). It is noteworthy that the acids EPA and DHA were the ones that obtained the highest amount, being these marine oils.

Figure 1 - Chromatographic characterization of the microalgae *C. homosphaera* oil.



Source: Santa Cruz do Sul University (UNISC).

Thus, the results found in the characterization of the oil are in accordance with studies conducted by Costa *et al.* (2006), which evaluated the fatty acid profile of the microalgae of the genus

Chlorella and observed the same fatty acids mentioned in table 7, varying the concentration of each constituent in a proportion of 0.06% to 27%. The variation in the composition and content of lipids and fatty acids in microalgae can be explained by the influence of some factors such as temperature, light, CO_2 concentration, nitrogen concentration, among other nutrients (FERNANDEZ *et al*., 2000; COLLA *et al*., 2004; ARAUJO; GARCIA, 2005).

Chemical physical analysis of nanocapsules

The three nanoformulations presented adequate chemical-physical characteristics compatible with the results found in formulations produced from the nanoprecipitation method. Table 3 presents the values of the four parameters evaluated in the characterization of the nanocapsules.

Table 3 - Average diameter, polydispersion index (PDI), zeta potential, and pH of nanocapsules.

Nanocapsules	Average diameter(nm)	PDI	Zeta potential (mV)	pH
NC1	221,8 0,74	0,21 0,01	-13,9 ± 0,69	7,1 0,01
NC2	191,1 1,46	0,11 0,00	-13,8 ± 1,07	0,01
NC3	194,5 2,24	0,11 0,01	-14,7 ± 0,69	0,01

Legend: NC1= nanocapsule containing *Chlorella* oil; NC2= nanocapsule containing *Chlorella* oil and vitamin E; NC3= nanocapsule containing vitamin E.
Source: author's construction.

Scientific literature reveals that there may be differences in the size of nanoparticles containing oils, these differences may be related to the nature of nanoencapsulated oil, viscosity, hydrophobicity, or surface tension of the materials used for the preparation of nanoparticles (FARIA, 2014). Therefore, among the different methods of preparation of nanocapsules, nanoformulations may have a particle size between 100 and 300 nm (SCHAFFAZICK *et al*, 2003), a fact that can be observed in the nanocapsules produced in this study. Regarding the polydispersion index, a parameter that indicates homogeneity of particle diameter and stability, the nanoformulations showed compatible results and within the range considered adequate (values from 0.15 to 0.3) for formulations that have the purpose of application in topical products (MOHANRAJ; CHEN, 2006).

In the characterization of the nanocapsules another parameter evaluated was the zeta potential, which indicates the surface load of the nanoparticles produced, their values vary between ± 30 mV, these results reveal that there is good colloidal stability in solution, being interfered with only, by the composition of the particle, the dispersant agent, the pH or the ionic force of the formulation (MELO *et al*., 2010). The zeta potential values found in nanocapsules produced with microalgae oil

and vitamin E fit the range established by the literature, indicating a very good colloidal stability in the nanoformulations. Finally, the pH of the nanocapsules was evaluated and this parameter may vary over time, being a very important factor, because it evaluates the stability of the nanoformulations produced (KISHORE *et al*., 2011). The pH of the nanocapsules showed values that are demonstrated in the previous table, which indicate that these nanoformulations can be used for topical administratin, when they aim at this function, and the pH of the skin varies in the range of 4 to 7 (ALVES *et al*., 2007).

IN VITRO TEST ANALYSES

Cellular viability and PicoGreen

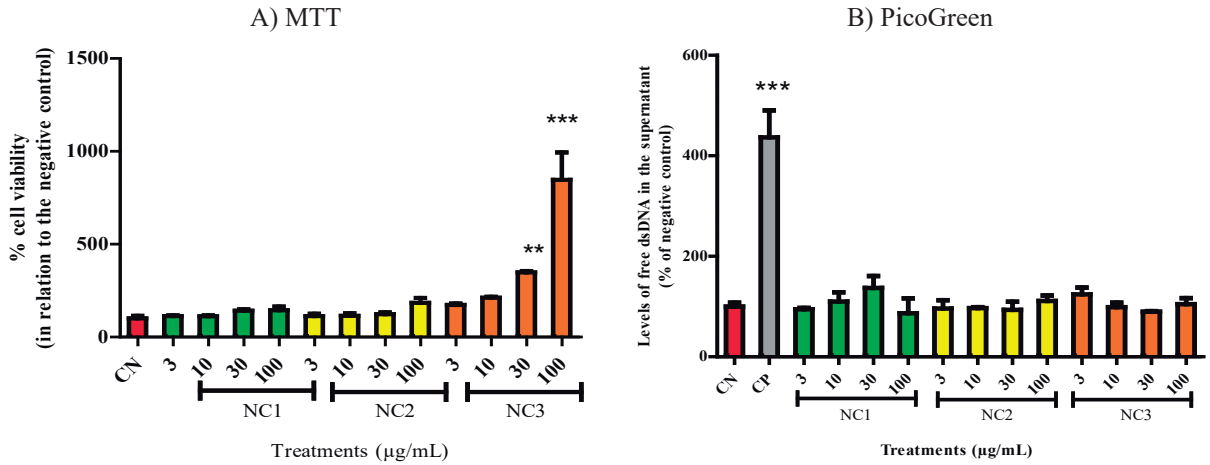
Nanocapsules can be easily absorbed by cells in the human body when applied topically, so it is necessary to verify the possible cytotoxicity potential of the nanocapsules produced to investigate and ensure the efficacy and safety of nanoformulations at the cellular level (FRIEDRICH *et al*., 2015).

Figure 2 (A and B) shows the results obtained by the MTT and PicoGreen assays, performed with the nanocapsules containing microalgae oil and vitamin E, incubated for 24h. The MTT and PicoGreen methods indicate cell viability and DNA damage, respectively, being the second complementary method to the first.

The results demonstrated by the MTT (Figure 2A) indicate that there was no decrease in cell viability in relation to negative control in the treatments of the nanocapsules produced. Cell viability can be determined in four ranges, cell viability greater than 90% of the formulation is not considered cytotoxic; viability between 60 and 90% mildly cytotoxic; viability between 30 and 59% moderately cytotoxic and viability less than or equal to 30% considered extremely cytotoxic (KONG *et al*., 2009).

It was observed concomitantly that at concentrations of 30 and 100 μg/mL, that is, the highest concentrations of NC3 there was a behavior of cell proliferation/overestimation compared to the negative control, leading to two possible hypotheses: the first, the number of particles contained in the formulation results in absorbance with the same wavelength that is used to measure the colored product, thus increasing the reading of absorbance by the equipment; the second, which by the very specific properties linked to the application of nanoscience, such as the large surface area, may result in high adsorption power, therefore, the nanocarriers can remove the colored reagent that is in the cellular environment thus maximizing the cellular viability (STONE *et al*., 2009).

Figure 2 - MTT and PicoGreen with 24h incubation assay.



Legend: NC1= nanocapsule containing *Chlorella* oil; NC2= nanocapsule containing *Chlorella* oil plus vitamin E; NC3= nanocapsule containing vitamin E.

The PicoGreen method was used to quantify fluorescently whether DNA tapes will be released in the middle, derived from cell membrane disruption, revealing whether there is toxicity when, therefore, free DNA tapes are found expressively in the supernatant of the tested nanoparticles.

PicoGreen test results demonstrated that there was no dsDNA in the supernatant (Figure 2B) in any of the nanocapsules produced and tested, this indicates a genoprotective capacity of treatments with nanoformulations, explained by the presence of vitamin E (tocopherol) in the production of nanoparticles, a fact observed and proven in another study, in which the α-tocopherol can protect cells against DNA damage.

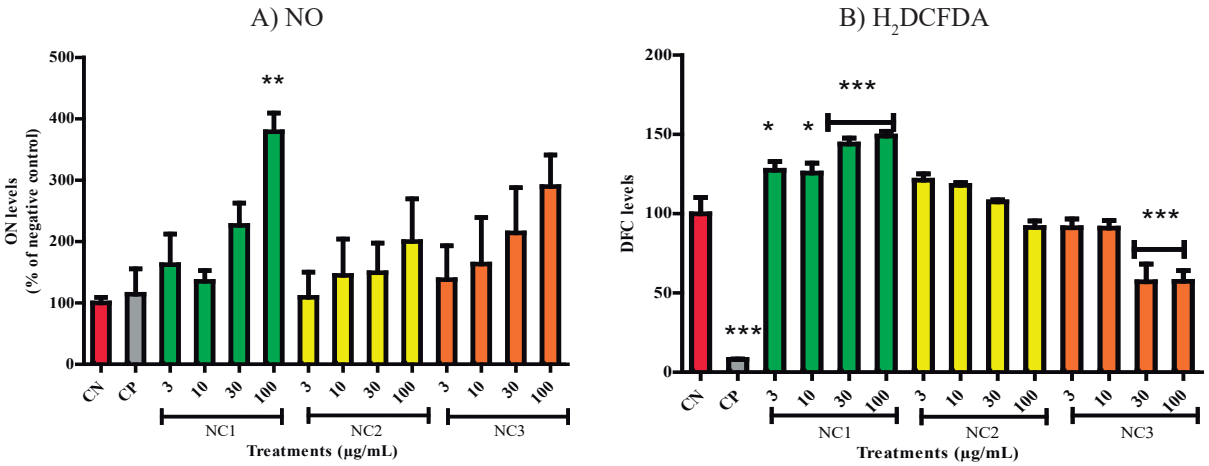
The study that bought this protective action used epidermal fibroblasts from young and elderly individuals, who underwent oxidative stress induced by hydrogen peroxide, after the administration of α-tocopherol, the damage was observed by the shortening of the fibroblast's telomeres in the untreated group, demonstrating an increase in oxidative stress, already in the treated group, protected the cells of young and elderly individuals (MAKPOL *et al.*, 2010). Therefore, the results found in this study corroborate the literature, since there is α-tocopherol in the nanocapsules produced (NC1, NC2, and NC3), either by adding vitamin E in the formulation, or in the composition of microalgae oil itself, which may contain this vitamin.

Nitric oxide production and DCFH-DA test

Through the evaluation of the results in Figure 3 (A), was observed that there was no detection of nitric oxide in relation to negative control in all treatments, although only in the highest concentration (100 µg/mL) of NC1, a significant difference was detected in relation to the control. Nitric oxide performs many biological activities, such as vasodilation in inflammatory processes (SRIRAM *et*

al., 2016). Taking this into consideration, higher levels of NO can be explained, since nitric oxide is one of the molecules that are present in lesions or wounds, playing an important role in healing and tissue repair, performing different physiological functions, such as vasodilation, inhibition of platelet aggregation and reduced adherence of endothelial leukocyte cell (RIZK *et al*., 2004).

Figure 3 - NO and DCFH-DA with 24h incubation assay.



Legend: NC1= nanocapsule containing *Chlorella* oil; NC2= nanocapsule containing *Chlorella* oil plus vitamin E; NC3= nanocapsule containing vitamin E.

Recognized as a healing phases modulator, nitric oxide shows the ability to modulate chemoattractive cytokines (IL 1, IL 6, IL 8, and TGF β1), to regulate collagen deposition, angiogenesis, and cell proliferation, and even apoptosis (AMADEU *et al*., 2008).

The dichlorofluorescein diacetate (DCFH-DA) test showed low levels of ROS figure 3 (B) by the analysis of 24h of incubation in relation to the negative control in NC2 and NC3, but there was a significant difference in the production of ROS at the highest concentrations of NC1. This fact corroborates with the nitric oxide levels test results, which shows a significant difference in the production of NO in NC1in higher concentrations. According to Barreiros *et al.* (2006) the human organism undergoes constant action of reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated from inflammatory processes, by some type of dysfunction, or even from food. Among the biomolecules targeted by these species are those found in cell membranes, DNA proteins, and RNA. It is well known that the action of oxidizing species on DNA causes mutation or even oncogenesis. However, the organism is protected by macro and micromolecules of the endogenous origin or from the diet. Therefore, the role of preventing attacks by ROS and RNS or regenerating damage caused in biological systems, are up to macromolecules such as tocopherols, carotenoids, and flavonoids. This fact may be a probable explanation for the low levels of ROS in NC2 and NC3 since they contain in the formulation vitamin E (micromolecule α tocopherol).

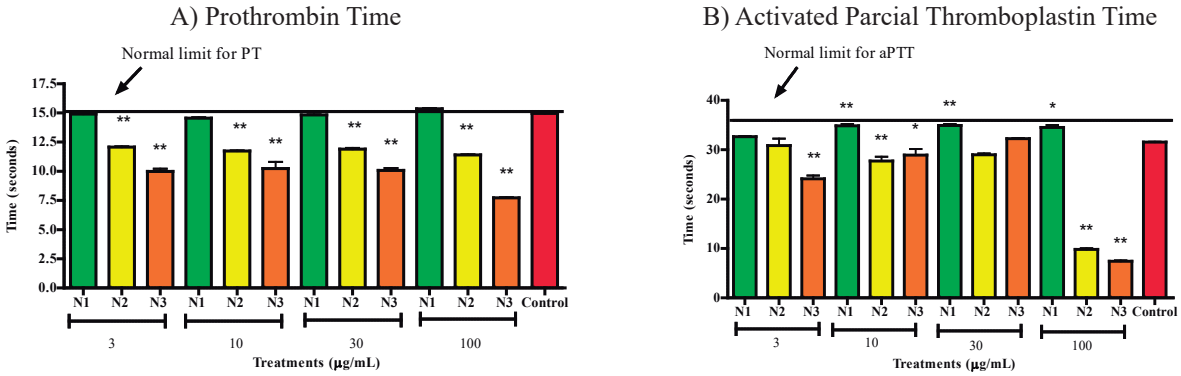
PT and aPTT

Prothrombin time and thromboplastin time are hemostasis tests that are important for the diagnosis of hemostatic abnormalities and in the monitoring of anticoagulant therapy. The "waterfall" model proposed by Macfarlane and David in 1964 to explain the physiology of blood coagulation, divides coagulation into an extrinsic pathway and an intrinsic pathway that converges at the activation point of factor X (beginning of the final or common pathway). Thus, the PT evaluates the extrinsic pathway of coagulation, then evaluates factors VII (extrinsic route) and X, V, II, and I (common pathway). aPTT assesses the intrinsic pathway of coagulation, then evaluates factors VIII, IX, XI, and XII, together with factors X, V, II, and I of the common pathway (FRANCO, 2001).

Baseline reference values for PT vary in an interval between 12 and 15 seconds and normal values for aPTT vary between 25 and 35 seconds, according to literature (SALVADOR-MORALES *et al*., 2009).

For the PT test, the nanocapsules tested showed significant variation in relation to the control, but remain within the allowed limit, and NC1 in all concentrations presented a value equal to the upper normal limit (average time 15.0 seconds), according to Figure 4 (A). In the aPTT test, NC1 showed a significant difference in the higher concentrations (10, 30, and 100 µg/mL), but remained at the normal limit of the test at all concentrations tested, according to Figure 4 (B). Between NC2 and NC3, significant variation was observed at different concentrations but remained within the permitted limit. Therefore, we can conclude that the nanocapsules produced and tested do not interfere with blood clotting in any of the pathways.

Figure 4 - Prothrombin time (PT) and Activated Parcial Thromboplastin time (aPTT) coagulation against the nanocapsules produced.



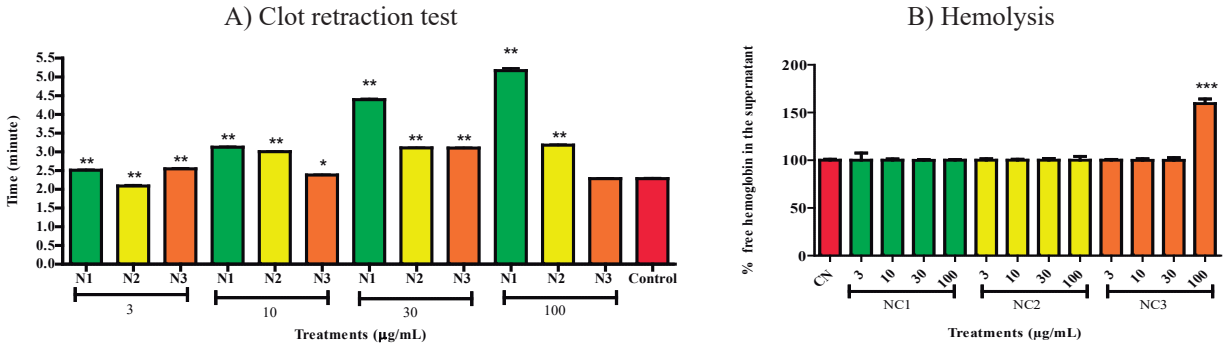
Legend: NC1= nanocapsule containing *Chlorella* oil; NC2= nanocapsule containing *Chlorella* oil plus vitamin E; NC3= nanocapsule containing vitamin E.

Clot retraction and hemolysis

According to Smyth *et al.* (2010), a long period of clot retraction may signal error at some point in the coagulation cascade, right after the interaction of integrin and the αIIbβ3 receptor with fribrin. Suggesting a long time of bleeding and an abnormal formation of platelet thrombus, a fact that can be observed in acquired or hereditary platelet disorders. The alteration of the αIIbβ3 integrin receptor is specifically involved in Glanzmann's thrombasthenia, hemorrhagic syndrome, also known as factor deficiency and platelet aggregation. A low number of platelets or fibrinogen, as well as high concentrations of red blood cells, extend the clot retraction time, and anti-platelet drugs can also prolong this retraction time. Low values or short retraction time suggest thrombosis or other pathologies.

Figure 5 (A) shows the results obtained by the clot retraction test, in which there was a small significant difference in the three nanocapsules tested and in the different concentrations in relation to the control, however the clot retraction test does not show that there may be alteration at some point of coagulation, corroborating the results found in the PT and aPTT tests.

Figura 5 - Clot retraction and hemolysis of the nanocapsules produced.



Legend: NC1= nanocapsule containing *Chlorella* oil; NC2= nanocapsule containing *Chlorella* oil plus vitamin E; NC3= nanocapsule containing vitamin E.

The hemolysis test is essential to define the degree of interaction that new materials can have with erythrocytes, as well as to define whether the interaction may compromise the structure of red cells and consequently occur in lysis and release of cytoplasmic content in the blood plasma (DOBROVOLSKAIA; MCNEIL, 2013). The hemolytic activity makes it possible to evaluate the viable potential of the action to be tested in causing lesions in the plasma membrane of cells (COSTA-LOTUFO *et al*., 2002).

The results obtained by the hemolysis assay showed that the percentage of free hemoglobin in the nanostructures supernatant resembles negative control, being non-toxic to blood plasma, only at the highest concentration of NC3 (100 µg/mL) there was a significant difference compared to the control, as can be seen in Figure 5 (B). Riéffel (2019) produced polymeric nanocapsules of oily nucleus, containing tucumã oil, by the nanoprecipitation method of the preformed polymer using PCL,

evaluated the toxicity profile by hemolysis assay, which demonstrated that there was a reduction in hemolytic activity in the same concentrations tested in this study (3, 10 and 30 μg/mL), and at the concentration of 100 μg/mL there was a significant difference in hemolytic activity compared to the negative control of the nanocapsules tested, corroborating the results found in this study.

Ratã *et al.* (2019) developed chitosan-based nanocapsules and functionalized with as1411 aptamer, with satisfactory results as average diameter between 100 and 267 nm and in cytotoxicity and hemolysis tests, results that prove the lack of toxicity and excellent hemocompatibility of the nanocapsules produced.

CONCLUSION

In this study, nanocapsules containing oil extracted from *Chlorella homosphaera* microalgae with the addition of vitamin E were produced in an innovative way, aiming at a possible topical application of this formulation in the future. They presented favorable and adequate chemical-physical characteristics for the nanoformulation produced. The *in vitro* evaluation of the nanocapsules revealed that they are not cytotoxic, as there was no decrease in cell viability, produced ROS and RNS at levels that can be explained by biological processes, and have not yet presented DNA damage. As well as the hemocompatibility assays were also satisfactory, without the interference of nanocapsules in the coagulation pathways. Therefore, it is concluded that the nanocapsules produced containing *Chlorella homosphaera* microalgae oil and vitamin E are viable and with a profile that does not present to genotoxicity. However, because it is innovative, it needs further studies and further research to confirm the beneficial properties of nanocapsules containing oil and vitamin E, evaluating their efficacy in cosmetic formulations of topical use.

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