

Production, characterization and biological evaluation of nanocapsules containing tricresol formalin and their comparison with the free form

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Abstract

Ethnopharmacological relevance: Tricresol formalin is composed of 90% formaldehyde and 10% cresols, highly volatile, has action at a distance, has been used in endodontics since the 20th century, and it remains widely used in Brazil in dental treatments, in necrotic teeth and with periapical lesions. However, there is still controversy regarding the biological compatibility under the conditions of clinical use of this drug, as the studies carried out on this substance and its components are not consistent with its clinical use. Formaldehyde is reported as a potential cytotoxic substance, because when in direct contact with cells it is responsible for a cytogenotoxic response, so an alternative to increase stability and ensure the safe administration of this compound in direct contact with cells would be nanoencapsulation. The use of nanomaterials provides numerous advantages, as the main interests are increased solubility and drug release control. **Study objective:** This study aimed to produce and characterize nanocapsules containing tricresol formalin as active, evaluating and comparing the in vitro cytotoxic effect of free and nanostructured forms. **Materials and methods:** a nanoparticle was produced, optimization of the preparation method and characterization of nanocapsules containing tricresol formalin. Were performed antimicrobiological tests, tests for cell viability through the tetrazolium method assay (MTT), free radical production, double strand DNA damage, and nitric oxide production. **Results:** The formulation used did not show toxic behavior against human peripheral blood mononuclear cells and showed a significant reduction in the toxicity of tricresol formalin in human fibroblast cells. The nanostructures showed values similar to the free form for antimicrobial activity. The nanoparticles showed mean particle size of 192.3 ± 2.5 nm, PDI of 0.101 ± 0.013 , zeta potencial of -17.7 ± 2.8 mV, and pH of 5.48 ± 0.3 . **Conclusion:** Thus, it is evident that nanocapsules containing tricresol formalin can become a safer alternative for use within endodontics.

Keywords: Antimicrobial activity; Cytotoxicity; Formaldehyde; Nanotechnology

1. Introduction

Tricresol formalin has been used in endodontics since the 20th century (RIBEIRO; MARQUES; SALVADORI, 2004; SILVA et al., 2012) and is indicated in cases of pulp necrosis with the presence of periapical lesion or in emergency procedures (LOPES; SIQUEIRA, 2004). Studies have been carried out on this drug and its components, but there is still controversy regarding its biological compatibility under conditions of clinical use.

Formaldehyde-based solutions, such as tricresol formalin, have been reported as potential cyto and genotoxic solutions due to the release of substances through their vaporization (RIBEIRO; MARQUES; SALVADORI, 2004). None of the experiments performed were able to determine the real biological response of cresols including tricresol formalin (HAUMAN; LOVE, 2003), however, despite their limitations, laboratory tests are adequate to verify mechanisms involved in the biocompatibility of this drug (LEIRSKAR; HELGELAND, 1981).

The introduction of formaldehyde in dentistry dates to the 18th century. It is difficult to accurately identify the mechanism(s) responsible for formaldehyde-induced microbial inactivation. The main substances derived from formaldehyde are formocresol, used in pediatric dentistry, in deciduous teeth on the vital pulp, and tricresol formalin, indicated in permanent teeth with pulp necrosis and periapical lesion. Tricresol formalin is a compound based on formaldehyde and cresol. Formaldehyde is a gas produced by the incomplete combustion of methanol, it is soluble in water, presenting an aqueous solution of approximately 38% to 40% of formaldehyde by weight, called formalin. Tricresol reduces the irritating properties of formaldehyde. This material is derived from "black coal", being a potent antiseptic and considered less toxic than formaldehyde (THOMAS et al., 2006).

Tricresol formalin, composed of 90% formaldehyde and 10% cresol, is still being used in Brazil in endodontic treatments of necrotic teeth with periapical lesions, it is used as a delay dressing in the pulp chamber of permanent teeth with pulp necrosis and periapical lesions. This medicine provides disinfection of the canal, providing conditions to repair periapical lesions. Bactericidal action occurs at a distance, by releasing formaldehyde vapors. This material is a potent antiseptic and is considered less toxic than formaldehyde (SOUZA et al., 1978).

Tricresol formalin was the drug that demonstrated effective antimicrobial action on a cocktail of germs collected from the root canals of infected human teeth, due to the release of vapors, which is directly related to the volume of medication used. Consequently, such vapors may extend to a more distant region, presenting a bactericidal action at the level of periapical tissue cells. Thus, as there is a direct relationship between the bactericidal power and the power of tissue irritation, germicides that act at a distance will certainly produce a lesion of drug origin in the periapical tissues, and this lesion is sometimes greater than that produced by micro-organisms. (SOUZA et al., 1978).

Tricresol formalin can control endodontic infection, but it can cause irritation or destruction of living tissue (THOMAS, 2006). It is used between endodontic treatment sessions teeth with pulp necrosis, to reduce the number of micro-organisms. It is soaked in a small cotton ball placed in the pulp chamber, removing the excess, on a sheet of absorbent paper, before adding it. The effect is exerted at a distance from the application site, through the volatilization of its components (MORAIS et al., 2001).

Esberard et al. (1993) clinically evaluated 50 human teeth with chronic periapical lesions that, after coronary opening, received dressings with tricresol formalin from different manufacturers. The teeth were analyzed clinically and radiographically every 30 days, in periods varying from 2 to 9 months, seeking to observe, mainly: the presence or absence of painful symptoms and signs of reduction in the lesion or repair. The authors could observe that, in 34 of the 50 cases observed, there was regression or disappearance of the periapical lesion and that tricresol formalin did not determine the appearance of painful symptoms, when used for long periods, in teeth with chronic periapical lesions.

Studies prove the cyto-genotoxicity of tricresol formalin when in direct contact with cells, but the mechanism of action of tricresol formalin is through the release of formaldehyde vapors, which is highly lipophilic and has an antimicrobial action. Cresol vapors have an anti-inflammatory action and this combination has excellent clinical results. In this context, this study proposes the preparation of a nanocarrier containing tricresol formalin as active, as there is evidence of a reduction in adverse effects when drugs are nanostructured, either by controlled release or even by avoiding direct contact with the cellular environment (GUTERRES; ALVES; POHLMANN, 2007).

Nanotechnology has begun to change the scale and methods of release of assets. Through it, new formulations and new routes for the release of drugs can be developed, which may have their therapeutic potential increased, as they are released in previously inaccessible places in the body (GROOT; LOEFFLER, 2006).

The use of nanomaterials enables the drug to be released in specific targets, providing numerous advantages, such as: increased solubility, resistance to gastric enzymes, release control or vectorization of active agents, thus directing research for the development of more sophisticated and personalized medicines (RAMOS; PASA, 2008).

When systems are manipulated at the nanoscale, new properties for materials emerge and thus enable innovations in energy, manufacturing and medical treatment. These nanometric systems have a high surface area, enabling a homogeneous and controlled release of drugs, providing a more effective therapeutic response directly at the site of action for a prolonged period, by increasing its contact area. Due to the vectorization in organs, tissues and cells, these systems have the advantage of reducing the side effects presented by the drug in the conventional way (GUTERRES; ALVES; POHLMANN, 2007).

There are several nanocarrier systems, such as nanoemulsions, polymeric nanoparticles (nanocapsules and nanospheres), liposomes and lipid complexes (solid lipid nanoparticles and nanostructured lipid carriers) (MORA-HUERTAS et al., 2010; DIMER et al., 2013).

Since the 1980s, several drug delivery systems have been developed (HANS; LOWMAN, 2002). Encapsulation has become an interesting option as a drug release strategy, due to the possibility of reducing systemic toxicity, protecting molecules that are vulnerable to degradation in a physiological environment, inducing, through specific properties, controlled release or even masking a taste unpleasant (WATTS; DAVIES; MELIA, 1990).

In dentistry, it is possible to use nanotechnology to promote local anesthesia in a less invasive way (PATIL; METHA; GUVVA; 2008), enhance root canal disinfection therapy (KISHEN et al., 2008), treat dentin hypersensitivity, increase efficacy of drugs and grafts in the treatment of periodontitis (PATIL; METHA; GUVVA; 2008).

This work is also justified by the scarcity of current studies on the action of tricresol formalin, since there are few reports described in the literature when compared to the use to the use itself in clinical procedures. It should be noted that this work is the first in the literature to convey tricresol formalin (formaldehyde) in nanostructures.

2. Materials and methods

2.1 Production of Nanostructures

The production of nanostructures containing tricresol formalin (NCTF) was performed on Nano Laboratories of the University Franciscan. The tricresol formalin used as active for the formulations is commercially available from IODONTOSUL, lot: 2223, manufactured on 07/31/2017, valid on 07/31/2019. The preparation of nanostructures was performed as suggested by Fessi et al. (1989), revised by Mora-Huertas et al. (2010), using the nanoprecipitation method, involving the mixing of an organic phase in another aqueous phase with the necessary adaptations. The choice of polymer that was used is based on the indication proposed by Andronescu and Grumezescu (2017).

After evaluating the reproducibility and stability of the NCTF formulation, the constituent concentrations were defined. The organic phase consisted of acid polymer poly- ϵ -caprolactone (PCL) (50 mg), acetone (25 mL) as solvent, the active ingredient tricresol formalin (25 mg), surfactant sorbitan monstearate (Span60®) (50 mg) and the triglyceride oil of caprylic/caproic acid (250 mg). The aqueous phase was composed of the stabilizer polysorbate 80 (Tween80®) (100 mg) and ultrapure water (50 mL).

The organic phase was added slowly over the aqueous phase with the aid of a small funnel. The resulting suspension was kept under stirring for 10 minutes and then concentrated under low pressure to a volume of 25 ml, with the aid of a rota-evaporator, to obtain a concentration of tricresol formalin equal to 1 mg/mL. The production of white nanostructures (NB) without the active tricresol formalin was also carried out. Tricresol formalin was handled using an activated charcoal mask, protective goggles and gloves. In Table 1, all the components that were used to produce the optimized formulation of the nanostructures are presented, formulation which was used to carry out the tests performed in the experimental design.

Table 1. Composition of suspensions of nanostructures containing tricresol formalin, for a final volume of 25 mL.

Organic phase	Concentration	Aqueous phase	Concentration
Tricresol Formalin	0.025 g	Polysorbate 80	0.100 g (0.4%)
PCL	0.050 g (0.2%)	Ultrapure water	50 mL
Sorbitan monstearate	0.050 g (0.2%)		
Caprylic acid triglyceride	0.250 g (1%)		
Acetone	25 mL		

2.2 Physical-chemical characterization of the suspension of nanostructures containing tricresol formalin

The characterization of nanostructures containing the (NCTF) was performed according to the following

physical-chemical parameters: mean particle size distribution, polydispersion index, zeta potential and pH determination.

2.2.1 Determination of particle diameter and polydispersion index

The diameter and polydispersion index (PDI) determinations of the produced nanoparticles were carried out by dynamic light scattering (Zetasizer® nano-ZS model ZEN 3600, Malvern), after dilution of the dispersions in water (500 times, v/v). The results were expressed in nanometers (nm).

2.2.2 Zeta potential

The zeta potential was determined using electrophoresis techniques using the equipment Zetasizer® nano-Zs model ZEN 3600, (Malvern, England), after dilution of the formulations in a 10 mMol/L NaCl solution (500 times v/v). The results are expressed in millivolts (mV) measurements from three different suspensions.

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2.2.5 pH determination

The pH of the formulations was determined using a previously calibrated Digimed® potentiometer (with pH 4.0 and 7.0 standard solutions). The results are expressed from the reading of three different suspensions.

2.2.6 Stability of formulations

To determine stability, nine NCTF formulations were prepared with a concentration of 1 mg of the active to 1 mL of solution (1 mg/mL), separated into 3 batches, stored in amber vials and under different conditions; climatic chamber (40 ± 2 °C) (CC), refrigeration (4 ± 2 °C) (RE) and room temperature (25 ± 2 °C) (TA) (ANVISA, 2004). At times 0, 7, 15, 30, 60 and 90 days, the following parameters were evaluated: determination of the mean particle diameter, polydispersion index, zeta potential and pH according to the methodology already described in the previous items.

2.3 Experimental design

Tests were performed with the techniques of disk-diffusion in agar and broth microdilution to evaluate the antimicrobial activity of NCTF, and NB (nanostructures that have the same constituents as NCTF, but

without the presence of the active Tricresol Formalin), comparing with free tricresol formalin (TFL), based on the results obtained, a prospective *in vitro* study was conducted in which the commercial strain, HFF-1 ATCC® SCRC-1041™ (human fibroblasts) and peripheral blood mononuclear cells (PBMC) were used as an experimental model in order to investigate the potential cytogenotoxic effect of tricresol formalin, comparing the forms of TFL, NCTF and NB.

2.4 Antimicrobial activity

2.4.1 Evaluation of antimicrobial activity by disk-diffusion

The disc-diffusion method was proposed by Bauer et al. (1966). Inocula containing *Enterococcus faecali* ATCC were seeded onto Petri dishes containing Mueller Hinton agar. Afterwards, 10 µL of the formulations containing the NCTF at concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.062 mg/mL, and 0.031 mg/mL, the white nanostructures, and free tricresol formalin were added to the discs, which in turn were deposited on the plate. After adding the disks, the plate was incubated for 24 hours at 37 °C in a bacteriological incubator, after 24 hours, the size of the halos formed was verified with the aid of a pachymeter.

2.4.2 Evaluation of antimicrobial activity by broth microdilution

The antimicrobial activity was determined by the Minimum Inhibitory Concentration of bacterial growth, according to the methodology of broth microdilution, as recommended by the protocol of the Clinical and Laboratory Standards Institute (CLSI) (2015), the microorganisms chosen were *Enterococcus faecali*, *Pseudomonas aeruginosa*, and *Escherichia coli*.

Sterile flat-bottom microplates containing 96 wells were used. Each well received 20 µL of the inoculum suspension on a 0.5 McFarland scale, that is, 1.5×10^8 CFU/mL, 100 µL of the culture medium, BHI broth, and 100 µL of treatment, being this or nanostructures containing Tricresol Formalin or Free Tricresol Formalin, concentrations of 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, 0.312 mg/mL, 0.156 mg/mL; 0.078 mg/mL; 0.039 mg/mL; 0.019 mg/mL, the positive control was the inoculum plus 200 µL of broth, without the addition of the treatment, and the negative control was only the culture medium, 1 well was tested with only 100 µL of broth plus 100 µL of white nanostructures and 20 µL of inoculum to evaluate the activity of white nanostructures.

The plates were incubated at $35 \text{ °C} \pm 2 \text{ °C}$ for 16-20 hours in an incubator, the interpretation of the result was made by checking the smallest amount of treatment that was able to inhibit the growth of microorganisms, the determination was made through visual method, by checking the turbidity of the medium or by the presence of cell clusters, and if the medium is cloudy it indicates that the treatment concentration was not sufficient to promote the inhibition of the growth of microorganisms.

To confirm the presence of viable microorganisms at non-inhibitory concentrations, the dye TCC (2,3,5-triphenyltetrazolium chloride) was added to each well of the plate in a volume of 10 µL, which reflects the activity of dehydrogenase enzymes involved in the breathing process. By hydrogenation of 2,3,5-triphenyltetrazolium chloride, a red, stable and non-diffusible substance, triphenyl formazan, is produced in living cells (GABRE, 1986). This made it possible to distinguish the live samples, colored red, from the dead ones that had no color change.

2.5 Cytotoxicity assessments

The use of “*in vitro*” tests, using cell cultures from different tissues, can be an alternative for evaluating the toxicity of various substances. Cell viability and damage to structures are used as analysis parameters, which can assess the biocompatibility of a substance in different materials (NOGUEIRA et al., 2014). For materials to be considered non-cytotoxic, through *in vitro* tests, they must keep cells alive and not affect their cellular functions (ARAÚJO et al., 2008).

The cells were grown in a 5% CO₂ incubator at 37 °C in the premises of the Cell Culture Laboratory of the Franciscana University. Lineages of Peripheral Blood Mononuclear Cells (PBMC) (obtained from waste samples from the School Laboratory of Clinical Analysis of the Franciscana University, under the approval of the Ethics Committee for Research with Human Beings of the institution (CAAE: 31211214.4.0000.5306) were cultivated in RPMI 1640 medium with the addition of 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen® and Sigma® products). Cell culture and treatment were carried out similarly to that described by Falanga et al. (1996).

After thawing the HFF-1 cells (ATCC ® SCRC-1041™), this strain was maintained in polystyrene bottles (TPP) in DMEM cell culture medium, according to ATCC, containing 10% inactivated fetal bovine serum (Invitrogen) at 56 °C for 1 hour, 100 U/ml penicillin (Invitrogen) and 100 U/ml streptomycin (Invitrogen), at 37 °C in a humid atmosphere containing 5% CO₂.

Weekly replicates were performed in laminar flow, so that each bottle received 5 mL of medium with a fixed number of cells at the time of the replicate (2.0×10^5 cells/mL). The volume, with the number of cells above, was transferred to a new bottle with fresh medium.

After obtaining a satisfactory confluence for carrying out the experimental assays, the cells were seeded in 96-well plates and left for 24 h to adhere.

After 24 h cells were treated with the following concentrations 5000 µg/mL; 2500 µg/mL; 1250 µg/mL; 625 µg/mL; 312 µg/mL; 156 µg/mL; 78 µg/mL; 39 µg/mL; 19 µg/mL of Free Tricresol Formalin (TFL), nanostructures containing Tricresol Formalin (NCTF) or white nanostructures (NB), the PBMC received the same treatment, but on the same day they were seeded on their plates. After the treatments, the plates were incubated in a CO₂ oven at 37 °C for 24 hours.

For the evaluation of *in vitro* cytogenotoxicity, the following tests were used: MTT, dichlorofluorescein diacetate, Picogreen DNA and Nitric Oxide.

2.6 Toxicity assessment

2.6.1 Evaluation of cell proliferation rate by MTT assay

The cell proliferation rate was investigated using the tetrazolium method assay (MTT) in a procedure similar to that described by Krishna et al. (2009). Cell viability and cell proliferation measurements are performed with this method. In MTT, yellow (3,4,5-dimethylthiazolyl-2)-2-tetrazolium and 5-diphenyltetrazolium bromide are chemically reduced through activation of the mitochondrial dehydrogenase enzyme that generates NADPH. Therefore, it is a marker of mitochondrial function. Thus, in the presence of a greater number of cells or greater viability, the MTT reaction produces a purple color that can be quantified by spectrophotometry. Absorbance values lower than the negative control indicate a reduction in cell proliferation rate. On the contrary, higher absorbance values indicate an increase in cell

proliferation. The reading was carried out with a wavelength of 570 nm.

2.6.2 Free radical production rate

It was monitored through the using of a fluorescent probe, 2,7'-dichlorofluorescein (DCF). This assay is based on the following chemical assumption: dichlorofluorescein diacetate (DCFH-DA) is capable of diffusing across cell membranes. Inside cells, this molecule is deacetylated by the action of intracellular esterase enzymes (ROTA et al., 1999). This reaction, in turn, forms a non-fluorescent product, dihydrochlorofluorescein (DCFH). DCFH in the presence of ROS is oxidized (preferably by peroxides, hydroperoxides and NO●) to a highly fluorescent form of dichlorofluorescein (DCF). Thus, the greater the fluorescence detected by the greater absorbance assessed by the fluorimeter, the greater the occurrence of oxidizing compounds.

2.6.3 Fluorimetric DNA Quantification Assay by DNA-PicoGreen® Reagent

In order to complement the determination of cell viability, a fluorimetric assay for the quantification of free DNA in the medium was conducted using the reagent DNA-PicoGreen®, brand Invitrogen (Life Technologies), which is a fluorescent dye that binds to DNA double tape. This procedure was performed in a culture medium where cells are treated in order to determine the presence of double-stranded DNA in this medium due to possible cell disruption and cell death.

The dye was added to the sample in a dark 96-well Elisa plate, with a 5-minute incubation and fluorescence reading in a spectrofluorimeter at 480 nm excitation and 520 nm emission, according to Sagrillo et al. (2015).

2.6.4 Nitric Oxide Test Protocol

The nitric oxide test detects the presence of organic nitrite in the sample. Nitrite is detected and analyzed by the formation of a pink color when Griess reagent is added to the sample containing NO₂⁻.

The sulfanilamide in Griess reagent is responsible for the formation of diazonium in the nitrite sample. When the compound *N*-(1-naphthyl)-ethylenediamine dihydrochloride interacts with the diazonium salts, the pink color appears in the sample.

The sample used was cell culture supernatant, where 100 µL of sample supernatant was pipetted into a 96-well plate. Added 100 µL of Griess reagent to the supernatant; left at room temperature for 15 minutes, then the reading was performed at 540 nm in the spectrophotometer (CHOI et al., 2012).

2.7 Statistical analysis

The results obtained were tabulated and analyzed by the one-way test of variance (ANOVA), followed by Dunnett's post hoc. The level of statistical significance was set at 5% ($\alpha=0.05$). Analyzes were performed using GraphPad Prism version 5.0.

3. Results and discussions

With the development of this work, the patent of the object of study was generated, under the registration number BR1020200148524, Registration institution: INPI - National Institute of Industrial Property. Deposit: 21/07/2020 (BARCELLOS, 2020).

3.1 Physical-chemical characterization of white nanostructures and nanostructures containing tricresol formalin

3.1.1 Preformulation Studies

Initial formulations were produced and characterized to verify the reproducibility of the formulations, as well as the necessary optimizations to obtain better results.

The characterization technique that allows describing the particle size distribution is light scattering (WEIS et al., 2009). The results obtained in the first formulation demonstrated that the technique selected for the production of NC resulted in the formation of nanometric particles. The NCTF showed mean particle size within the expected 319.4 ± 5.30 nm, and a homogeneous suspension with PDI of 0.205 ± 0.012 , as seen in figure 1.

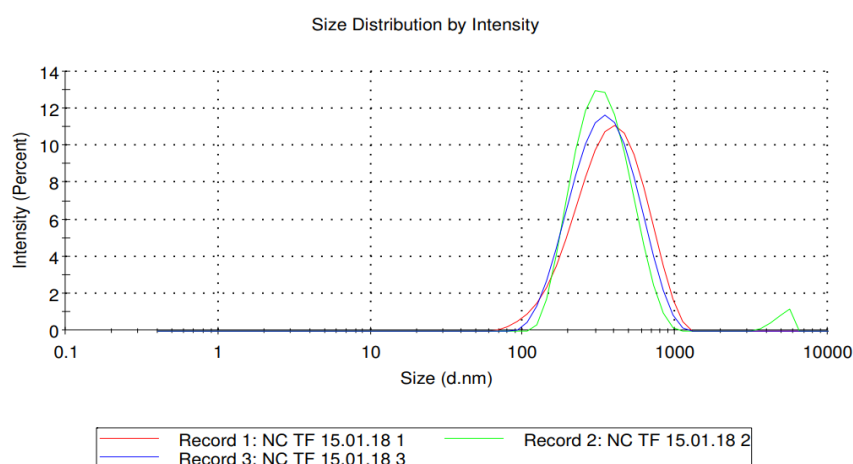


Figure 1. Average size analysis of the first NCTF formulation., particle size distribution by intensity graph, obtained by reading three samples of nanostructures.

The determination of the zeta potential is an important indicator of suspension stability. For there to be no aggregation and much less precipitation between the nanoparticles, it is necessary to repulsion between the charges (electrostatic) (CONTRI, 2011).

The values found in this characterization of NC had negative charges. The NCTF zeta potential was -26.3 ± 2.5 mV, as seen in Figure 2.

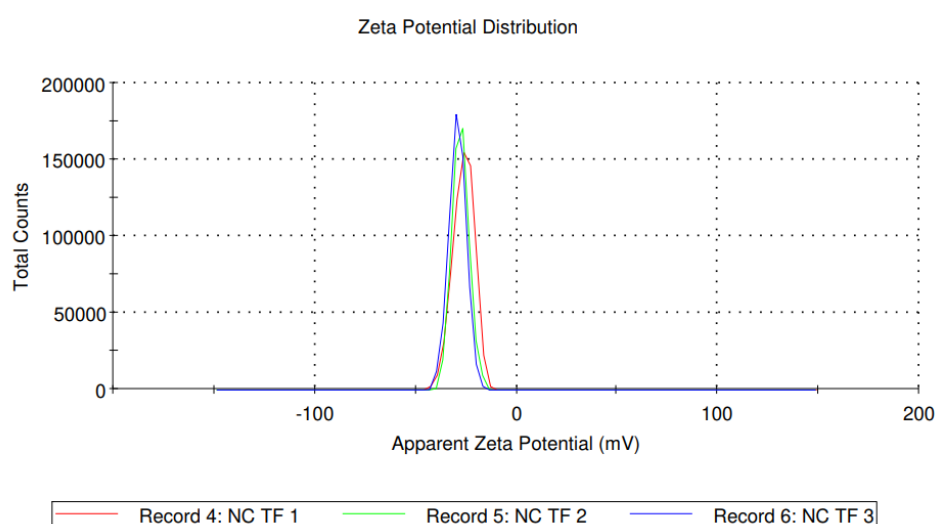


Figure 2. Zeta potential analysis of the first NCTF formulation, zeta potential graph obtained by reading three samples of nanostructures.

Another parameter that can characterize a suspension with adequate stability is the pH, since if the formulation presents an excessively acidic pH, deterioration of the polymer or one of its constituents can occur (SHAFFAZICK et al., 2003). In this research, the pH values of the NCTF were 5.2 ± 0.90 . The formulation had an acidic pH, a characteristic from the PCL polymer.

The NCTF showed macroscopically a smooth, milky appearance, the incorporation of tricresol formalin to a nanostructure considerably reduced the characteristic odor that it presents.

New formulations were produced to optimize the size and reduce the amount of surfactants, considering that the cytotoxicity of Polysorbate 80 is already known and highlighted in the literature (SANTOS et al., 2010).

An attempt was made to change the solvent, where acetone was replaced by ethanol, but ethanol was not able to solubilize the chosen polymer, PCL, so we kept using acetone.

The NCTF produced with 0.2% polysorbate 80 showed satisfactory results in terms of size (188 ± 3.90 nm), PDI (0.139 ± 0.011) and zeta potential (-19 ± 4.7 mV), however in the test of MTT, the formulation containing 0.4% polysorbate 80 showed less decrease in cell viability.

Table 2 shows the characterizations of the formulations produced until obtaining the formulation which was used.

Table 2. Characterization of NCTF formulations.

Formulation	Average size (nm)	PDI	Zeta potential (mV)	pH
NCTF 01	319.4 ± 5.3	0.205 ± 0.012	-26.3 ± 2.5	5.20 ± 0.9
NCTF 02	303.7 ± 8.3	0.214 ± 0.031	-14.2 ± 1.4	5.38 ± 0.8
NCTF 03	231.7 ± 11.2	0.185 ± 0.012	-7.89 ± 7.4	5.67 ± 0.1
NCTF 04	373.0 ± 20.9	0.205 ± 0.090	-29.2 ± 3.3	4.99 ± 0.8
NCTF 05	349.3 ± 21.6	0.311 ± 0.087	-11.4 ± 2.6	5.50 ± 0.7
NCTF 06	249.1 ± 11.7	0.154 ± 0.051	-13.9 ± 2.7	5.68 ± 0.4
NCTF 07	186.4 ± 1.9	0.180 ± 0.020	-17.8 ± 3.8	5.49 ± 0.3

NCTF 08	195.8 ± 4.4	0.097 ± 0.004	-18.1 ± 1.9	5.32 ± 0.1
NCTF 09*	192.3 ± 2.5	0.101 ± 0.013	-17.7 ± 2.8	5.48 ± 0.3
NCTF 10	188.4 ± 3.9	0.139 ± 0.011	-19.0 ± 4.7	5.45 ± 0.1

NCTF 09 * formulation was chosen as a working formulation.

The NB had values similar to the NCTF, with a small reduction in size when compared to the NCTF (177.2 ± 2.87 nm), PDI (0.084 ± 0.009), the zeta potential was the magnitude in which the greatest difference was observed compared to NCTF, where NB presented -9.00 ± 3.2 mV.

It is noteworthy that the methodology proposed and used by Fessi et al. (1989) has differences mainly in the concentrations of constituents compared to the formulation produced in this work, as shown in table 3, in which the constituents of the formulation developed by Fessi et al. are described (1989).

Table 3. Composition of indomethacin benzyl-benzoate nanocapsule suspensions, for a final volume of 10 mL.

Oil phase	Concentration	Watery phase	Concentration
Indomethacin	0.0125 g	Poloxamer	0.250 g (2.5%)
PLA	0.125 g (1.25%)	Ultrapure water	50 mL
Benzyl benzoate	0.500 g (5%)		
Epikuron 170	0.250 g (2.5%)		
Acetone	25 mL		

The formulation produced in this work has a lower concentration of polymers, oils and surfactants, as can be compared in the table 4.

Table 4. Comparison of percentage of constituents between the formulation of Fessi et al. (1989) and the NCTF formulation.

Componente	Fessi Concentration	Author concentration
Active	0.0125 g	0.025 g
Polymer	1.25%	0.2%
Oil	2.5%	1%
Surfactant	-	0.2%
Organic solvent	25 mL	25 mL
Stabilizer	2.5%	0.4%
Non-organic solvent	50 mL	50 mL
Final Volume	10 mL	25 mL

Another relevant difference in the formulation developed in this work regarding the formulation by Fessi et al. (1989) is the average size. The formulation produced by Fessi and collaborators had a size of 229 ± 29 nm, while the formulation of this work had an average size equal to 192 ± 2.50 nm.

3.1.2 Stability of Nanostructures

Nine NCTF formulations were produced with a concentration of 1 mg of the active to 1 mL of solution (1 mg/mL), and followed for a period of 90 days, with the mean particle size, polydispersion index, pH, and

zeta potential being verified. The nine formulations were divided into three groups to perform storage under different conditions, these being storage conditions at room temperature $25 \pm 2 \text{ }^\circ\text{C}$, refrigerator $4 \pm 2 \text{ }^\circ\text{C}$ and climate chamber $40 \pm 2 \text{ }^\circ\text{C}$.

The results can be seen in figures 3 to 6. The NCTF showed no significant difference in mean nanoparticle size and PDI after 90 days regardless of the storage condition. Refrigerated storage was the only one that showed variation in zeta potential, there was a significant change in pH of NCTF when stored at room temperature and climatic chamber. After a period of 30 days in a climatic chamber, the NCTF lost their characteristic but discreet odor of tricresol formalin, indicating that there was a release or detachment of the drug from the nanostructure.

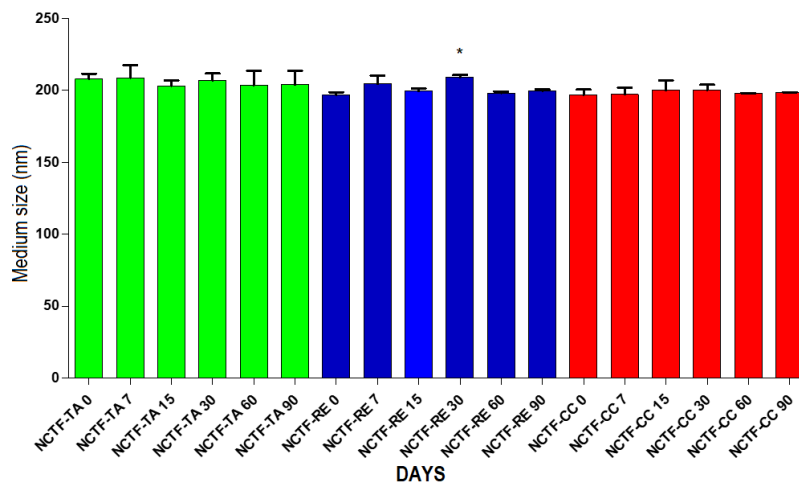


Figure 3. NCTF stability at three temperatures up to 90 days: medium size, TA – Room temperature ($25 \pm 2 \text{ }^\circ\text{C}$); RE – refrigerator ($4 \pm 2 \text{ }^\circ\text{C}$); CC – Climatic chamber ($40 \pm 2 \text{ }^\circ\text{C}$). NCTF (Nanostructures containing tricresol formalin). Data expressed as mean ($n=3$) \pm standard deviation (SD). Analyzes were followed by one-way Anova, followed by Dunnett's post hoc test, comparing with NCTF at time 0. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

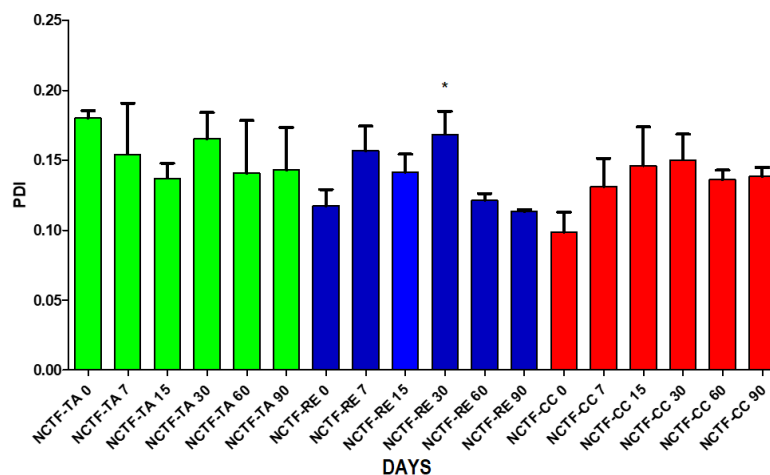


Figure 4. NCTF stability at three temperatures up to 90 days: polydispersity index, TA – Room temperature ($25 \pm 2 \text{ }^\circ\text{C}$); RE – refrigerator ($4 \pm 2 \text{ }^\circ\text{C}$); CC – Climatic chamber ($40 \pm 2 \text{ }^\circ\text{C}$). NCTF (Nanostructures containing tricresol formalin). Data expressed as mean ($n=3$) \pm standard deviation (SD).

Analyzes were followed by one-way Anova, followed by Dunnett's post hoc test, comparing with NCTF at time 0. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

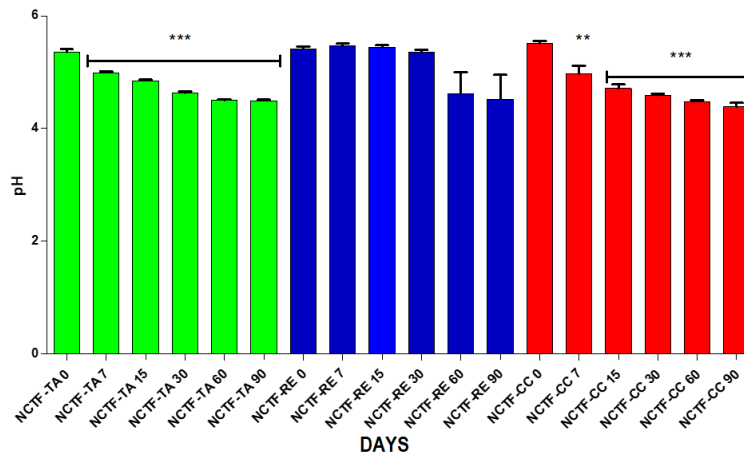


Figure 5. NCTF stability at three temperatures up to 90 days: pH, TA – Room temperature ($25 \pm 2 \text{ }^\circ\text{C}$); RE – refrigerator ($4 \pm 2 \text{ }^\circ\text{C}$); CC – Climatic chamber ($40 \pm 2 \text{ }^\circ\text{C}$). NCTF (Nanostructures containing tricresol formalin). Data expressed as mean ($n=3$) \pm standard deviation (SD). Analyzes were followed by one-way Anova, followed by Dunnett's post hoc test, comparing with NCTF at time 0. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

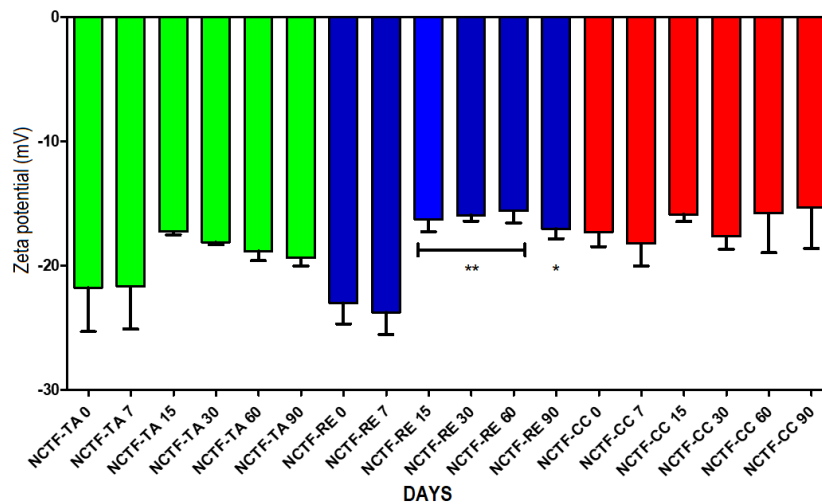


Figure 6. NCTF stability at three temperatures up to 90 days: zeta potential, TA – Room temperature ($25 \pm 2 \text{ }^\circ\text{C}$); RE – refrigerator ($4 \pm 2 \text{ }^\circ\text{C}$); CC – Climatic chamber ($40 \pm 2 \text{ }^\circ\text{C}$). NCTF (Nanostructures containing tricresol formalin). Data expressed as mean ($n=3$) \pm standard deviation (SD). Analyzes were followed by one-way Anova, followed by Dunnett's post hoc test, comparing with NCTF at time 0. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Based on the results obtained, we can see that refrigerated storage is the best alternative to maintain the stability of NCTF, because even after 90 days, they only showed changes in the zeta potential, and did not show changes in the other characterization tests and their organoleptic properties such as color and odor.

3.2 Antimicrobial activity

3.2.1 Disk diffusion test

In the diffusion disk test, NCTF and NB did not induce the formation of an inhibition halo in any of the concentrations tested in *Enterococcus faecalis*, while free tricresol formalin was able to form a 14 mm halo in *Enterococcus faecalis*. Table 5 shows the results obtained, this result is attributed to two factors, mainly the concentration tested, where the TFL is pure as determined by the methodology, but the NCTF are in known concentration and of small value, another factor is regarding the mode of active release, which when in free form is highly volatile, while the nanostructure promotes a controlled release.

Table 5. Results of the agar diffusion disc test with *E. Faecalis*.

Material	Result
NCTF 0.031 mg/mL	Did not induce halo formation
NCTF 0.062 mg/mL	Did not induce halo formation
NCTF 0.125 mg/mL	Did not induce halo formation
NCTF 0.25 mg/mL	Did not induce halo formation
NCTF 0.5 mg/mL	Did not induce halo formation
NB	Did not induce halo formation
Free tricresol formalin	14 mm halo

3.2.2 Microdilution Test

The antimicrobial activity analysis technique in broth microdilution is sensitive and provides more reliable results as the actual ability of the antimicrobial activity of an asset.

The choice of the concentration of 5000 µg/mL is due to the lack of previous results in the literature indicating the activity of tricresol formalin when tested by this methodology, the treatments tested presented results described in table 6 for NCTF and in table 7 for TFL.

NB were tested following the same methodology as NCTF but were tested without dilution. After performing this assay, it was observed that NB did not show antimicrobial activity. In the TFL plate, H₂O was used as the equivalent of the NB, since the NCTF are the combination of Nanostructures containing the active and the active, while the TFL is only the active diluted in H₂O.

The minimum inhibitory concentration (MIC) for *Enterococcus faecalis* was 312 µg/mL for both NCTF and TFL, for *Pseudomonas aeruginosas* was 2500 µg/mL for both NCTF and TFL, for *Escherichia coli* was 1250 µg/mL for both NCTF and TFL, indicating that the antimicrobial activity remained the same even after nanoencapsulation of the active.

Table 6. MIC results for NCTF.

	NC	PC	NB	19	39	78	156	312	625	1250	2500	5000
E.F.	O	X	X	X	X	X	X	●	O	O	O	O
E.F.	O	X	X	X	X	X	X	●	O	O	O	O
P.A.	O	X	X	X	X	X	X	X	X	X	●	O
P.A.	O	X	X	X	X	X	X	X	X	X	●	O
E.C.	O	X	X	X	X	X	X	X	X	●	O	O
E.C.	O	X	X	X	X	X	X	X	X	●	O	O

Legend: E.F= *Enterococcus faecalis*, P.A.= *Pseudomonas aeruginosas*, E.C.= *Escherichia coli*, NB= white nanostructures, NC: Negative Control, CP: Positive Control, “O” = negative growth, “X” = positive growth, concentrations in µg/mL.

Table 7. MIC results for TFL.

	NC	PC	H ₂ O	19	39	78	156	312	625	1250	2500	5000
E.F.	O	X	X	X	X	X	X	O	O	O	O	O
E.F.	O	X	X	X	X	X	X	O	O	O	O	O
P.A.	O	X	X	X	X	X	X	X	X	X	O	O
P.A.	O	X	X	X	X	X	X	X	X	X	O	O
E.C.	O	X	X	X	X	X	X	X	X	O	O	O
E.C.	O	X	X	X	X	X	X	X	X	O	O	O

Legend: E.F= *Enterococcus faecalis*, P.A.= *Pseudomonas aeruginosas*, E.C.= *Escherichia coli*, NB= white nanostructures, NC: Negative Control, PC: Positive Control, “O” = negative growth, “X” = positive growth, concentrations in µg/mL.

A study by Silva et al. (2012), using a methodology to assess the antimicrobial activity of tricresol formalin at a distance from the culture medium containing *Enterococcus faecalis*, concludes that tricresol formalin was effective to exert an antimicrobial effect at a distance, producing a halo inhibition of 8.7 ± 2.5 mm.

Mattos (2008) conducted research at the Staty University School of Dentistry of Londrina to evaluate the antimicrobial effects of some materials used in the root canals of primary teeth, among the study materials, tricresol formalin was used. Seven types of microorganisms were selected (*Bacillus cereus*, *Clostridium difficile*, *Escherichia coli*, *Fusobacterium nucleatum*, *Staphylococcus aureus*, *Staphylococcus epidermidis* e *Candida albicans*) obtained from the ATCC. Species were inoculated into Mueller Hinton meat agar and broth and incubated at 35 °C for 24 hours. Cotton rods carrying the micro-organisms or fungi in suspension were inoculated onto Mueller Hinton agar plates. At the end of the research, he found that all materials induced growth inhibition halos.

3.3 Toxicity assessment

3.3.1 Evaluation of the cytotoxicity of White Nanostructures formulation

To evaluate the cytotoxicity of white nanostructures, PBMC and HFF-1 ATCC® SCRC-1041™ fibroblasts were used. These cells were chosen according to Goldberg and Smith (2004), as fibroblasts are present in the dental pulp, as well as in the periodontal ligament there are several blood vessels, which have an abundance of PBMC.

The results for PBMC cultures after 24h in contact with NB at concentrations from 19 µg/mL to 5000 µg/mL are shown in Figure 7. The same curve performed in the microdilution test was used to perform a comparative analysis between the effective dose and the dose that would cause cell death.

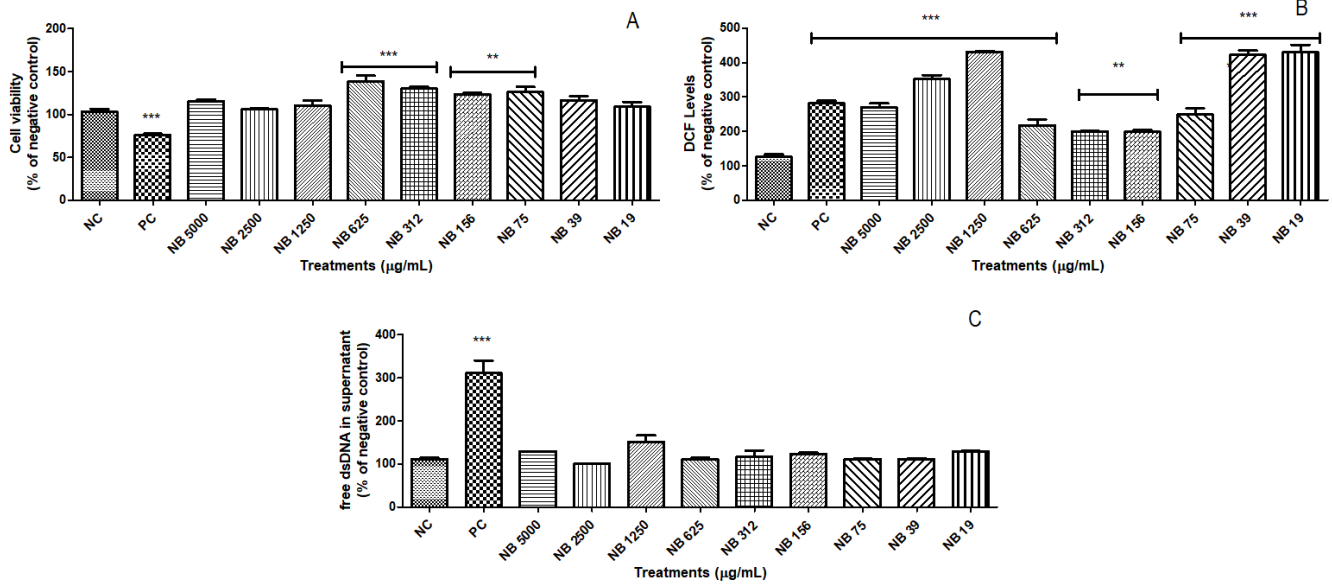


Figure 7. MTT, DCF, DNA-PicoGreen® test in PBMC after 24 hours with NB. A = MTT assay, B = DCF, C = DNA-PicoGreen® with 24 hours of incubation. Results expressed as percentage of negative control (100%). Data were expressed as mean ± standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

The results for cultures of HFF-1 fibroblasts after 24h in contact with NB at concentrations from 5000 µg/mL to 19 µg/mL are shown in Figure 8.

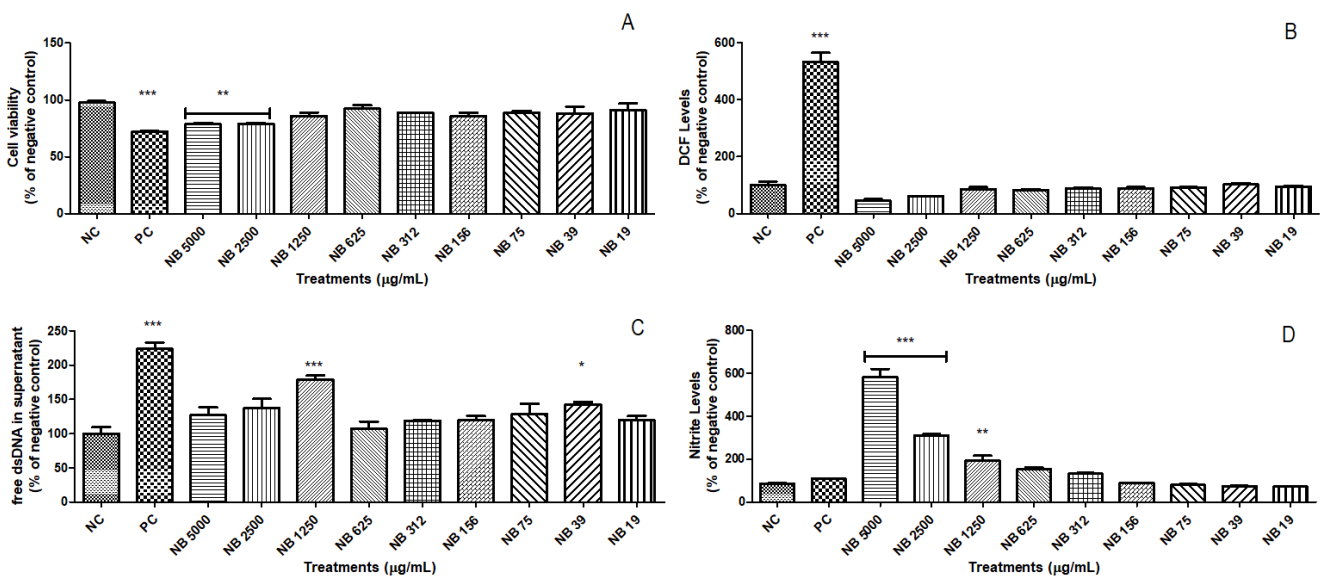


Figure 8. MTT test, DCF, DNA-PicoGreen®, nitric oxide in HFF-1 after 24 hours with NB. A = MTT assay, B = DCF, C = DNA-PicoGreen®, D = nitric oxide with 24 hours of incubation. Results expressed as percentage of negative control (100%). Data were expressed as mean ± standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In peripheral blood mononuclear cells, white nanostructures induced cell proliferation at concentrations from 625 to 75 µg/mL, in fibroblasts, NB caused a decrease in cell viability at concentrations of 5000 and

2500 $\mu\text{g/mL}$.

Studies evaluating the toxicity of traditionally marketed mouthwashes, which contained Polysorbate 80 as a surfactant, and using it as a positive control, concluded that the emulsifier is susceptible to cytotoxicity at different concentrations. In this study, the MTT technique against cells was used of gingival fibroblasts L-929 (SANTOS et al., 2010).

The reactive species, mainly hydrogen peroxide, were analyzed using the fluorescent product formed through the dichlorofluorescein diacetate (DCF) test. The test indicates that in PBMC the white nanostructures induced the production of ROS at all concentrations tested, in HFF-1 cells, the NB did not produce ROS at any concentration.

In PBMC, NB did not indicate the presence of dsDNA in the supernatant at any concentration tested, in HFF-1 cells it was observed the presence of dsDNA at concentrations of 1250 and 39 $\mu\text{g/mL}$.

The nitric oxide test performed on HFF-1 cells showed a positive result for the production of organic nitrite in NB at concentrations from 5000 to 1250 $\mu\text{g/mL}$.

3.3.2 Evaluation of the cytotoxicity of free Tricresol formalin

To assess the cytotoxicity of free tricresol formalin, PBMC and HFF-1 fibroblasts (ATCC® SCRC-1041™) were used, the cells were exposed to TFL in a 96-well plate. TFL was diluted in the culture medium for the selected strains, complete RPMI for PBMC, and DMEM for HFF-1.

The results for PBMC cultures after 24h in contact with TFL at concentrations from 5000 $\mu\text{g/mL}$ to 19 $\mu\text{g/mL}$ are shown in Figure 9.

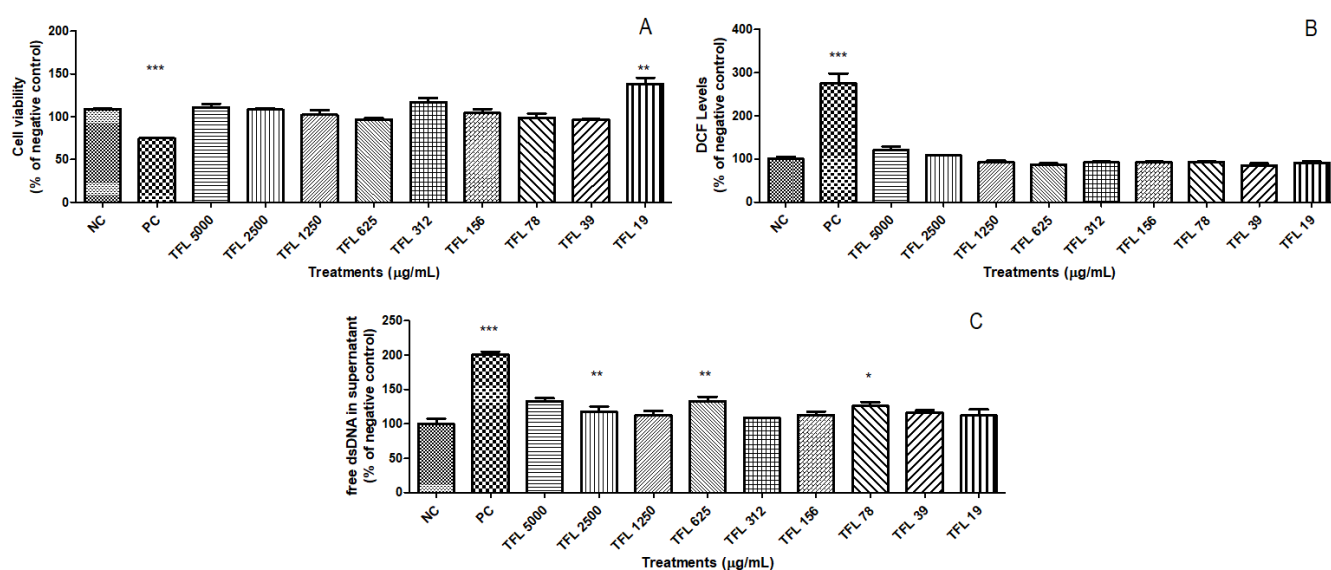


Figure 9. MTT, DCF, DNA-PicoGreen® test in PBMC after 24 hours with TFL. A = MTT assay, B = DCF, C = DNA-PicoGreen® with 24 hours of incubation. Results expressed as percentage of negative control (100%). Data were expressed as mean \pm standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The results for cultures of HFF-1 fibroblasts after 24h in contact with TFL at concentrations from 5000 $\mu\text{g/mL}$ to 19 $\mu\text{g/mL}$ are shown in Figure 10.

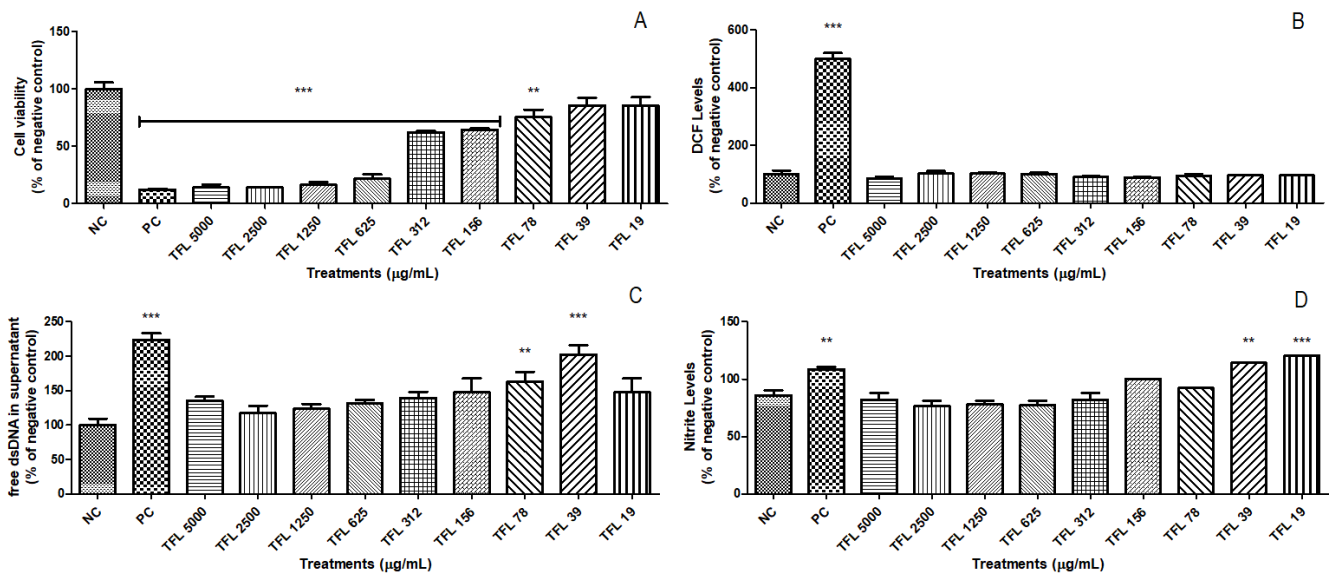


Figure 10. MTT test, DCF, DNA-PicoGreen®, nitric oxide in HFF-1 after 24 hours with TFL. A = MTT assay, B = DCF, C = DNA-PicoGreen®, D = nitric oxide with 24 hours of incubation. Results expressed as percentage of negative control (100%). Data were expressed as mean ± standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In PBMC, free Tricresol formalin caused significant proliferation at a concentration of 19 µg/mL, which suggests that perhaps TF, in small concentrations, is an inflammatory response inducer. On the other hand, in HFF-1 cells, TFL only at concentrations of 39 and 19 µg/mL did not decrease cell viability, which demonstrates that HFF-1 cells are more sensitive to the action of TF than PBMC. After calculating the IC_{50} , we reach the result that the IC_{50} for the TFL is 406.54 µg/mL IC_{50} .

IC_{50} calculation was performed using absorbance values at concentrations of 1250 µg/mL, 625 µg/mL and 312 µg/mL, which produced a scatter plot, figure 11, to obtain the result, the y is replaced by 50.

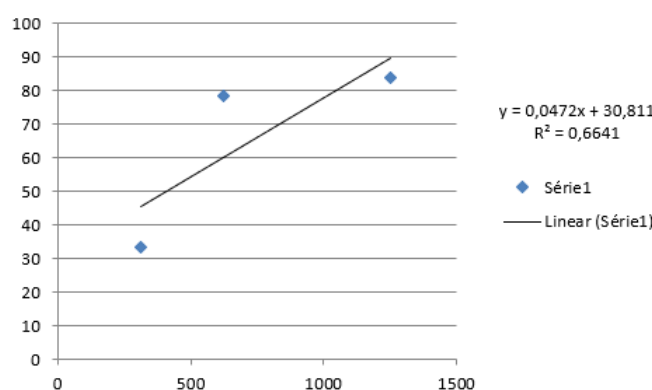


Figure 11. TFL scatter plot of cell viability inhibition in HFF-1

The TF performs a function at a distance, by releasing vapors (SOUZA et al., 1978). A study that used adequate methodology regarding the action of TF at a distance was carried out by Thé, Bauer, De Grood in 1976, where formalin, the majority substance present in the formulation of tricresol formalin, was used, evaluating the action against VERO cells. The results obtained in the tests indicated that with a distance of

10 mm between the VERO cells in culture medium and the pure formalin applied to a cotton in the amount of 5 μ L, they did not present different results from the negative control.

It can be highlighted the fact that the study mentioned above did not take into account the concentration of the medication to be used, only its quantity in the practical use formula.

As can be seen in the study by Thomas (2006), the author performed the tests using tricresol formalin, formocresol and formaldehyde, which she also did not take into account the concentration of medications, only the exposure times in direct contact with the cells tested.

According to Thomas (2006), formocresol, tricresol formalin and formaldehyde were cytotoxic at the different exposure times tested (1, 2, 3, 4, and 5 minutes) in Hep2, NIH3T3 and HeLa tumor cell lines. The cytotoxic effect remained after 24 hours, 48 hours and 7 days of post-treatment incubation, in the three cell lines tested.

Lovschall et al. (2002) found in a study that, under experimental conditions, human tissue cells seemed to be more sensitive to formaldehyde toxicity than HeLa tumor cells.

It was verified by Cardoso et al. (2005) the interaction of formocresol in Swiss mouse macrophages, the study worked with drug concentrations from dilutions. The dilutions studied were 1:10, 1:50, 1:100, 1:500, 1:1000 and 1:5000. The result was that the most concentrated ones such as 1:10 and 1:50 decreased cell viability.

In a more recent study by Ko, Jeong and Kim (2017), the cytotoxicity of formocresol in CHO-K1 cells was evaluated using a methodology which took into account the drug concentrations to be tested, these being 1, 10 and 100 μ g/ml. The results of this work indicated that the concentration of 100 μ g/mL was able to markedly reduce cell viability, and all concentrations caused damage to the DNA, verified by DNA alkaline comet assay.

In PBMC the DFC test indicated that TFL did not produce free radicals at any concentration, as well as in HFF-1 cells, the negative results for free radical production rates in TFL can be attributed to the antioxidant action of tricresol.

According to a study by Yeung et al. (2002), the three cresol isomers, *o*-cresol, *m*-cresol e *p*-cresol, are H_2O_2 , scavengers and superoxide radicals generated by xanthine / xanthine oxidase. The cresols *o*-(*orto*), *m*-(*meta*) e *p*-(*para*) showed protective effects on DNA damage. The results indicate that cresol isomers are effective ROS scavengers and can prevent ROS-induced damage when used as pulpotomy agents or as intracanal medications. Due to the difference in the position of the functional hydroxyl group in the three cresol isomers, *m*-cresol is the most effective ROS scavenger.

For the double-stranded DNA damage verification test in PBMC, TFL showed results for the presence of dsDNA in the supernatant at concentrations of 2500, 625, 78 μ g/mL, indicating that there was damage to the cells at these concentrations. In HFF-1 cells, TFL showed results that indicated DNA damage at concentrations of 78 and 39 μ g/mL.

The nitric oxide test performed on HFF-1 cells showed a positive result for the production of organic nitrite by TFL at concentrations 78 and 39 μ g/mL.

It is important to highlight the cell lines chosen for carrying out the experimental assays, as there are many cell types, the cell line to be chosen will depend on the exposure trajectory and potential target organs. It is noteworthy that the type of cell defined can "relevantly influence the results, increasing or reducing the

possibility of providing measurable and reliable signals" (STONE; JOHNSTON; SCHINS, 2009).

3.3.3 Evaluation of the formulation of Nanostructures containing Tricresol Formalin

To evaluate the cytotoxicity of nanostructures containing tricresol formalin, a formulation with a concentration of 20 mg/mL was produced, and from it to carry out the necessary dilutions, PBMC and HFF-1 ATCC® SCRC-1041™ fibroblasts were used.

The cells were exposed to nanostructures containing tricresol formalin at different concentrations, as a study involving nanostructures and tricresol formalin is unprecedented in the literature, it was chosen to work with a high initial concentration.

The results for PBMC cultures after 24h in contact with NCTF at concentrations from 5000 µg/mL to 19 µg/mL are shown in the figure 12.

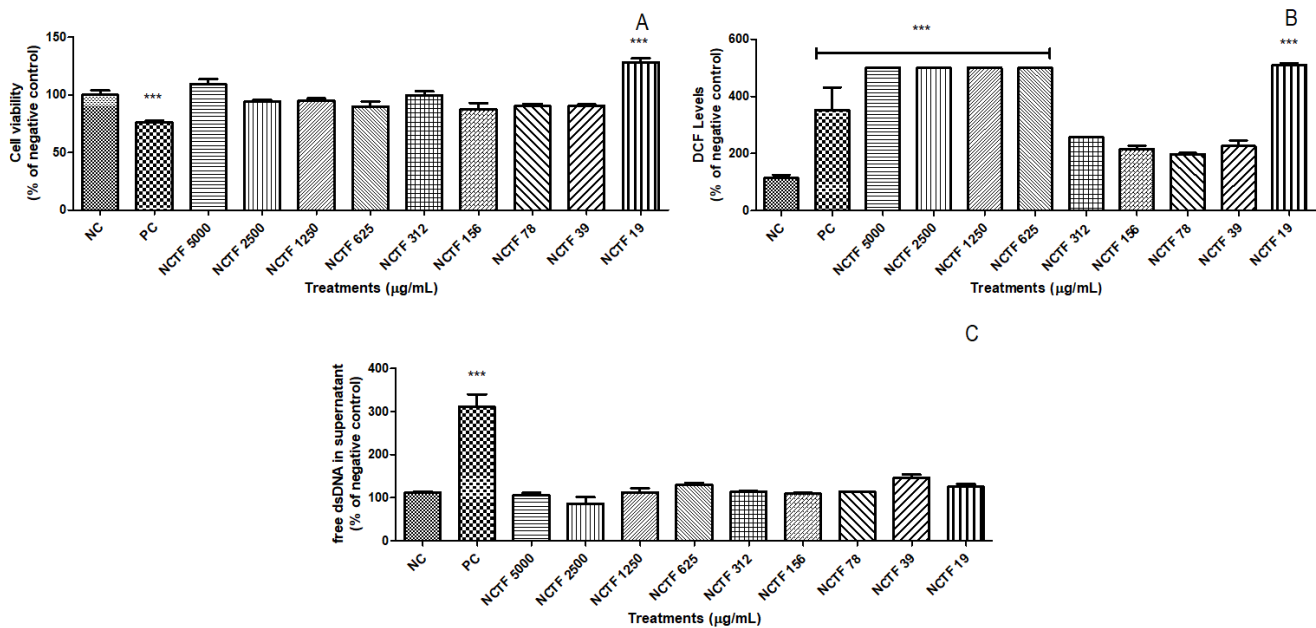


Figure 12. MTT, DCF, DNA-PicoGreen® test in PBMC after 24 hours with NCTF. A = MTT assay, B = DCF, C = DNA-PicoGreen® with 24 hours of incubation. Results expressed as percentage of negative control (100%). Data were expressed as mean ± standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The results for cultures of HFF-1 fibroblasts after 24h in contact with NCTF at concentrations from 5000 µg/mL to 19 µg/mL are shown in Figure 13.

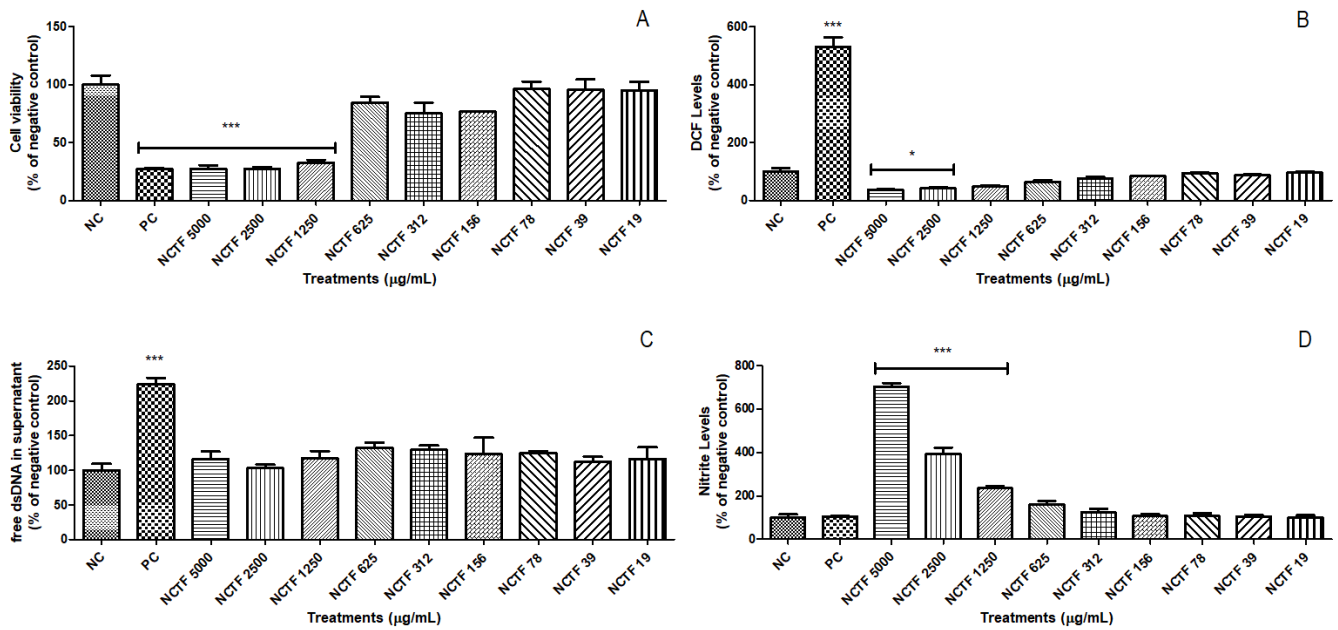


Figure 13. MTT test, DCF, DNA-PicoGreen®, nitric oxide in HFF-1 after 24 hours with NCTF. A = MTT assay, B = DCF, C = DNA-PicoGreen®, D = nitric oxide with 24 hours of incubation. Results expressed as percentage of negative control (100%). Data were expressed as mean ± standard deviation (SD).

Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In peripheral blood mononuclear cells, nanostructures containing Tricresol formalin, as well as free Tricresol formalin, showed a significant proliferation at a concentration of 19 µg/mL, this suggests that TF in small concentrations is an inducer of a pro-inflammatory response.

In fibroblasts, NCTF at concentrations of 5000, 2500, 1250 µg/mL significantly decreased cell viability, on the other hand, TFL only at concentrations of 39 and 19 µg/mL did not decrease cell viability. When the NCTF IC₅₀ is calculated, we reach the result that the NCTF IC₅₀ is 1029.03 µg/mL.

IC₅₀ calculation was performed using absorbance values at concentrations of 1250 µg/mL, 625 µg/mL and 312 µg/mL, which produced a scatter plot, figure 14, to obtain the result, the y is replaced by 50.

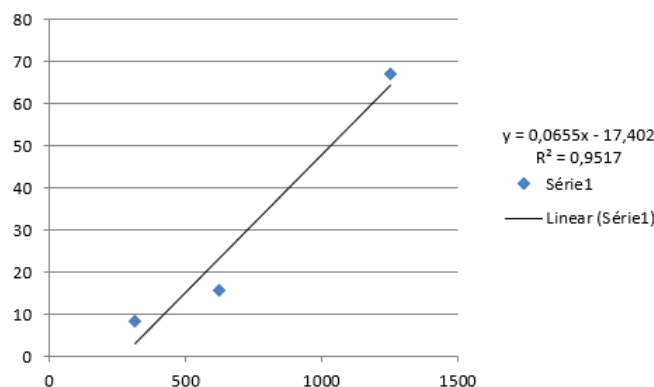


Figure 14. NCTF scatter plot of cell viability inhibition in HFF-1.

In PBMC, NCTF indicated that there was a significant production of free radicals at concentrations from 5000 to 625 µg/mL and 19 µg/mL, and in HFF-1 cells, NCTF did not produce free radicals.

In the double-stranded DNA damage test, both PBMC and HFF-1 cells did not indicate the presence of

dsDNA in the supernatant at any tested concentration of NCTF.

The nitric oxide test showed a positive result for the production of organic nitrite in NCTF at concentrations from 5000 to 1250 µg/mL.

Relating the results of MTT in HFF-1 cells from NB, NCTF and TFL, figure 15, we can see that NB at higher concentrations has mild toxicity, NCTF at higher concentrations has severe toxicity, together with TFL, demonstrating that the response of toxicity presented in the MTT test is responsible for the presence of tricresol formalin and not for the constituents of the nanostructure.

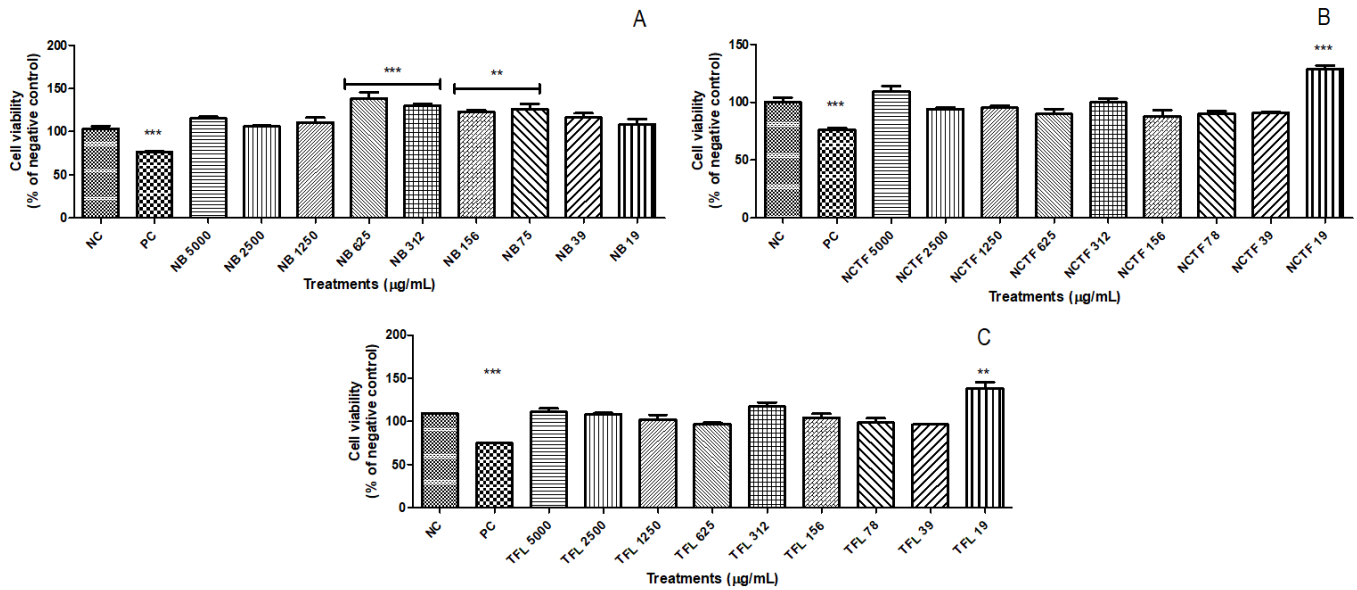


Figure 15. MTT test in HFF-1 after 24 hours with NB, NCTF, TFL. MTT assay with 24 hours of incubation, A = NB, B = NCTF, C = TFL. Results expressed as percentage of negative control (100%). Data were expressed as mean ± standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

As already evaluated in the microdilution test to verify which concentration has antimicrobial activity, the NCTF presented results within safe values according to the MTT test in HFF-1 cells, with its IC_{50} calculation being equal to 1029.03 µg/mL, while its minimal inhibitory activity was 312 µg/mL for the main microorganism of clinical interest, *Enterococcus faecalis*. While TFL has an IC_{50} equal to 406.54 µg/mL, even though it also has antimicrobial activity at the same concentration as its nanoencapsulated form for *Enterococcus faecalis*, TFL has a significantly lower safety when compared to NCTF, that is, owes to the controlled release mechanism that the nanostructured form makes possible.

When comparing NB, NCTF and TFL, it appears that there was no production of reactive oxygen species in HFF-1 cells, but in PBMC, NB and NCTF induced the production of reactive oxygen species, as can be seen in figure 16.

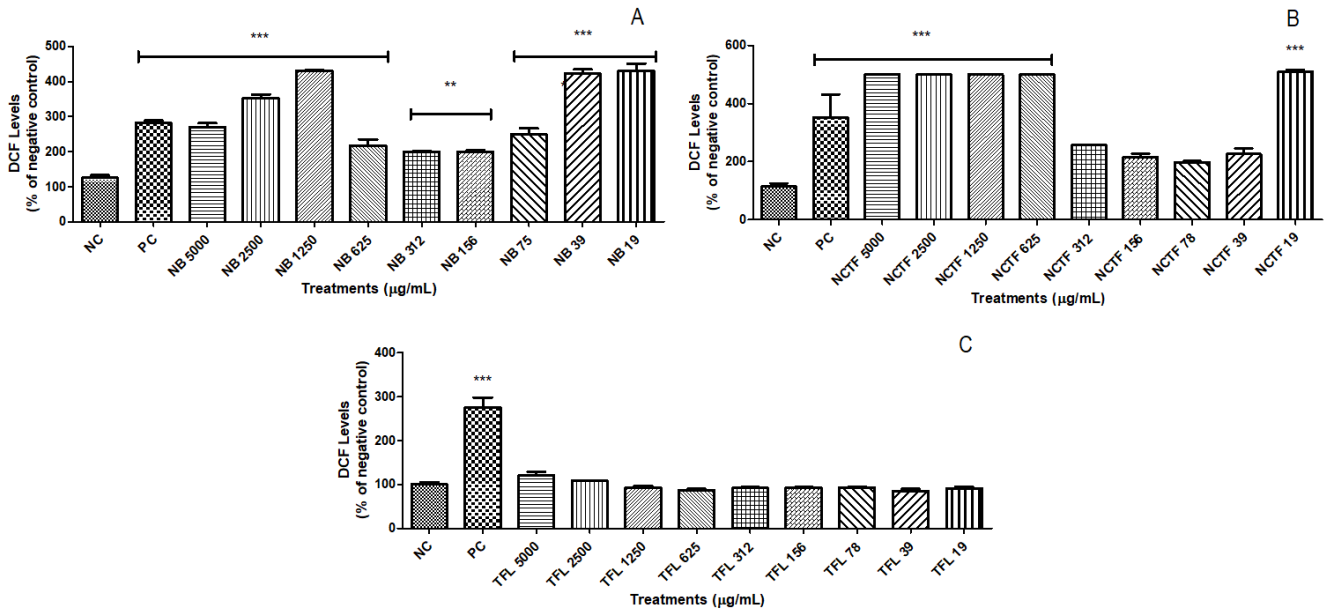


Figure 16. DCF test in PBMC after 24 hours with NB, NCTF, TFL. DCF assay with 24 hours incubation, A = NB, B = NCTF, C = TFL. Results expressed as percentage of negative control (100%). Data were expressed as mean ± standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

TFL causes greater damage to double strand cellular DNA (rupture of membranes) when compared to NB or NCTF in HFF-1 cells, in PBMC only TFL causes damage, as can be seen in Figure 17, which allows us to conclude that the action of tricresol formalin is capable of generating damage to the double strand of DNA.

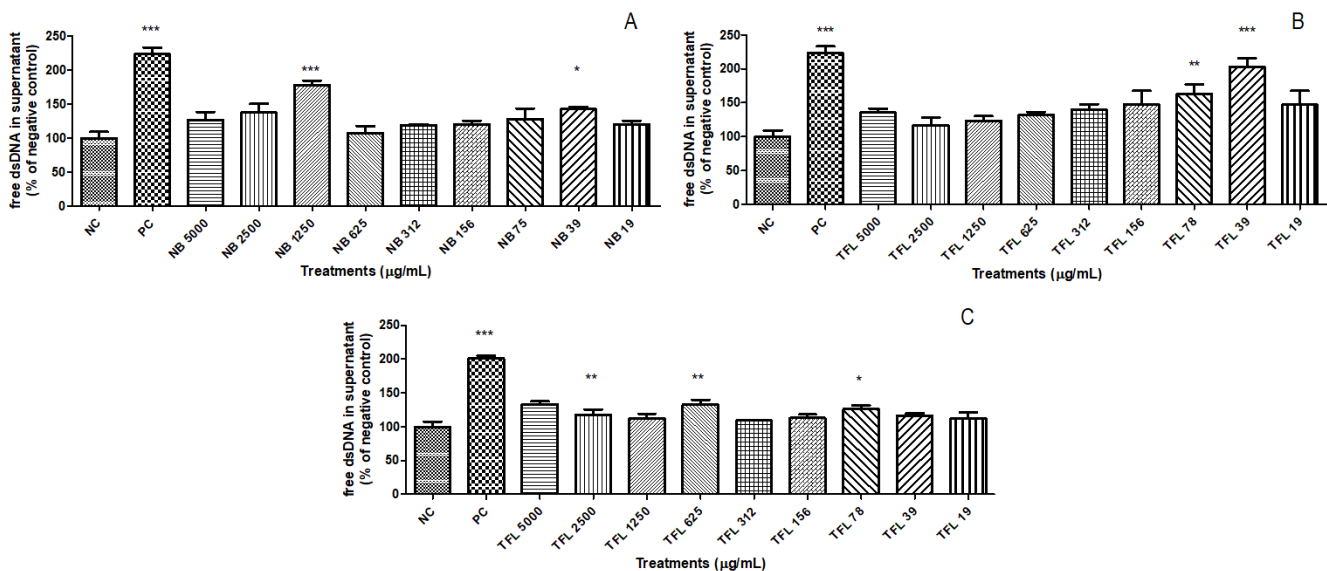


Figure 17. DNA-PicoGreen® test in HFF-1 and PBMC after 24 hours with NB, TFL. dsDNA test with 24 hours incubation, A = NB in HFF-1, B = TFL in HFF-1, C = TFL in PBMC. Results expressed as percentage of negative control (100%). Data were expressed as mean ± standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$

were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

It is believed that the result presented in the nitric oxide test is a response to the volatilization of tricresol formalin, as NB and NCTF presented high nitrite production, while TFL induced nitrite production only at lower concentrations, as can be seen in figure 18.

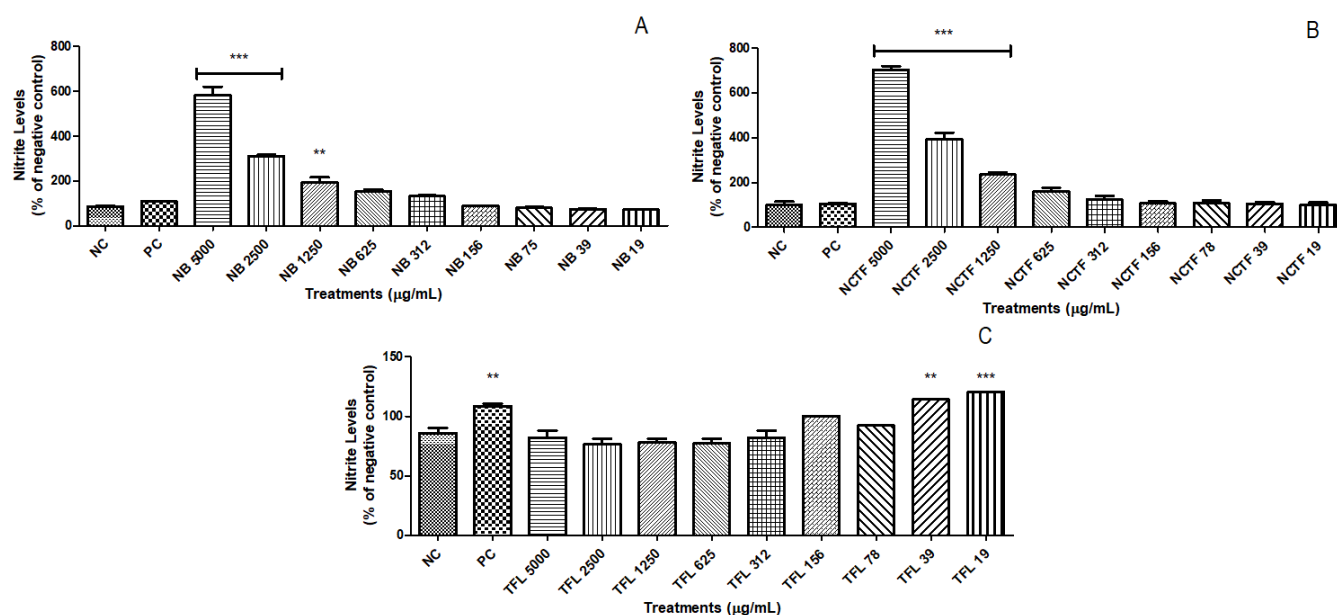


Figure 18. Nitric oxide test in HFF-1 after 24 hours. Nitric oxidation assay with 24 hours of incubation.

Results expressed as percentage of negative control (100%). Data were expressed as mean \pm standard deviation (SD). Analyzes were performed by one-way ANOVA followed by Dunnett's post hoc test.

Values with $p < 0.05$ were considered statistically significant. Where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Relating to the data presented in the figure above, it is observed that NO is very important for the function of the immune system, playing a key role in the activity of macrophages and cellular defenses against intracellular pathogens (DURNER et al., 1999). A remarkable aspect of this molecule is its ability to be beneficial or potentially toxic depending on the tissue concentration or depuration (FLORA FILHO et al., 2000).

Table 8 shows a comparison of the results obtained for the TFL compared to the result obtained for the NCTF.

Table 8. Comparison of TFL and NCTF results.

Assay	TFL result	NCTF result
Antimicrobial activity for <i>E. faecalis</i>	312 $\mu\text{g/mL}$	312 $\mu\text{g/mL}$
Decrease in cell viability (PBMC)	NP	NP
Production of ROS's (PBMC)	NP	625 to 5000 $\mu\text{g/mL}$
Damage to double stranded DNA (PBMC)	2500; 625 e 78 $\mu\text{g/mL}$	NP

Decreased cell viability (HFF-1)	78 to 5000 µg/mL	1250 to 5000 µg/mL
Production of ROS's (HFF-1)	NP	2500 and 5000 µg/mL
Damage to double stranded DNA (HFF-1)	39 and 78 µg/mL	NP
NO production (HFF-1)	19 and 39 µg/mL	1250 to 5000 µg/mL
IC ₅₀	406.54 µg/mL	1029.03 µg/mL

Legend: NP = not presented.

4. Conclusion

The production of nanostructures containing tricresol formalin got satisfactory results, the formulation presented good stability at room temperature, but presented better results when stored under refrigeration for up to 90 days.

The antimicrobial activity of tricresol formalin remained the same after drug encapsulation.

The evaluation of cell viability by the MTT assay represents that for PBMC free tricresol formalin and NCTF do not decrease its viability, but for HFF-1 cells, TFL is significantly more cytotoxic than NCTF, this indicates that NCTF are safer and can be used at higher concentrations and in direct contact with the cells, not requiring the use of cotton soaked in tricresol formalin, thus avoiding discomfort with the odor of gases volatilized by the active.

Based on the above, we can conclude that after some more investigations, nanostructures containing tricresol formalin may come to replace free tricresol formalin, as they have pharmacological safety and excellent antimicrobial efficacy.

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