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LAVANDULA ANGUSTIFOLIA ESSENTIAL OIL-LOADED NANOCAPSULES AND BIOLOGICAL ACTIVITY ON FIBROBLASTS

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ABSTRACT

Lavender essential oil (LEO) has shown promising properties for use in anti-aging cosmetics because of its ability to enhance collagen synthesis in fibroblasts. The nanoencapsulation of LEO may not only improve the permeation of the active ingredient into the skin, but also helps to prevent loss of volatile ingredients and to increase the formulation stability. The aim of this study was to develop LEO-loaded nanocapsules and to evaluate its biological activity. LEO-loaded nanocapsules were prepared using a 2^3 full factorial design by the preformed polymer interfacial deposition method. An analytical method for the evaluation of the efficiency of lavender oil encapsulation in nanocapsules was developed and validated by High Performance Liquid Chromatography analysis and demonstrated a maximum encapsulation efficiency of 43.5%. Analysis by Atomic Force Microscopy revealed spherical morphology of nanocapsules. LEO-loaded nanocapsules were also characterized by Dynamic Light Scattering technique and Streaming Potential. Hydrodynamic Diameter Average and Polydispersity Index were 259.5±7.4 nm and 0.148±0.03, respectively. The zeta potential observed was -32.12±0.97 mV. The LEO-loaded nanocapsules safety was demonstrated by cytotoxicity test in fibroblasts. Furthermore, the essential oil of *L. angustifolia* showed cytoprotection of fibroblasts towards the oxidation promoted by the peroxide solution. Therefore, the produced LEO-loaded nanocapsules demonstrated great stability and biological safety to be used as a cosmetic ingredient for anti-aging purpose.

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INTRODUCTION

The aging of the dermis, the deepest layer of the skin, is characterized mainly by abnormal production as well as by the increased degradation of collagen and elastin fibers (Kim, Chang & Lee, 2019; Bocheva, Slominski & Slominski, 2019), which results in wrinkles and sagging, the most visible signs of skin aging (Eckhart, Tschachler & Gruber, 2019; Im *et al.,* 2019). This complex biological process is directly related to intrinsic and extrinsic factors, the best known of which are exposure to UV radiation, tobacco smoke and pollution, and the consequent oxidative stress (Ha *et al.,* 2019; Silva *et al.,* 2019). Oxidative stress is caused by the excessive generation of reactive oxygen species (ROS), such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2).

In this situation, these ROS cause damage to DNA, lipids and cellular proteins (Im *et al.,* 2019; Kozics *et al.,* 2017). Damage to the skin is also a consequence, as ROS increases the inflammatory process and consequently activates transcription factors, causing damage to the extracellular matrix (ECM) due to the deregulation of proteolytic degradation (Pillai, Oresajo & Hayward, 2005). ROS are usually fought by an antioxidant defense system present in the body (Zillich *et al.,* 2015). Its effects can be balanced by antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), as well as by other antioxidants, which maintain the balance of this oxidative balance, neutralizing or removing these species (Kozics *et al.,* 2017). Antioxidant molecules derived from natural products has the property of eliminating or neutralizing these species, protecting against damage mediated by radicals (Kim *et al.,* 2019). Aged and photoaged skin has a decreased antioxidant defense mechanism; consequently it has an increased inflammatory process that promotes damage to the

skin, inducing wrinkling (Kim, Chang & Lee, 2019). Therefore, cosmetics developed to reduce aging and photo-aging damage to the skin must act as antioxidants to prevent the action of ROS (Bocheva, Slominski & Slominski, 2019; Pillai, Oresajo & Hayward, 2005). A recent study showed that Lavender essential oil has a protective antioxidant effect in cell culture, reducing DNA-induced oxidation (Kozics *et al.,* 2017). Belonging to the Lamiaceae family, *Lavandula angustifolia*, is commonly known as lavender and is widely used for its relaxing and antibacterial properties (Im *et al.,* 2019). Several variable chemical substances constitute essential oils, which are volatile and complex liquid mixtures predominantly biosynthesized by aromatic plants. Essential oils are generally composed of diterpenes, monotepenes, and sesquiterpenes (Carvalho, Estevinho & Santos, 2016). The properties of lavender essential oil are primarily related to the presence of linalool and linalyl acetate, and secondarily to the presence of lavandulol, geraniol, bornyl acetate, borneol, terpineol, and eucalyptol or lavandulyl acetate (Białoń *et al.,* 2019).
There are also some dermatological uses of lavender essential oil, such as for skin conditions as eczema, psoriasis, dermatitis and allergies (Cavanagh & Wilkinson, 2002). Mori *et al*. (2016) demonstrated that topical application of *L. angustifolia* essential oil increased collagen synthesis by fibroblasts, predominant cells in the dermal layer. This is precisely the goal of most anti-aging interventions (cosmetic products and aesthetic procedures) (Sifaki *et al.,* 2020; Drąg-Zalesińska *et al.,* 2019).

However, components of essential oils are volatile (Drąg-Zalesińska *et al.,* 2019) so their nanoencapsulation can prevent their evaporation and protect against degradation while allowing their controlled release (Lopes *et al.,* 2018), increasing the effectiveness of the product. Nanocapsules have diameters smaller than 1 μm and this high surface area is responsible for their biological activities (Frank *et al.,* 2015). They are extensively used for topical applications due to these advantages (Sallam & Elzoghby, 2018). Nanocapsules consist of a polymeric shell enveloping an oily core (Ephrem *et al.,* 2014) and are composed of the active ingredient, polymer, surfactant, and water. The polymeric shell controls the release of the active ingredient. Poly(ε-caprolactone) (PCL) is among the polymers the most used in nanocarriers (Lopes *et al.,* 2018) because it is biocompatible, biodegradable and easily fabricated (Lee *et al.,* 2017). Since LEO-loaded nanocapsules were not found in the literature, the aim of this study was to develop and to evaluate polymeric nanocapsules containing *Lavandula angustifolia* essential oil aiming at its application as a cosmetic ingredient.

MATERIAL AND METHODS

Material: *Lavandula angustifolia* essential oil was obtained from WNF (São Paulo, São Paulo, Brazil); poly(e-caprolactone) (PCL) from Tai'an Health Chemical Co., Ltd.; acetone PA from Neon Comercial (Suzano, São Paulo, Brazil); Span 80® from from Sigma-Aldrich (St. Louis, Missouri, USA); Tween 80®, standard of ± linalool (96%), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), cell culture medium (RPMI 1640), antibiotics (penicillin /streptomycin) and fetal bovine serum (SBF) from Sigma-Aldrich (St. Louis, Missouri, USA); acetonitrile HPLC-grade from J.T.Baker (Phillipsburg, New Jersey, USA) and ultrapure water purified by a Milli-Q purification system from Millipore (Milford, Connecticut, USA).

Methods

Encapsulation efficiency analysis: The linalool quantification was carried out using an Agilent 1100 series (Santa Clara, California, USA) HPLC system coupled to diode-array detector (DAD). The chromatograms were recorded and analyzed through Agilent Chemstation version B.04.03-16 and the chromatographic separation was achieved in Zorbax® Eclipse XDB-C18 (4.6x150 mm, 5 μm). The mobile phase water (mobile phase A) and acetonitrile (mobile phase B) eluted at a flow rate of 1 ml/min at 25º C. A linear gradient of 30% to 65.9% B from 0-20 minutes, held at 100% B from 20-25 minutes and then set to initial conditions of 30% B for 5 minutes. The injection volume was 10 μL, being the analytes detected at 210 nm. The method was validated according ICH Q2 (R1) guidelines. The following parameters were evaluated: selectivity, linearity, matrix effect, limits of detection and quantification (LoD and LoQ), accuracy, precision and robustness. All statistical analysis were carried out by using Minitab version 17.1.0, (LEAD Technologies, Inc. North Carolina, USA) and Prism version 6.01 (GraphPad Software, Inc. California, USA). The solution stability was performed to evaluate the potential loss of linalool content over time in a prepared sample. To assess it, a triplicate linalool was monitored during every hour for five hours. After the development of the analytical methodology, the encapsulation efficiency (EE%) was achieved by indirect quantification of linalool in the supernatant after centrifugation (2854 g, during 1 h) of the nanocapsules dispersion in comparison with the essential oil.

Preparation of Lavandula angustifolia essential oil-loaded nanocapsules: *Lavandula angustifolia* essential oil-loaded nanocapsules dispersions (NCs) were prepared by the interfacial deposition of preformed polymer method (Fessi *et al.,* 1989). Briefly, an organic phase composed by PCL, *Lavandula angustifolia* essential oil, Span 80® and acetone was dropped into an aqueous phase composed by Tween 80® and ultrapure water, under magnetic stirring. Then, the obtained dispersion was evaporated until reaching a final volume of 30 mL. Blank nanocapsules (BNCs) were prepared using capric/caprylic triglycerides (CCT) instead of *L. angustifolia* essential oil. In order to optimize the preparation of nanocapsules a 2^3 full factorial design was used. The three factors evaluated were the concentration of PCL, Span 80® and Tween 80®, while adding a fixed amount of acetone (20 g), essential oil (500 mg) and water (50 g). Each factor was set at a low (-) and high (+) level according to its concentration, with the following independent variables: 40 mg (-) and 80 mg (+) for PCL, 60 mg (-) and 100 mg (+) for Span 80®, and 150 mg (-) and 250 mg (+) for Tween 80®. In total, eight nanocapsules were prepared according to design, with EE% being the monitored variable. Additionally, a response surface was built in order to illustrate the optimization, along with the significance of effects through ANOVA. The analysis was carried out using Design Expert® 11 Stat-Ease, Inc. (Minneapolis, USA).

Fourier Transform Infrared Spectroscopic Evaluation: The chemical characterization of raw materials and dispersion of blank nanocapsules were performed by Fourier Transform Infrared Spectroscopy (FTIR) in Alpha-P (BRUKER®) equipment, in transmittance mode, using the Attenuated Total Reflectance (ATR) technique and the following conditions: 24 scans, 4 cm^{-1} resolution and spectral range of 4000 – 400 cm^{-1}.

Characterization of nanocapsules: The formulation of nanocapsules that presented the highest encapsulation efficiency was characterized in terms of morphology, hydrodynamic diameter, polydispersivity, zeta potential and cytotoxicity. Topographic images of nanocapsules were obtained by Atomic Force Microscopy (AFM) using an Agilent Scanning Probe Microscope (Model 5500) with tapping mode. The images were obtained in 1x1μm area using a NSC15 (MikroMash) cantilever with a resonance frequency of 320 kHz and Force Constant of 40 N/m. The particles were diluted to 0.020% (w/w) with ultrapure water, and 200 μL were deposited on freshly mica. Hydrodynamic diameter and polydispersity index of the nanocapsules dispersion were determined by Dynamic Light Scattering (NanoDLS, Brookhaven®). Prior to the analysis, the samples were diluted 1:100, in ultrapure water. Zeta potential was determined by streaming potential (Particle Charge Titration Analyzer, Stabino®). Prior to the analysis, the samples were diluted 1:1000, in ultrapure water.

Cell culture: The NIH 3T3 mouse embryo fibroblast cell line was cultivated in an RPMI culture medium supplemented with 10% of fetal bovine serum (FBS) and 100 $U.mL^{-1}$ of an antibiotic solution (penicillin/streptomycin). Then, the cells were incubated in a humidified athmosphere with 5% CO_2 at 37 ^oC.

Cytotoxicity Assay: Cytotoxicity evaluation of the nanocapsules was performed *in vitro* using MTT assay (Mosmann, 1983). The cells were seeded into 96 well culture plates (1.10^3 cells/well) with supplemented medium followed by incubation during 24h for adhesion. Then, the same supplemented medium was added containing *Lavandula angustifolia* essential oil-loaded nanocapsules (10, 50, 100, 150 and 200 µg.mL^{-1}) followed by incubation. 24 hours later, the medium was replaced by the MTT solution (0.05 mg.mL^{-1}) and cells were incubated for 3 h. Then, 100 uL of DMSO were added for the solubilization of formazan crystals formed and the absorbance was measured at 540 nm using a microplate reader (Thermo Scientific Multiskan®FC, Thermo Fisher Scientific, Vantaa, Finland). The optical density (OD) values were converted to % of cell viability using the Equation 1:

(Cell viability % = ($OD_{treatment}$/$OD_{control}$).100)

Cytoprotection Assay: The cells were plated in a 96-well plate (1.10^3 cells/welll) and incubated for 24 hours with supplemented medium. Then, after removing the medium, the cells were exposed to *Lavandula angustifolia* essential oil (50, 100, 250, 500, 1000 µg.mL^{-1}) for another 24 hours. Then, the cells were exposed to oxidation by adding a solution of hydrogen peroxide (H_2O_2) to pre-established concentration of 20 µM in phospate buffered saline (PBS) that promoted a 50% reduction in cell viability. Then, the solution with peroxide was removed and the cells had their viability assessed by the MTT method (0.05 mg.mL^{-1}) (Maluf *et al.,* 2018).

Statistical Analysis: The statistical analysis of the data was performed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test at a significance level of $p < 0.05$. GraphPad Prism 6.01 was used for graphical analysis (GraphPad Software Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

Encapsulation efficiency analysis: The proposed method was selective since the linalool does not share the retention time with another compound present in matrix with 99.68% of peak purity. The linear regression analysis showed that the method is linear at the range of 1294 and 99.6 µg.mL^{-1}. The coefficient of determination (R-squared) was 0.9983. The regression fit (p-value < 0.05) and lack-of-fit (p-value = 0.316) indicate that the linear model is significant and the error is random, respectively. The data has shown to be homoscedastic (p-value = 0.735, Brown-Forsythe test) and with residual normality (p-value = 0.311, Anderson-Darling test). Through F-test (Table 1), the comparison between calibration curve of linalool in matrix and in solution showed equal variances at high (p-value = 0.106), medium (p-value = 0.909) and low (p-value = 0.158) levels of concentration. Then, the comparison between angular coefficient of calibration curve in solution and in matrix was not statistically significant (p-value = 0.496, Student's t-test), implying in the absence of matrix effect. The LoD obtained from the regression was 41.07 µg.mL^{-1} for linalool and the LoQ was fixed at the lower concentration of calibration curve (99.6 µg.mL^{-1}, s/n 234.9). Accuracy ranged from 98.92 to 101.19%. The RSD obtained in repeatability were between 2.50 and 1.56%. The intermediate precision between different days showed no statistical significance (p-value > 0.05 through F-test). From the Pareto chart and half-normal plots of robustness tests, it was possible to detect a significant effect in linalool content (wavelength detection) and retention factor (temperature and gradient slope). The change of ± 0.1 mL.min^{-1} of the flow rate does not influences the content and retention factor significantly. Within five hours, the linalool content showed no statistical significance through Student's t-test (p-value = 0.116). Therefore, the chromatographic samples were considered stable in a period of five hours. The validated chromatographic method was used to quantify linalool on supernatant of eight different nanocapsules formulations according to the full factorial design. The EE% obtained from all eight nanocapsules were used to build the response surface (Figure 1), which varied within the range of 3.68% to 43.51%.

This encapsulation efficiency range is similar to the range obtained by Pinto *et al.* (2016) who produced PCL nanocapsules containing L. sidoides essential oil by the emulsion-diffusion method.

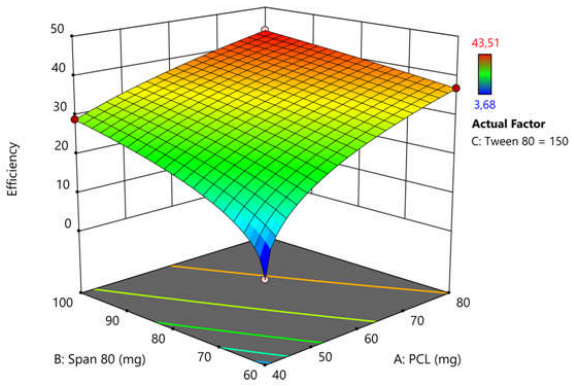

Figure 1. Surface response for encapsulation efficiency using PCL, Span 80 and Tween 80 in mg

All data of the surface response regression model were transformed using a power of 2.81. All effects up to second order were significant (p-value < 0.05). The R-squared and R-squared adjusted obtained were 1, with predicted R-squared of 0.9997, suggesting suitability of the regression. The regression coefficients are shown in equation 2. Based on the results obtained, all factors (PCL, Span 80 and Tween 80) affect the yield of encapsulation efficiency. The combination that yielded the best efficiency (43.51%) consisted in 80 mg of PCL, 100 mg of Span 80 and 150 mg of Tween 80 along with 20 g acetone, 50 g water, 500 mg essential oil, resulting in the combination of NC05 nanocapsule. The NC05 undergone to further characterization.

($EE\%^{2.48}$ = 5905.22A + 3183.63B - 2557.94C + 520.50AB - 7265.49AC - 3735.90BC + 17076.14)

Spectroscopic Evaluation: From the FTIR spectrum obtained (Figure 2) it was possible to observe the characteristic bands of the raw materials (Csp^3-H stretch bands in the region of 2900 cm^{-1} and C=O stretch bands in the region of 1700 cm^{-1}) and the total removal of the organic solvent from the dispersion of nanocapsules at the end of the process. Furthermore, it is possible to verify that there were no chemical reactions between the raw materials, since we did not observe the formation of new peaks in the spectrum of nanocapsules.

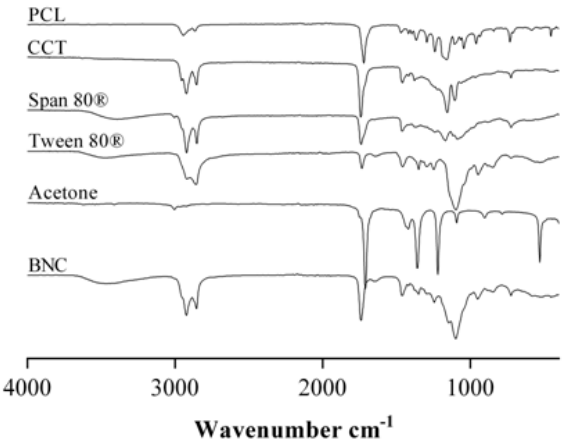

Figure 2. FTIR spectra of PCL, CCT, Span 80®, Tween 80®, Acetone and BNCs

Nanocapsules characterization: NC05 presented a spherical shape (Figure 3) and mean hydrodynamic diameter of 259.5 ± 7.4 nm, close in size to those found by Ephrem *et al*. (20) who produced PCL nanocapsules containing rosemary essential oil.

Table 1. Equation coefficients and significance of the effects through ANOVA

	Coded coefficient	Sum of Squares	df	Mean Square	F-value	p-value
Model	$(Efficiency)^{2.81}$ =	$9.485x10^8$	6	$1.581x10^8$	30523.82	0.0044
A	+ 5905.22 * A	$2.790x10^8$	1	$2.790x10^8$	53870.10	0.0027
B	+ 3183.63 * B	$8.108x10^7$	1	$8.108x10^7$	15657.37	0.0051
C	- 2557.94 * C	$5.234x10^7$	1	$5.234x10^7$	10107.78	0.0063
AB	+ 520.50 * AB	$2.167x10^6$	1	$2.167x10^6$	418.53	0.0311
AC	- 7265.49 * AC	$4.223x10^8$	1	$4.223x10^8$	81546.34	0.0022
BC	- 3735.90 * BC	$1.117x10^8$	1	$1.117x10^8$	21560.78	0.0043
Intercept	+ 17076.14	-	-	-	-	-

Note: A = PCL; B = Span 80; C = Tween 80.

The polydispersion index value obtained was 0.148 ± 0.03, which characterizes a monodisperse formulation, and the zeta potential value found was -32.12 ± 0.97 at pH 4.29 ± 0.14, which indicates stability of the formulation since it prevents the aggregation of nanocapsules (Coviello *et al.,* 2015).

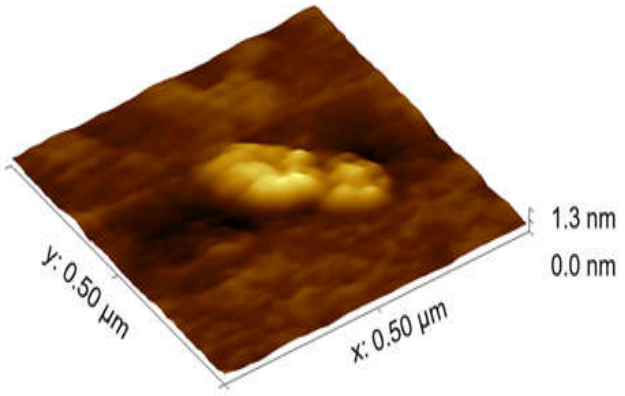

Figure 3. NC05 image by Force Atomic Microscopy

Cytotoxicity and Cytoprotection Assay: According the cytotoxicity analysis of NC05 by the MTT method (Figure 4) there was no statistically significant difference between the results obtained at the concentrations tested and the control test (NIH 3T3 mouse embryo fibroblast cell line treated only with the supplemented culture medium).

Similar results were found by Balasubramanian and Kodam (2014), who evaluated the cytotoxicity of the essential oil of *L. angustifolia* in the same cell line. As a result, NCs can be considered safe for skin application.

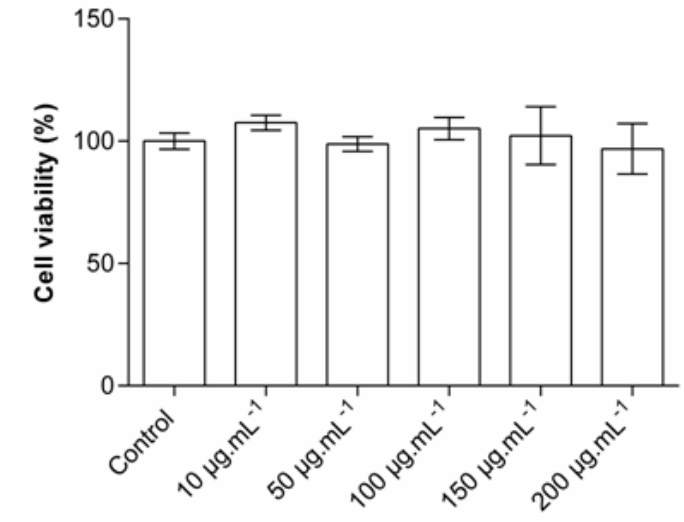

Figure 4. Cell viability (%) of NIH-3T3 mouse embryo fibroblast cell line treated with *Lavandula angustifolia* essential oil-loaded nanocapsules dispersions (MTT assay)

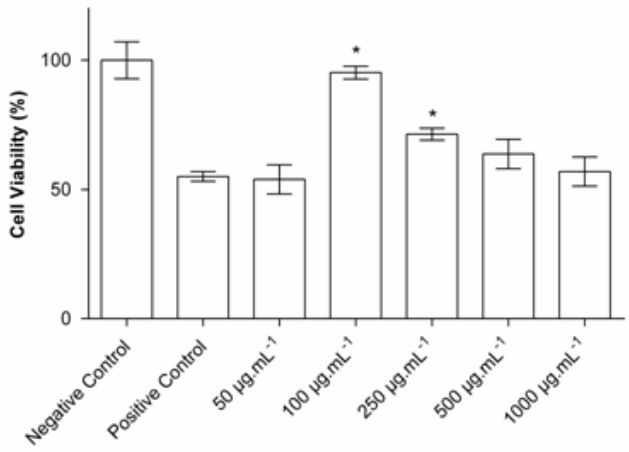

Figure 5. Cell viability (%) of NIH-3T3 mouse embryo fibroblast cell line treated only with culture medium (negative control), peroxide solution H_2O_2 (positive control) and with *Lavandula angustifolia* essential oil; * indicates P < 0.0001 against positive control

The cytoprotection assay evaluated the antioxidant protection offered by lavander essential oil to fibroblast cells after exposure to the oxidative damage induced by hydrogen peroxide. Results demonstrated by Kozics *et al*. (7) have already shown the DNA-protective effect against DNA strand breaks induced by H_2O_2 of *L. angustifolia* oil in hepatocytes cells. In this study, lavender essential oil showed a cytoprotective effect on the fibroblast cell line (Figure 5), being statistically significant (P <0.0001) at concentrations of 100 and 250 ug.mL^{-1} when compared to the positive control group (cells treated only with a 20 µM H_2O_2 solution).

As is known, hydrogen peroxide is an endogenous reactive specie of oxygen responsible for the oxidative damage of proteins, lipids and nucleic acids, inducing cellular damage. As a form of protection, the body has an antioxidant enzymatic mechanism against damage caused by free radicals, degrading H_2O_2 in water and O_2 (Murphy & Friedman, 2019). As a result, the essential oil of *L. angustifolia* prevented cell death induced by hydrogen peroxide, and can be considered a promising active ingredient for anti-aging cosmetics, preventing damage caused to dermal cells.

CONCLUSION

Lavender oil-loaded nanocapsules were obtained through factorial planning with an average hydrodynamic diameter of approximately 250 nm, unimodal distribution (polydispersion index 0.148 ± 0.03) and great stability (zeta potential -32.12 ± 0.97).

An analytical method for the evaluation of the efficiency of lavender oil encapsulation in nanocapsules was developed and validated. Furthermore, the essential oil of *L. angustifolia* showed interesting cytoprotective activity in fibroblasts against free-radical induced damage. As well, lavender nanocapsules showed absence of cytotoxicity in the same cell line. Hence, lavender oil-loaded nanocapsules proved to be a potential tool as a cosmetic ingredient for anti-aging purpose.

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