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Avaliação físico-química e biológica de nanoemulsão contendo clotrimazol como alternativa terapêutica para o tratamento de candidíase vulvovaginal

Physicochemical and biological evaluation of clotrimazole loaded nanoemulsion as a therapeutic alternative for treatment of vulvovaginal candidiasis

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RESUMO

A candidíase vulvovaginal é uma infecção de alta incidência, atingindo 75% das mulheres adultas. No entanto, os microrganismos desenvolvem mecanismos de proteção, e por isso, é importante o desenvolvimento de estratégias. O objetivo do presente estudo foi o desenvolvimento de formulação nanoemulsionada contendo clotrimazol (CTZ) visando o tratamento tópico da candidíase vulvovaginal. As nanoemulsões (NE-CTZ) foram preparadas pelo método de temperatura de inversão de fase e caracterizadas em relação ao diâmetro hidrodinâmico médio (DHm), índice de polidispesividade (PdI), potencial Zeta (pZ), Eficiência de Encapsulação (EE), Espectroscopia no Infravermelho Médio (FT-MIR), morfologia, citotoxicidade em linhagem de fibroblastos (NHI/3T3) e atividade antifúngica frente às cepas de *Candida albicans* sensível (ATCC 24433) e resistente à múltiplas drogas (ATCC 10231). O DmH das nanoemulsões foi de aproximadamente 260 nm, com PdI característico de sistemas monodispersos (~ 0.3) e pZ de -26 mV. As nanoemulsões propiciaram elevada incorporação do CTZ (EE ~ 100%) e os ensaios de FT-MIR puderam corroborar a encapsulação desse ativo. As análises morfológicas mostraram nanogotículas com tamanho semelhante ao encontrado para o DHm e com formato arredondado irregular. No ensaio de viabilidade celular foi constatado que em todas as concentrações testadas (4,8-0,15µg/mL) a nanoemulsão demonstrou menor potencial citotóxico quando comparada ao fármaco livre. Em relação aos ensaios antifúngicos, a NE-CTZ apresentou atividade inibitória frente às cepas testadas na faixa de concentração de 4,8- 0,6µg/mL (referente ao CTZ). Pelo exposto, a formulação desenvolvida apresenta características físico-químicas e biológicas promissoras para utilização no tratamento da candidíase vulvovaginal, sobretudo nos casos de resistência antifúngica a outros fármacos azólicos.

Palavras-chave: nanoemulsão, clotrimazol, candidíase vulvovaginal.

ABSTRACT

Vulvovaginal candidiasis is a high incidence infection, affecting 75% of adult women. However, microorganisms develop protective mechanisms, so it is important to develop strategies. The aim of the present study was the development of clotrimazole (CTZ) loading nanoemulsion for topical treatment of vulvovaginal candidiasis. The nanoemulsions (NE-CTZ) were prepared by the phase inversion temperature method and characterized in relation to the average hydrodynamic diameter (Zav), polydispersity index (PdI), Zeta potential (Zp), Encapsulation Efficiency (EE), Mid Infrared Spectroscopy (FT-MIR), morphology, cytotoxicity in fibroblast cell line (NHI/3T3) and antifungal activity against susceptible (ATCC 24433) and multidrug resistant (ATCC 10231) strains of *Candida albicans*. The Zav of nanoemulsions was approximately 260 nm, with PdI characteristic of monodisperse systems (~ 0.3) and Zp was equal to -26 mV. Nanoemulsions provided high CTZ incorporation (EE ~ 100%) and FT-MIR assays could corroborate the encapsulation of this active. Morphological

analysis showed droplets similar in size to those found for Zav and with irregular round shape. In the cell viability assay it was found that for all concentrations tested (4.8- 0.15µg/ mL) nanoemulsion showed lower cytotoxic potential when compared to free drug. Regarding antifungal assays, NE-CTZ showed inhibitory activity against strains tested in the concentration range of 4.8-0.6µg/ mL (referring to CTZ). Therefore, the developed formulation presents promising physicochemical and biological characteristics for use in the treatment of vulvovaginal candidiasis, specially in cases of antifungal resistance to other azole drugs.

Keywords: nanoemulsion, clotrimazole, vulvovaginal candidiasis.

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a fungal infection considered the second most common vaginal infection that affects about 70-75% of the female population of childbearing age (XU et al., 2018). It is estimated that VVC reaches 138 million women, annually, and that 372 million women are affected throughout their life by this disease (DENNING et al., 2018). Although candidiasis does not cause the patient's death, it can cause physical, psychological and sexual complications (AHANGARI et al., 2019).

Despite there are 71 types of yeast, *Candida albicans* is largely responsible for most infections (AMARAL et al., 2019). About 90% of VVC cases caused by *C. albicans* are treated with oral or topical antifungals (FARHAN et al., 2018). Oral preparations are chosen for systemic infections or immunosuppressed patients (KANG et al., 2018). Current treatment of VVC uses azole antifungals including fluconazole, miconazole, itraconazole, ketoconazole, thioconazole and clotrimazole (CALVO et al., 2018). It is important to consider that the use of azoles in the treatment or prevention of *Candida* infections has increased the occurrence of resistance of the microorganism to these antifungals, especially fluconazole (SOUZA et al., 2019). In addition, fluconazole is likely to favor adverse events such as gastrointestinal disorders, headaches, nausea and skin rash (ALSAAD et al., 2015). To overcome these drawbacks, clotrimazole may be the drug of choice for VVC therapy.

Clotrimazole has better tolerability and fewer adverse effects when compared to other azoles. This antifungal acts by inhibiting lanosterol 14-alpha demethylase in *C. albicans* cells. This inhibition occurs due to the binding of the unsubstituted nitrogen (N-3 or N-4) of the imidazole moiety to the ferric heme group and also by the binding of the substituted N-1 to cytochrome P450 apoprotein. Inhibition of this enzyme promotes blockage of the conversion of lanosterol to ergosterol, that alters the membrane permeability and causes the microorganism death (QUINDÓS et al., 2019).

For the treatment of vulvovaginal candidiasis, lotrimazole-containing creams are commercially available. However, the development of modified release formulationsmay be a therapeutic alternative for greater treatment efficacy. In this sense, the development of nanotechnology-based release systems is increasingly being explored in pharmaceutical development and research (AMARAL et al., 2019; VERMA et al., 2019). In this sense, studies shows that the use of nanostructured devices provide greater drug delivery efficiency by releasing it specifically to the target and improving its bioavailability (SOSNOWSKA et al., 2017). Therefore, sub-toxic drug concentrations can be used, which helps to reduce the development of resistance by microorganisms. In addition, nanoparticles allow a lower release of the encapsulatedactive, that promotes a longer action of the drug, reduced toxicity and reduced side effects, which improves the patient's life quality (JAIN, THAREJA , 2019).

Nanoemulsions are gaining prominence due to their properties that allow the incorporation of hydrophobic or hydrophilic actives. This is due to the fact that its constitution is made by two immiscible liquids (SOSNOWSKA et al., 2017). Besides that, nanoemulsions have a small droplet size, easy tissue penetration, high drug permeation rate and are easily prepared at low cost (SORIANO-RUIZ et al., 2018).

With these considerations in mind, the objective of this study was to develop and characterize nanoemulsions containing clotrimazole as a therapeutic alternative for the treatment of vulvovaginal candidiasis.

MATERIAL AND METHODS

Nanoemulsion preparation

Nanoemulsions loaded CTZ (NE-CTZ) were prepared the phase inversion temperature method. Briefly, the materials were separated into aqueous phase consisting of water (80 %) (continuous phase) and oil phase containing Caprylic/Capric Triglyceride (Miglyol®, 10%), a mixture of non-ionic surfactant (10%) [polysorbate 80 (TWEEN® 80, 60%) and sorbitan monostearate (SPAN® 60, 40%)] and clotrimazole (2.5 mg/mL) (dispersed phase) and heated at 75°C. Next, the aqueous phase was dripped onto the oil phase at 600 rpm and the emulsion formed was cooled in an ice bath to 25 °C under constant stirring. For comparison purposes nanoemulsion without clotrimazole (NE) was prepared by the same method.

Average hydrodynamic diameter (Zav), polydispersity index (PdI) and Zeta potential (Zp)

The determination of the Zav as well as the PdI was performed using the Dynamic Light Scattering (DLS) technique. Zeta potential values were determined using the electrophoretic mobility technique. Measurements were performed on Zetasizer Nano ZS90 equipment (Malvern®, Malvern, UK), at 25 °C, after dilution of samples (400x) in ultrapurified water. Measurements were performed in triplicate and values were expressed as mean ± standard deviation.

Encapsulation Efficiency (EE)

The EE was determined indirectly by quantifying the free drug present in the supernatant after centrifugation of NE-CTZ. Samples were centrifuged on Amicon centrifuge filters (MWCO 10.000, Millipore, Germany) at 19.000 G for 30 minutes. The filtrate was analyzed on the Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific Inc., USA), after proper dilution in ethanol, at 208nm. The percentage of encapsulation efficiency (% EE) was calculated according to the following equation:

$$(I)\ \%\ EE = \frac{total\ drug\ content - free\ drug}{total\ drug\ content} \times 100$$

Fourier Transform Mid-Infrared Spectroscopy (FT-MIR)

FT-MIR analyzes were performed on the Perkin Elmer® SPECTRUM TWO equipment (São Paulo, Brazil) which has a zinc selenide attenuated total reflectance (ATR) enhancement with a resolution of 4 cm-1. NE, NE-CTZ and pure clotrimazole were analyzed. For this, the suspended samples (20 µL) were dried at 40°C directly on the equipment. The spectrum was obtained using OPUS® Software for wavelengths in the range of 4000-550 cm -1 with 16 spectrum scans (RUTZ, 2017).

Transmission Electron Microscopy

The morphological characterization of nanoemulsions was made by transmission electron microscopy (MET) at the Central Electron Microscopy Laboratory of the Federal University of Santa Catarina using the JEM-1011 (Jeol LTD., Tokyo, Japan) equipment operated at accelerating voltage of 80kV. For this, an aliquot

of the NE and NE-CTZ was placed on an amorphous carbon-coated Parlodion® 200 mesh (CF200-Ni, SEM) nickel grid and then dried for 24h at room temperature. Thus, the bright field option was selected for morphology analysis up to 50,000x magnification.

Cell viability

In a 96-well plate NIH/3T3 cells were seeded at a density of $5x10^3$ cells per well with DMEM with 10% fetal bovine serum and 1% antibiotic (penicillin/streptomycin). Subsequently, the plate was incubated overnight at 37°C in 5% CO2. NE, NE-CTZ and free drug solutions were prepared and added to the wells (n=3) at the following concentrations: 0.15, 0.3, 0.6, 1.2 and 2.4µg/mL of CTZ. Cells with culture medium were considered negative control and treatment with 5% DMSO was considered positive control. Also, the solvent control (DMSO 0.5%, used for solubilization of CTZ) was added. The plate was incubated under the same conditions as above for 24h. After this time, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added and the plates were incubated for 4h. The precipitate formed was dissolved with DMSO and their absorbance was measured on a Spectramax 190 (USA) spectrophotometer at 540 nm. Negative control was considered as 100% viable cells. Analysis of variance (ANOVA) was performed to test the effects of the treatments and the Tukey test was used for comparisons between the individual treatment groups using GraphPad Prism 5.0 software (GraphPad Software, Inc). p values less than 0.05 ($p < 0.05$) were considered statistically significant.

In vitro **antifungal activity**

The antifungal activity of NE-CTZ was performed according CLSI (2017) in order to determine the Minimal Inhibitory Concentration (MIC). Sensitive (ATCC 24433) and multidrug resistant *C. albicans* (ATCC 10231) was cultured at 35 °C for 24 h in Sabouraud Dextrose agar (SDA). Sample stock solution of NE, NE-CTZ and CTZ were diluted from 0.0375 to 4.8 µg/mL (final volume = 80 µl) with DMSO (final concentration ≤ 1%). After, 100 µL of Sabouraud Dextrose Broth (SDB) was added to the wells. Finally, 20 µl of 103 CFU/mL (according to 0.5 McFarland turbidity standards) of standardized fungal suspension was inoculated in a 96-well plate and the test was performed in a volume of 200 µL. Plates were incubated at 35 °C for 24 h.

Similar tests were performed simultaneously for growth control (SDB + microorganisms + DMSO) and sterility control (SDB + DMSO). The MIC values were calculated as the highest dilution showing complete inhibition of tested strain. The analyses were performed in duplicate.

To determine the Minimum Fungicidal Concentration (MFC), samples (NE-CTZ and CTZ) from each well that showed no visible fungus growth in the MIC assay was plated on freshly prepared SDA plates and incubated at 35 °C for 24 h (CAMPOS et al., 2018).

The MFC was expressed as the concentration of the samples that did not show any growth on a new set of agar plates.

RESULTS AND DISCUSSION

Average hydrodynamic diameter (Zav), Polydispersity Index (PdI) and Zeta potential (Zp)

The Zav, PdI and Zp values of the NE and NE-CTZ are shown in Table 1.

Table 1 - Average hydrodynamic diameter, polydispersity index (PdI) and zeta potential of nanoemulsions (n=3)*

	Zav (nm)	PdI	Zp (mV)
NE	265.7 ± 1.84	0.30 ± 0.05	-26.5 ± 1.1
NE-CTZ	274.2 ± 3.50	0.32 ± 0.08	-26.0 ± 0.6

*Results are expressed as average ± standard deviation

The mean diameter value found for the nanoemulsion formulations were consistent with literature data (HASANI et al., 2015). Besides, the values for the NE-CTZ was in accordance with those indicated for topical application (50-300 nm) (COSTA et al., 2018). This size is important for nanoparticle retention at the specific site of therapeutic action, ensuring that it does not cross the deepest layers of the skin and enter the systemic circulation (GARCÊS et al., 2018). From the results found for PdI it is possible to suggest the formation of monodisperse systems, which contributes to the stability of nanoemulsions. In this sense, scientific data report that values less than or equal to 0.3 are characteristic of nanoparticles with unimodal size distribution and low size variability (AMARAL et al., 2019).

Zeta potential indicates the surface charge of the nanoparticles and also suggests the stability of the suspension. This stability can be reached by the use of nonionic molecules, which promotes steric stability, but also from the electrical repulsion of the surface charges of each nanoparticle (JAIN, THAREJA, 2019). The values found for NE and NE-CTZ are of about -20mV. This charge promotes repulsion between the droplets and consequently, promotes the system's stability. The negative charge found may be due to ionization of fatty acids present in the oil phase.

Encapsulation Efficiency (EE)

The results of free CTZ quantification after nanoemulsion centrifugation led to EE close to 100% (99,995% ± 0,005) and may be justified due to the high solubility of clotrimazole in the oily phase of nanoemulsion, which allows most of the drug molecules to be encapsulated within the droplets. From this perspective, Souto et al (2004) used the same oil to prepare CTZ-loading nanostructured lipid carriers. Besides that, similar result was also found by Das, Ng and Tan (2014) that developed clotrimazole-containing solid lipid nanoparticles and lipid nanocarriers with EE between 80-90%.

Fourier Transform Mid-Infrared Spectroscopy (FT-MIR)

Infrared spectrometry was used to corroborate drug encapsulation and possible nanoparticle changes. The free drug spectrum was recognized by the library of the equipment used to do the analysis (Figure 1). Peaks at 3050 and 1560 are characteristic of the clotrimazole molecule and can be attributed to the heteroatom ring (KAUR et al., 2018). The peaks around 300 may be due to the C-H bond belonging to its aromatic ring, and the peaks around 600-800 are characteristic of clotrimazole´s aromatic region. These characteristics were also reported by Patel and colleagues (2018) and Bilensoy and colleagues (2006).

By observing the nanoemulsion spectrum it is possible to verify the presence of a broad band around 3400 that can be attributed to OH bonds present in polysorbate 80 and caprylic acid molecules. The two peaks around 3000 are characteristic of C-H bonds present in all molecules used in nanoemulsion formation. Another peak found around 1700 may be characteristic of C = O bonds found in polysorbate 80, sorbitan monostearate

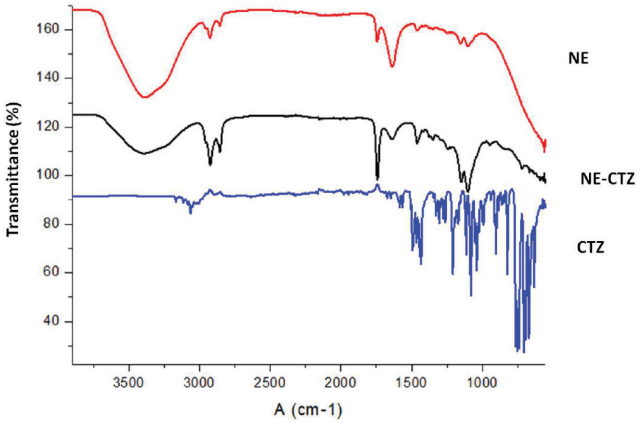

Figure 1 - FTIR spectrum of nanoemulsion with clotrimazole (black), nanoemulsion without clotrimazole (red) and free clotrimazole (blue)

and caprylic acid molecules. Beside this peak, a C = C bond can be observed and it is present in the surfactants (PAIVA et al., 2015). Regarding the NECTZ, although presenting a similar profile to the NE, it is possible to notice that some peaks are displaced or enlarged, which may suggest interactions between the nanoemulsion constituents and the CTZ. In addition, it is possible to observe that clotrimazole's aromatic region peaks at around 600 to 800 cm–1 have disappeared.

These data may suggest the occurrence of CTZ encapsulation in nanoemulsions, which corroborates the EE results.

Transmission Electron Microscopy (TEM)

TEM analyzes were conducted to evaluate the morphology of nanoemulsions. The images obtained (Figure 2) showed round shaped nanoparticles and can confirm the size found by the DLS technique. Compared to NE, NE-CTZ microscopy images reveal that the presence of the drug in nanoemulsions does not lead to changes in droplet morphology. This observation is similar to that observed for the Zav results and may indicate that encapsulation of CTZ, a small molecule (344.8 Da), does not increase these droplets.

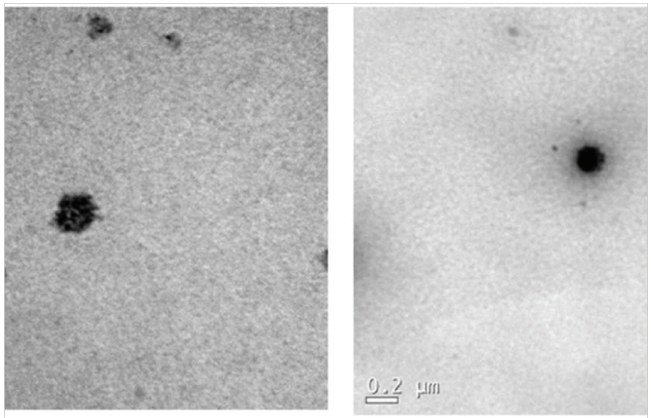

Figure 2 - NE (left) e NE-CTZ (right) - 200 nm increase.

Cell viability

The cytotoxicity evaluation of the NE, NE-CTZ and free drug and controls were evaluated using NIH/3T3 cell for 24h by the MTT assay and the results are expressed in Figure 3. As result, no significant reduction of cell viability was observed for the NC treatment at any concentration tested. Treatment with NC showed more than 95% of viable cells for all the concentrations. This reflects positively on the formulation, since it does not affect healthy cells.

When compared to the control group, treatment with free CTZ led to significantly reduced cell viability at all concentrations tested. The viability reduction was of 20%, 33%, 36% and 46% for the concentrations 0.3, 0.6, 2.4 and 4.8µg/mL, respectively. However, the potential cytotoxic effect of the drug is minimized after its encapsulation in nanoemulsions. In this sense, at the highest concentration of CTZ (4.8µg/mL) it was observed 56% reduction of cell viability compared to the nanoencapsulated drug. The same was observed for the remaining concentrations 0.15, 0.3, 0.6, 1.2, 2.4µg/mL that showed 28%, 34%, 28%, 29% and 41% reduction on viable cells compared to NE-CTZ treatments.

This difference in cell viability can be attributed primarily to the fact that the drug is protected inside the droplet, minimizing its direct action on cells. In addition, it can be an indication of a controlled CTZ release profile. Results of low cytotoxicity by nanoparticles were also found by Martinez-Perez (2017) in which the polymeric nanoparticles of poly(lactic-co-glycolic acid) and chitosan protected the cells from the clotrimazole action, presenting a cell viability greater than 75%. This finding corroborates one of the basic premises of nanotechnology: the potential for reducing possible toxic effects of drug molecules and may suggest that the system developed is a promising alternative for CTZ delivery.

In vitro antifungal activity

Resistance to azole antifungals is a major public health problem as the number of clinical infections caused by resistant *C. albicans* has increased considerably worldwide (CAMPOS et al., 2018). Azole antifungals, especially fluconazole, are the most prevalent drugs in clinical practice for treating vulvovaginal candidiasis. However, this treatment has been shown to be inefficient due to the increase in resistant strains (CHEN et al., 2010). Thus, the search for therapeutic alternatives is imperative. In this sense, this study aimed to evaluate the possibility of using nanostructured CTZ to increase antifungal efficacy, especially in cases of multidrug resistant *C. albicans strains*.

The results of *in vitro* antifungal activity show that NE-CTZ showed a MIC value of 0.6 µg / mL for both strains tested. In contrast, the MIC for the free drug was > 0.075 µg /mL.

This may be due to the fact that free drug is already available for use, and suggests that nanoemulsion release CTZ slowly and prolongedly. Otherwise, Soriano-Ruiz et al. (2018) observed that MIC values of NE-CTZ were lower than free CLT in solution. This discrepancy may be due the difference between the oil phases used in the preparation of the formulations.

In this sense, it is possible that the oil used in our study provides a more gradual release of the active.

In addition, it can be verified that the treatment with NE did not present inhibition of *C. albicans strains*, as observed by Soriano-Riz et al. (2018). This is because its constitution does not have any asset that could confer antimicrobial activity. Also, the DMSO control

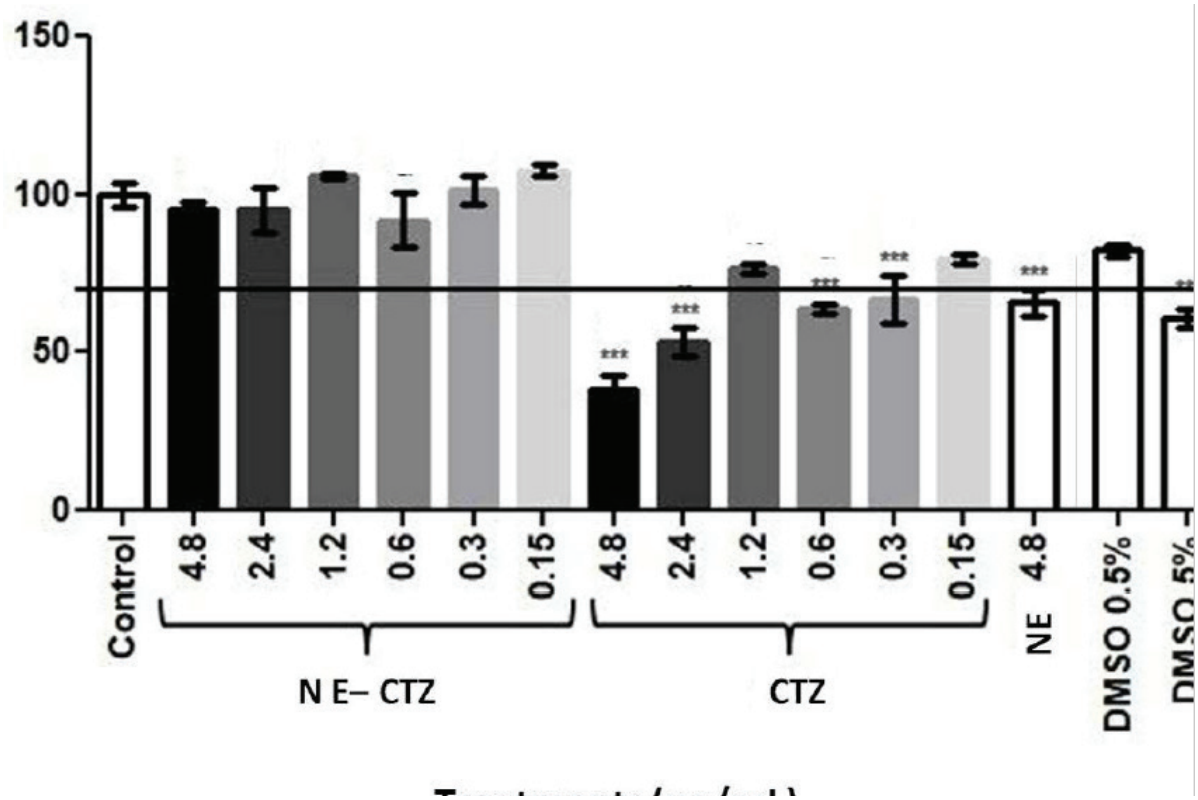

Figure 3 - Cell viability assay by MTT method on NIH/3T3 cells after 24h of treatment with CTZ, NE and NE-CTZ. Results are expressed as average of cell viability ± standard deviation.

showed no fungal growth, revealing the reliability of the test.

Finally, the Minimum Fungicidal Concentration for the NE-CTZ and CTZ were performed at concentrations of 0.6, 1.2, 2.4 and 4.8 µg/mL. All concentrations tested showed fungistatic action against sensitive and resistant *C. albicans*. This finding may indicate that nanostructuring maintains CTZ activity and thus may be a promising tool for topical treatment of vulvovaginal candidiasis.

CONCLUSION

The results presented in this work indicate that the developed nanoesmulsions have satisfactory physicochemical properties, low cytotoxic potential against the studied cell line and promising *in vitro* antimicrobial activity against *C. albicans strains*. Thus, it may constitute an innovative therapeutic option for the treatment of vulvovaginal candidiasis, especially in cases of strains resistant to other azole drugs. For the continuity of the study, we are conducting *in vivo* efficacy tests and stability evaluation of semisolid formulation containing the developed nanosystem aiming to prove the potentialities of its future use.

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