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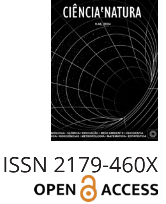

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Chemistry

Development, characterization, and cytogene-ecotoxicological bioassay of different free and nanoestrutured formulations containing *trans*-anethole

Desenvolvimento, caracterização e bioensaio citogenoecotoxicológico de diferentes formulações livres e nanoestruturadas contendo *trans*-anethole

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ABSTRAT

Trans-anethole is an aromatic compound found in large amounts in the essential oils of various plants, such as star anise. It is recognized for its culinary and traditional medicinal uses. Despite its potential, its low solubility and complex characteristics hinder its absorption in the human body. To overcome this challenge, nanotechnology has emerged as a promising solution. In this study, nanocapsules and nanoemulsions containing *trans*-anethole were developed and characterized for physical-chemical parameters, including particle diameter, zeta potential, pH, polydispersity index, encapsulation rate, and content stability over a 90-day period. Biological assessments were conducted using cytogenotoxicity tests on PBMC cells and a toxicological bioassay with *Artemia salina*. The results demonstrated that the nanostructured systems remained stable for 90 days under refrigeration, exhibiting compatible pH, polydispersity index, zeta potential, and particle diameter for biomedical applications while also exhibiting no cytotoxic or genotoxic activity. The toxicological assays revealed significant protection against the toxicity of *trans*-anethole when delivered in nanostructured systems, as compared to its free form.

Keywords: Nanotechnology; Biotechnology; Toxicity; Anethole

RESUMO

O *trans*-anethole é um composto aromático encontrado, em grandes quantidades, no óleo essencial de diversas plantas, como o anis estrelado. É reconhecido por seus usos culinários e medicinais tradicionais. Apesar de seu potencial, a sua baixa solubilidade e as características complexas dificultam

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sua absorção no corpo humano. Para superar esse desafio, a nanotecnologia surgiu como uma solução promissora. Neste estudo, nanocápsulas e nanoemulsões contendo *trans*-anethole foram desenvolvidas e caracterizadas quanto aos parâmetros físico-químicos, incluindo diâmetro de partícula, potencial zeta, pH, índice de polidispersão, taxa de encapsulamento e estabilidade por um período de 90 dias. As avaliações biológicas foram realizadas usando ensaios de citogenotoxicidade em células PBMC e bioensaio toxicológico em *Artemia salina*. Os resultados demonstraram que os sistemas nanoestruturados produzidos permaneceram estáveis por 90 dias sob refrigeração, exibindo pH compatível, índice de polidispersão, potencial zeta e diâmetro de partícula para aplicações biomédicas, além de não exibirem atividade citotóxica ou genotóxica. Os ensaios toxicológicos revelaram proteção significativa contra a toxicidade do *trans*-anethole quando veiculado nos sistemas nanoestruturados, em comparação com sua forma livre.

Palavras-chave: Nanotecnologia; Biotecnologia; Toxicidade; Anetol

1 INTRODUCTION

Star anise, along with other medicinal plants and spices, finds extensive usage in gastronomy and traditional medicine. Apart from enhancing the taste, color, and aroma of food, these plants possess pharmacological properties and potential, including antioxidant and antimicrobial effects (Sharafan et al., 2022). Aromatic plants serve as valuable reservoirs of bioactive substances, which have been widely utilized by the pharmaceutical industry for their numerous beneficial effects in combating viral and bacterial infections, as well as in addressing conditions like cancer and diabetes (Patra et al., 2020, Sharafan et al., 2022, Yu et al., 2019). Additionally, these plants exhibit analgesic and digestive properties (Pérez-Rosés et al., 2015). Star anise (*Illicium verum*), a native plant of Asia, is highly regarded for both its medicinal and culinary uses. It shares a similar aroma with anise due to the presence of the same oil, albeit in a more concentrated form, with *trans*-anethole being its most significant active compound (Yu et al., 2019).

The chemical characteristics of *trans*-anethole, including its low solubility and the presence of numerous phytochemicals, make it challenging for the human body to absorb (Patra et al., 2020). This issue has drawn the attention of the pharmaceutical industry, which is actively seeking new methods to enhance anethole's absorption and delivery. Consequently, nanotechnology has emerged as a promising solution to overcome this limitation (Nikam et al., 2018).

Nanoencapsulation offers an alternative method for protecting active substances or drugs by shielding their constituents from chemical degradation, photosensitization, and volatility. Additionally, it enhances their solubility and bioavailability, optimizing their biological properties and, most importantly, reducing their toxicity (Durán, Mattoso, Morais, 2006).

Nanoemulsions offer an alternative method for the delivery and preservation of lipid-based actives. These colloidal nanoparticles, ranging in size from 10 nm to 1000 nm, possess a solid spherical structure with an amorphous and lipophilic surface. Additionally, they carry a negative charge, enhancing their stability. By utilizing nanoemulsions, lipid-based actives can be efficiently delivered and preserved, providing an innovative solution for their application in various industries such as pharmaceuticals, cosmetics, and food (Nikam et al., 2018).

Toxicology is a multidisciplinary field encompassing various aspects, and the concept of a toxic substance is highly relative, influenced by the characteristics and properties of the agent, dosage, and the nature of the surrounding environment, whether biological or otherwise (Gu, 2019). Bioassays are employed to assess the toxicity of substances under investigation, aiming to evaluate their effects on biological systems (Salles et al., 2022, Gambelas, 2003). These tests, which seek to predict the toxic potential and interactions with the environment, can be conducted through different approaches, involving the analysis of data obtained from microorganisms, cells, laboratory animals, or human subjects (Rios, 1995, Viana, Salles, Bruckmann, 2019).

Given the increasing emphasis on minimizing animal use in laboratory settings, there is a demand for the development and standardization of tests that reduce the reliance on guinea pigs while still detecting the toxicity of substances used in clinical applications, such as biomaterials, to ensure patient safety and prevent adverse reactions (Ciapetti et al., 1996). *In vitro* methods offer advantages over *in vivo* testing, as they allow for better control of experimental variables, easier acquisition of meaningful data, and often shorter test periods, which ultimately reduces costs (Rogero et al., 2003).

In vitro cytotoxicity tests are classified in ISO 10993-1 (international standardization body) as the first test to evaluate the biocompatibility of any material. These tests promote preliminary results related to the interaction between the material and the biological environment. They are fast, efficient, and inexpensive tests (Rogero et al., 2003)

Given that trans-anethole exhibits very low solubility in water, which is the primary solvent in intra and extracellular environments, nanostructures can enhance its properties and enable its application in Biomedicine compared to its free form, thereby reducing its potential toxicity. In this study, the nanostructured systems were thoroughly characterized, and their stability, pharmacological safety, and ecotoxicity were analyzed. Cytogenetic and ecotoxicity tests were conducted as essential measures to determine the safety of the developed nanostructured systems concerning living organisms and the environment.

2 METODOLOGY

2.1 Development of polymeric nanocapsules containing *trans-anethol*e (NCTA), and nanoemulsions (NETA)

The deposition method of the preformed polymer proposed by Fessi, Puisieux and Devissaguet (1988) was used. In the nanoemulsions there was no addition of polymers to the formulation, according to Table 1. The components of the organic phase and the aqueous phase were placed separately in flasks and kept under magnetic stirring in a water bath at controlled temperature (35 ± 2 °C) for 60 minutes. After this period, the organic phase was slowly poured into the aqueous phase and kept under magnetic stirring for 10 minutes. The obtained solution was taken to the rotavaporator to eliminate the organic solvent and reach the expected final volume for the concentration of 1mg/mL of *trans*-anethole.

Table 1 – Composition of the nanocapsules and *trans*-anethole nanoemulsions formulation produced in this study (for 25 mL)

Components	**(NCB)**	**NCTA**	**NEB**	**NETA**
		Oil phase		
Trans-anethole (g)		0.025		0.025
Eudragit L 100 (g)	0.25	0.25	0.25	0.25
Spam 60 (g)	0.1915	0.1915	0.1915	0.1915
Crodamol (g)	0.7755	0.775	0.7755	0.7755
Ethanol (mL)	66.75	66.75	66.75	66.75
		Aqueous phase		
Tween 80 (g)	0.1915	0.1915	0.1915	0.1915
Ultrapure water (mL)	133.25	133.25	133.25	133.25

Source: Authors (2023)

(NCB): Blank Polimeric Nanocapsule, **NCTA**: *Trans*-anethole Polymeric Nanocapsule, **NEB**: Blank Nanoemulsion, **NETA**: *Trans*-anethole Nanoemulsio(

2.2 Characterization of the suspensions containing the nanocapsules and the nanoemulsions

The suspensions containing the nanocapsules were carried out through the analysis of their physical appearance, pH measurements, the mean particle size distribution, the polydispersity index (PDI) and the zeta potential, performed at the Laboratory of Nanotechnology of the Franciscan University (UFN). To determine the mean particle diameter and the polydispersity index, the photon correlation spectroscopy technique, also known as dynamic light scattering (DLS) was used. A 20 μL aliquot of the nanoparticle suspension was collected after the rotavaporation process and dispersed in 4mL of ultrapure water, in other words, dispersed in the ratio 1: 200 (v/v). The cuvette containing the sample was inserted into the analyzer chamber of the Zetasizer® Nano-ZS (Malvern) equipment so that the light beam passed through the dispersion throughout its length. Each sample was measured ten times, and the mean and standard deviation were recorded for further statistical treatment (factorial

design). The test conditions were as follows: scattering angle: 90 °C, temperature: 25 °C, laser wavelength: 660 nm. Additionally, based on the same sample, the polydispersity index recorded as mean and standard deviation (n = 3) was measured.

The determination of the hydrogen potential (pH) was carried out directly in the suspensions in a Digimed potentiometer previously calibrated with pH 4 and 7 buffer solutions. The zeta potential of the nanocapsule suspension and nanoemulsions was obtained by means of the electrophoretic mobility technique in the Zetasizer apparatus ® Nano-ZS, (from Malvern). The samples were previously diluted 500-fold in 10 mM sodium chloride and filtered through 0.45 μm membrane. The results were expressed in millivolts (mV) from the mean of the triplicates. To determine the stability of the suspensions the samples were packed in amber glass and stored in a refrigerator (4 °C ± 2 °C) for 90 days. All samples were analyzed for 0, 3, 7, 20, 30, 60 and 90 days for pH, PDI, zeta potential and average particle diameter parameters. The total concentration of the active agent in the nanocapsules was determined by UV/VIS, considering the difference between the total concentration of *trans*-anethole in the formulation and the concentration present in the aqueous phase of the suspension. The total concentration was determined by determining the free active present in the aqueous phase of the suspension and performed by ultrafiltration-centrifugation of the suspensions using Microcon® MC Millipore 10,000 Da membranes for 5 minutes at 5000 rpm until 40 μL of ultrafiltrate was obtained, in which the non-associated (free) *trans*-anethole was quantified, using the analytical methodology described by Guterres et al., (1995).

2.3 Cytogenotoxicity tests performed on peripheral blood mononuclear cells (PBMCs)

Testes to verify the pharmacological safety of the different free and nanostructured formulations, the samples were obtained from peripheral blood, from the Laboratory of Clinical Analysis (LEAC) of the Franciscan University, under the Ethics Committee of the institution and CAAE registration: 31211214.4.0000.5306. Samples were collected

by venipuncture using Vacutainer (BD Diagnostics, Plymouth, UK) in 8 mL heparinized tubes. PBMCs were cultured in a 5% CO_2 incubator at 37 °C in premises of the Franciscan University Center Cell Culture Laboratory in appropriate culture medium RPMI 1640, with addition of 10% fetal bovine serum and 1% penicillin / streptomycin and 1.5 g L^{-1} sodium bicarbonate (Invitrogen® and Sigma® products). Cell culture and treatment were conducted similarly to that described by Falanga et al*.,* (1996).

Cells were cultured with the presence of *trans*-anethole in its free and nanostructured forms. A negative (no treatment) and positive control group (cells treated with Hydrogen Peroxide - H_2O_2) were maintained and analyzed in all protocols developed. All tests were run in triplicate at a concentration of $2x10^5$ cells/mL. The curve was constructed according to the molecular weight thereof: 10, 50, 200 and 500 µg/mL.

2.4 Cell proliferation investigated using the tetrazolium method (MTT)

Assay in a procedure similar to that described by Krishna et al., (2009). Measurements of cell viability and cell proliferation were performed with this method. Yellow MTT 2-(3,4,5-dimethylthiazolyl-2)-tetrazolium and 5-diphenyltretazolium bromide are chemically reduced through the activation of the enzyme dehydrogenase which generates NADPH. Therefore, it is a marker of the mitochondrial function, thus, in the presence of more cells or greater viability the reaction of MTT produces a purple coloration that can be quantified by spectrophotometry. Absorbance values lower than the negative control, indicate a reduction in the rate of cell proliferatio(In contrast, higher absorbance values indicate increased cell proliferation (Krishna et al., 2009). The samples were analyzed in a spectrophotometer at 570 nm. The experiment was performed in triplicate and the results were expressed as a percentage of the negative control.

2.5 The DNA Comet Test

Was performed according to Singh et al*.,* (2008). This test has high sensitivity and makes it possible to quantify the levels of single strand breaks of DNA. On a glass

slide previously covered with a 1.5% agarose layer, the cells are deposited in low melting agarose. The material was immersed in lysis solution (89 mL lysis solution to 10 mL Dimethyl sulfoxide and 1 ml Triton X-100), for the removal of membranes and cytoplasm from the cells. In sequence, the slides were then incubated in alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA in distilled water) and subjected to electrophoresis for about 30 minutes at 25 V and 300 mA. Subsequently, the neutralization, fixation and staining processes were performed so that the genetic material could be analyzed. The analysis of each slide was done under an optical microscope and the cells were classified according to the image format in four damage classes (Fronza, 2010).

For the evaluation of toxicity with A. salina, 10 mg of 90% viability cysts were placed in a beaker containing 350 mL of pre-aerated artificial marine water for 15 minutes and maintained for 48 hours under constant aeration at 28 °C and photoperiod of 12 hours/day, 12 hours / night (DA SILVA et al., 2018). Artificial marine water was prepared which, 1 L of distilled water, 23 g of NaCl, 11.0 g of $MgCl_2.6H_2O$, 0.4 g of Na_2SO_4, 1.3 g of $CaCl_2.2H_2O$ and 0.7 g of KCl was adjusted to pH 9.0 by the addition of Na_2CO_3.

2.6 Determination of acute toxicity and LC50

The test substances were diluted in 10 mL of artificial marine water at concentrations of 5.0, 10, 15 and 20 µL/10mL. The assay was conducted in triplicate tubes with two controls: a negative control, consisting of artificial marine water and a positive control consisting of sodium Dimethyl Sulphate ($(CH_3)_2SO_4$) in the concentration of 20 µL/10 mL diluted in artificial marine water. In each experimental unit (test tube) 10 nauplii were arranged and the tubes were kept under experimental conditions of 25 °C, photoperiod of 12 hours / day and 12 hours / night for 48 hours, when the number of survivors was counted by taking notes of any abnormal behavior (e.g., difficulties in swimming). LC50 was calculated using the probit method in which the concentrations tested are transformed

into logarithms and the percentage of deaths in probits. These data were submitted to simple linear regression analysis, obtaining a straight line and, by the obtained equation, the value was estimated for 50% of mortality after being transformed into antilog.

2.7 Statistical Analysis

The statistical software GraphPad Prism version 5.0 was utilized for statistical analyses, and the data were presented as mean ± standard deviation (SD). All *in vitro* experiments were conducted in triplicate, and treatments were compared using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Statistical significance was defined as $p<0.05$, represented as * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$. LD50 calculations were performed using the Biostat® software, and the results were reported as mean ± SD. All tests were conducted in triplicate.

3 RESULTS

The cytogene-ecotoxicity data are listed in the tables and graphs followed by the respective discussions.

3.1 Characterization and stability results of *trans-anethole*-containing nanostructured systems

The nanostructured systems produced in this study were evaluated for ninety days on the physicochemical parameters determining the stability of the suspensions: pH, PDI, zeta potential and average particle diameter. The results obtained are described in Tables 2 to 5 below.

Table 2 – Stability of Blank Nanocapsules (NCB), on physical-chemical parameters

	Days	pH	Averege diameter (nm)	PDI	Zeta potential (mV)
(NCB)	0	5.9 ± 0.1	231 ± 1.57	0.21± 0.01	-21±0.5
	3	5.8± 0.05**	199 ± 1.52***	0.21± 0.01	-16 ±2.6
	7	5.33±0.05**	200 ± 1.0***	0.23 ± 0.04	-16 ± 2.6
	20	5.53 ± 0.05**	200 ± 1.0***	0.23 ± 0.04	-16 ± 2.6
	30	5.53 ± 0.05**	200 ± 1.0***	0.28 ±0.01*	-16 ± 2.6
	40	5.53 ± 0.05**	200 ± 1.0***	0.28 ± 0.01*	-16 ± 2.6
	60	5.33 ± 0.15***	202 ± 2.0***	0.28 ± 0.01*	-16 ±2.6
	90	5.31 ± 0.15***	202 ± 2.0***	0.29 ± 0.01*	-16 ±2.6

Source: Authors (2023)

Table 3 – Stability of *trans*-anethole nanocapsules (NCTA) on physicochemical parameters

	Days	pH	Averege diameter (nm)	PDI	Zeta potential (mV)
NCTA	0	5.5±0.0	95 ± 0.00	0.21± 0.1	-11± 0.00
	3	5.3±0.0	95,9± 0.00	0.23± 0.00	-13± 0.00
	7	5.3±0.0	95± 0.02	0.23± 0.00	-13± 0.00
	20	5.0±0.2	96± 0.00	0.20± 0.00	-12± 0.00
	30	4.7±0.0	101± 0.00	0.23± 0.00	-12± 0.00
	40	4.8±0.0	102± 0.00	0.33± 0.00	-13± 0.00
	60	4.4±0.1	101± 0.02	0.38± 0.00	-13± 0.00
	90	4.4±0.2	103± 0.02	0.41± 0.00	-13± 0.00

Source: Authors (2023)

Table 4 – Stability of nanoemulsions with *trans*-anethole (NETA) on physical-chemical parameters

	Days	pH	Averege diameter (nm)	PDI	Zeta potential (mV)
NETA	0	6.0±0.0	82.1±0.0	0.26±0.03	-11± 0.00
	3	6.0±0.0	83±0.0	0.27±0.03	-9.9± 0.00
	7	5.9±0.1	82±0.0	0.26±0.1	-12± 0.00
	20	5.6±0.01	81±0.0	0.24±0.03	-12± 0.00
	30	5.5±0.03	83±0.0	0.24±0.0	-13± 0.00
	40	4.8±0.01	80±0.2	0.20±0.1	-11± 0.00
	60	4.5±0.0	83±0.0	0.25±0.01	-9.9± 0.00
	90	4.5±0.0	83±0.0	0.25±0.01	-10± 0.00

Source: Authors (2023)

Table 5 – Stability of the Blank Nanoemulsions (NEB) on the physical-chemical parameters, represented in the table

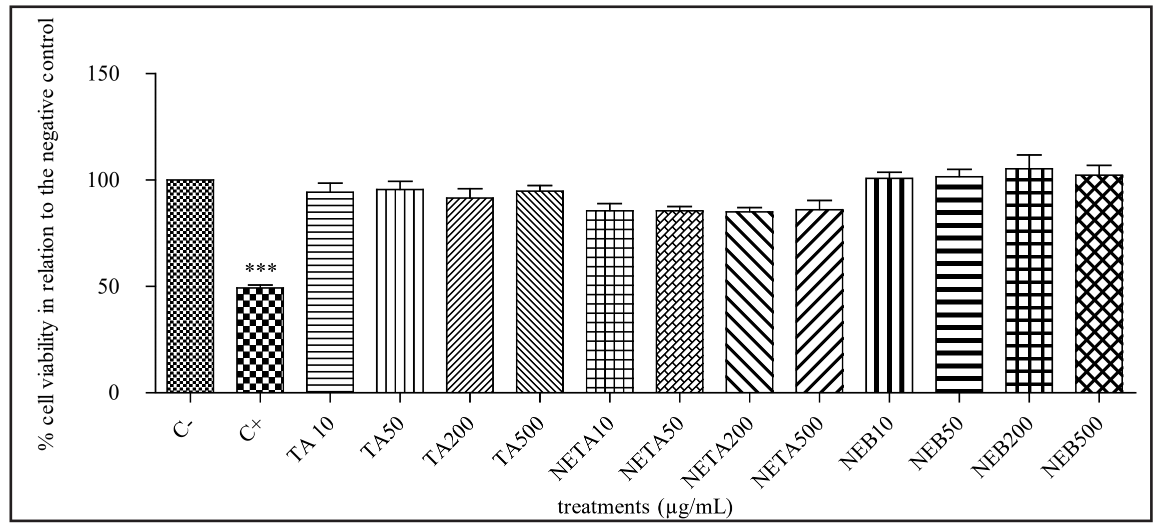
	Days	pH	Averege diameter (nm)	PDI	Zeta potential (mV)
NEB	0	6.3 ± 0.0	198 ± 1.0	0.13 ± 0.02	-20 ± 1.0
	3	6.3 ± 0.0	200 ± 1.0	0.15 ± 0.01	-20 ± 1.0
	7	6.3 ± 0.0	199,6 ± 0.57	0.15 ± 0.01	-20 ± 1.0
	20	6.3 ± 0.0	201 ± 3.2	0.15 ± 0.01	-20 ± 1.0
	30	6.3 ± 0.0	201 ± 3.2	0.16 ± 0.02	-20 ± 1.0
	40	6.3 ± 0.0	202 ± 2.0	0.16 ± 0.03	-20 ± 1.0
	60	5.9 ± 0.05***	203 ± 2.0	0.16 ± 0.03	-21 ± 1.0
	90	5.7 ± 0.05***	202 ± 2.0	0.16 ± 0.03	-21 ± 1.0

Source: Authors (2023)

3.2 Results of the MTT pharmacological safety evaluation of free formulations and nanostructured systems containing *trans-anethol*e

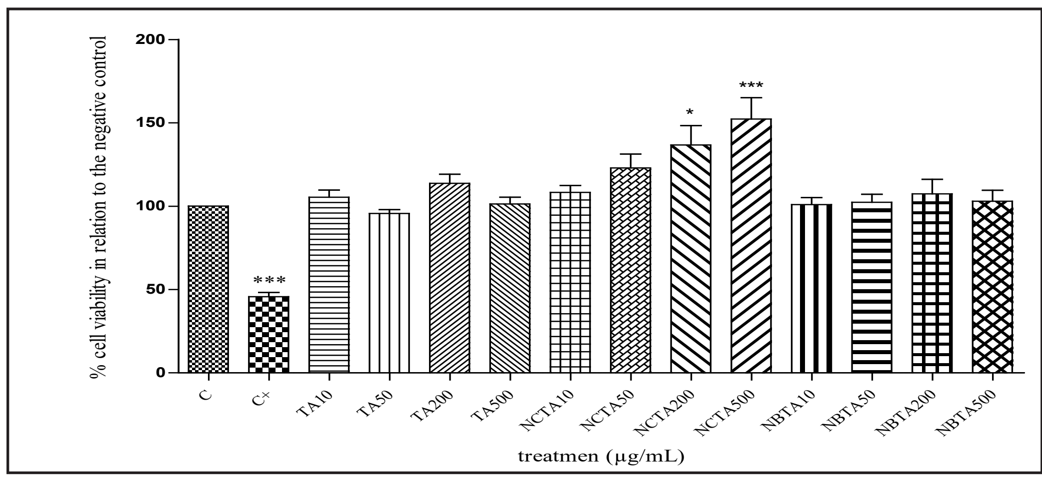
The pharmacological safety assessment of free *trans*-anethole and nanostructured systems produced were analyzed for cell viability via the MTT assay. The results are expressed as mean and standard deviation relative to the percentage of cell viability compared to the negative control and represented in figures 1 and 2, as per legend below.

Figure 1 – Results obtained from the evaluation of in vitro cytotoxicity of free *trans*-anethole and nanoemulsions, on PBMC by MTT



Source: Authors (2023)

Figure 2 – Results obtained from the evaluation of cytotoxicity of free *trans*-anethole and in polymer nanostructures in vitro on PBMC by MTT



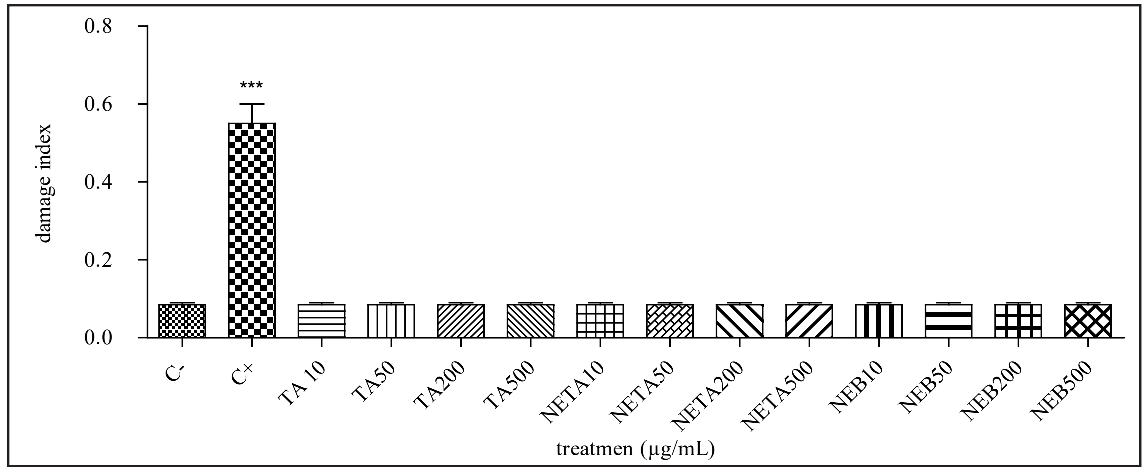
Source: Authors (2023)

3.3 Results of the evaluation of cytogenotoxicity of free formulations and nanostructured systems containing *trans*-anethole

The evaluation of the genotoxicity of free *trans*-anethole and nanostructured systems was performed by the Comet assay. The results are expressed as mean and

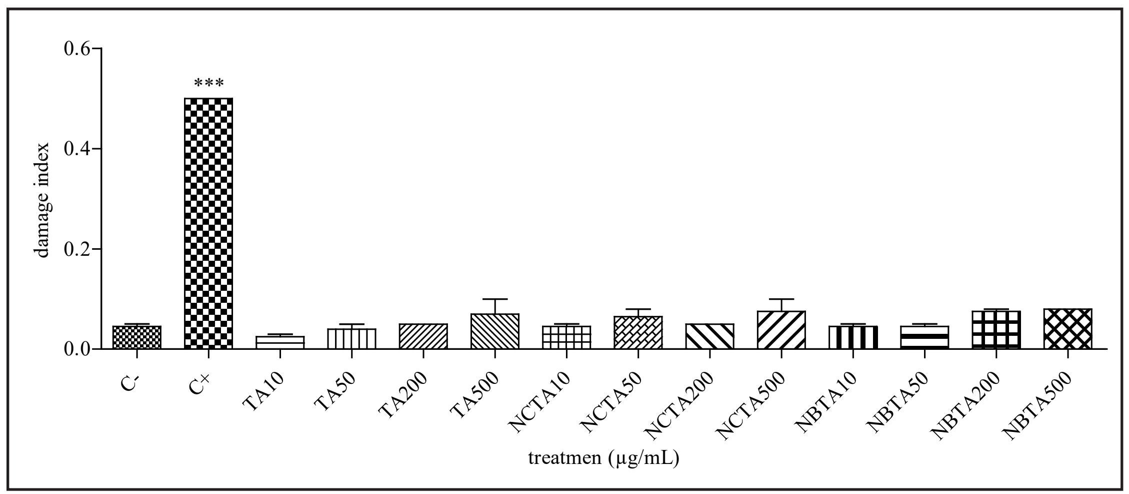
standard deviation relative to the percentage of cell viability compared to the negative control, these results are represented in figures 1-4, as per legend below.

Figure 3 – Results obtained from the evaluation of in vitro genotoxicity through the comet test



Source: Authors (2023)

Figure 4 – Results obtained from the evaluation of genotoxicity in vitro through the comet test



Source: Authors (2023)

3.4 Results obtained for the calculation of LC50 (dose required to kill 50% of the population of the test sample)

The lethal concentration of free *trans*-anethole and the nanostructured systems

produced was calculated by applying simple linear regression, where the data obtained for intercept (a) and regression coefficient (b) were used in the formula: Y1 = a = bX. The results obtained are shown in Table 6, below.

Table 6 – Values obtained for LC50 (%) of the samples tested

Sample tested	CL50
TA	2.75 μL/mL
NEB	6300 μL/mL
NETA	147 μL/mL
(NCB)	6300 μL/mL
NCTA	63 μL/mL

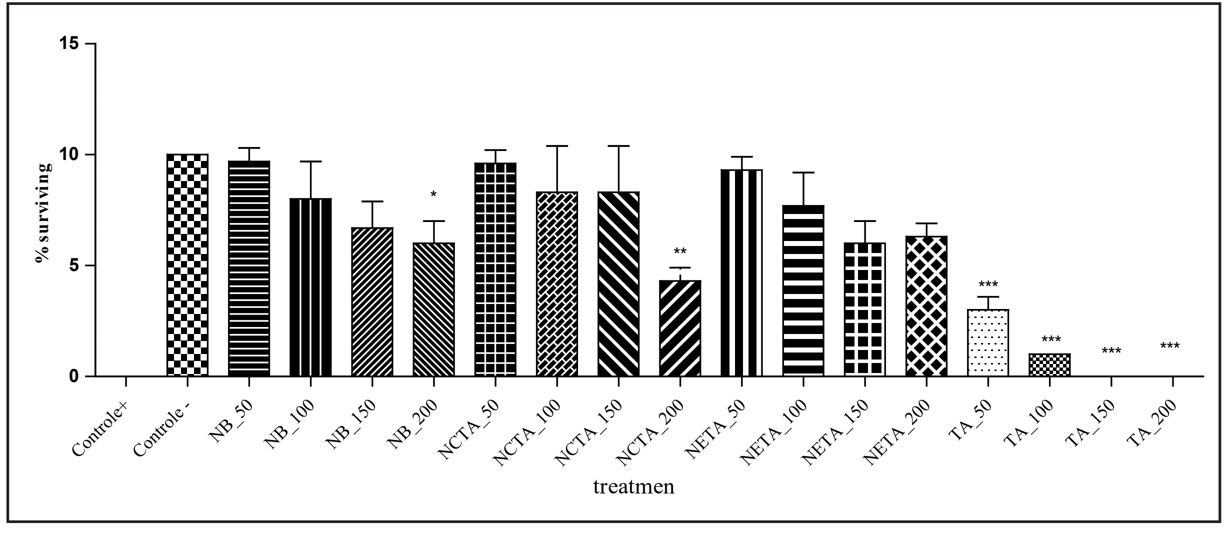
Source: Authors (2023)

(NCB): Blank Polimeric Nanocapsule, **NCTA**: *Trans*-anethole Polymeric Nanocapsule, **NEB**: Blank Nanoemulsion, **NETA**: *Trans*-anethole Nanoemulsion, **TA:** *Trans*-anethol

3.5 Results of the evaluation of the ecotoxicity bioassay using *Artemia salina* of the free formulations and nanostructured systems containing *trans*-anethole

The evaluation of the acute ecotoxicity was performed using the bioassay with *Artemia salina*, the results were expressed through the direct relation between the number of Artemias surviving after exposure of 48 hours to the free *trans*-anethole and in the nanostructured systems produced in this study and are represented in Figure 5.

Figure 5 – Results of bioassay analysis with saline artemia for 48 hours



Source: Authors (2023)

4 DISCUSSIONS

The nanostructured systems developed in this study were blank nanocapsule and blank nanoemulsions (NCB and NEB) and nanocapsules and nanoemulsions containing TA (NETA and NCTA) were prepared by adapting the interfacial deposition technique of the preformed polymer (Colusso, 2016, Fessi et al., 1989).

The nanocapsules and nanoemulsions presented the pH between 4.0 and 6.3, demonstrating an acidic character similar to the pH found in related works (Alves et al., 2007, Schaffazick et al., 2003). In our tests, we obtained highly promising results in terms of the parameters of PDI (Polydispersity Index), zeta potential, and mean particle diameter. The polydispersity index was consistently below 0.3, indicating good uniformity in size distributio (The average zeta potential obtained was -16 mV, which is noteworthy. Notably, both the nanocapsule and nanoemulsion containing *trans*-anethole demonstrated a reduction in size. This can be attributed to the physical and chemical interactions that occur when an active ingredient is incorporated into the formulation).

Incorporating an active ingredient such as our trans-anethol, can lead to changes in surface tension and emulsion stability, which in turn affects the interaction between the molecules present. Additionally, as observed, the charge of the formulation was reduced, altering the distribution of electric charges at the emulsion interface. In the case of the nanocapsule, this reduction in charge may have further promoted compaction and stabilization of the structure. These findings highlight the significant impact of incorporating *trans*-anethol ingredient, resulting in favorable changes in the physicochemical properties of the nanocapsule and nanoemulsion (Nunes et al., 2020).

This size reduction is considered adequate to prevent aggregation and precipitation within the nanostructures, ensuring the stability of the system (Nel et al., 2009). The average diameter obtained in our nanostructured systems ranged from 80 nm to 210 nm. Similar findings have been reported in related studies (Angeli, 2007, Schaffazick et al., 2003), providing further evidence that our results are consistent with the existing literature data.

In this study, the nanocapsules and nanoemulsions were stable in the first 30 days and showed an expected pH drop for the type of nanostructured system developed, which agrees with the work described by (Müller-Goymann, 2004, Schaffazick et al., 2003).

In the MTT cell viability test results, it was observed that only the nanocapsules with the highest concentrations of TA displayed a significant difference compared to the negative control. These nanocapsules exhibited an increase in cell proliferation, indicating a positive effect on cellular growth. Conversely, the other treatments did not demonstrate this characteristic.

Based on the results obtained from the MTT assays, it can be concluded that the nanostructures containing TA are not cytotoxic. In fact, they exhibit a potential for cellular protection, as evidenced by their positive impact on cell proliferation (Bruckmann et al., 2022, Oliveira et al., 2022). Moreover, the genotoxicity evaluation using the Comet assay in this study revealed no significant damage to the genetic

material. These findings indicate the pharmacological safety of the utilized systems and align with the results reported by Choo et al. (2011), Franceschi et al. (2011) and Galicka et al. (2014).

An alternative approach to cytotoxicity testing that is quick, efficient, and cost-effective involves the use of mammalian cells, specifically macrophages. Macrophages are a group of cells that share a common lineage originating from the bone marrow. They circulate in the blood as monocytes and, upon settling in tissues, mature into macrophages, exhibiting phenotypes that are directly influenced by their surrounding environment (Fujiwara, Kobayashi, 2005). In the absence of inflammation, the primary role of macrophages is to remove dead and damaged cells. Given their unique characteristics, macrophages serve as valuable indicators of cytotoxicity and genotoxicity.

In this regard, cell cultures offer a vital tool for investigating the cytotoxicity of compounds with potential therapeutic activity (Costa-Lotufo et al., 2010). By utilizing macrophages, researchers can gain valuable insights into the impact of these compounds on cellular health and viability. This approach provides an important avenue for assessing the potential therapeutic effects and safety of various compounds.

The results of the bioassay with *Artemia salina* used as an ecotoxicity bioindicator clearly demonstrate the reduction of the toxicity of nanostructured systems containing trans-anethole when compared to the negative control. The free *trans*-anethole showed toxicity at all concentrations tested while only the nanocapsules with the highest concentration (200 μL/mL) showed a significant difference.

The utilization of bioassays for toxicity analysis, especially involving organisms positioned at the base of food chains like *Artemia salina*, presents a compelling approach (Carvalho et al., 2009). *Artemia salina*, a species of microcrustacean found in saltwater environments, is widely employed in initial toxicological tests due to its sensitivity to environmental variations, making it a valuable bioindicator (Tipping, Abel, 1990). Consequently, the mortality or immobility of this organism has been utilized in bioassays for assessing environmental (Meyer et al., 1982).

In addition to their effectiveness as bioindicators, Artemia tests offer the advantages of being rapid, practical, and cost-effective (Rios, 1995, Carvalho et al., 2009). These characteristics make them highly suitable for assessing the potential toxicity of substances and environmental safety in a timely and efficient manner.

Thus, we can conclude that the nanostructured systems reduced the ecotoxicity of the tested asset, (Oliveira et al., 2022, Pérez, Giling, 2001, Rodriguez et al., 2009). The great sensitivity of *Artemias salina* to substances dispersed in the aquatic environment may be related to the results obtained by other researchers such as Rand and Petrocelli (1985) already reported that toxic substances had effects on cell membranes and interference in the formation of lysosomes. Sánchez leal, (1995) reports effects on cell membranes, such as osmotic shocks and changes in cellular permeability, as well as transport and mitochondrial inhibitors (Dawson et al., 1989). These reports highlight the sensitivity of marine organisms, confirming their potential as indicators of ecotoxicity.

5 CONCLUSIONS

The results obtained in this study lead us to the conclusion that the production, development, and characterization methods employed were effective in the creation of nanostructured systems containing *trans*-anethole. These formulations exhibited stability for a period of 90 days under refrigeration between 2 °C and 8 °C.

The MTT cell viability analysis did not yield statistically significant results for the formulations, indicating that they did not have adverse effects on cell viability. Moreover, the evaluation of genotoxicity using the Comet assay demonstrated no significant DNA damage, suggesting their promising potential for use in Biomedicine.

The bioassay conducted with *Artemia salina* showed a substantial reduction in the toxicity of the nanostructured systems containing *trans*-anethole compared to the free *trans*-anethole. This finding clearly indicates that the nanostructured systems, whether in the form of nanoemulsions or polymer nanocapsules, provided protection

to the active ingredient and minimized its toxic effects in its free form. Notably, the toxicity of the free active ingredient was significantly higher compared to the tested nanostructured systems. However, further complementary tests such as chronic toxicity and biological activity assessments will be necessary to determine their full pharmacological potential, which motivates the need for additional investigations.

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