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Diphenyl Diselenide and Clotrimazole Co-loaded into Eudragit® RS 100 Nanocapsules Formulation Has Superior Antioxidant Potential and Promising Anti-candida Activity

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HIGHLIGHTS

- Clotrimazole and diphenyl diselenide were successfully co-loaded into nanocapsules.
- The nanocarriers showed no toxic effect in the alternative model of chorioallantoic membrane.
- Nanoencapsulation improved *in vitro* antioxidant action in comparison to the free ingredients.
- The formulation containing both actives and coconut oil showed the most promising properties.

Abstract: In the current study, nanocapsules (NC) formulations containing a co-load of clotrimazole (C), a highly prescribed antifungal drug, and diphenyl diselenide [$(PhSe)_2$], an organoselenium compound with a promising scope of pharmacological actions, were prepared. Formulations were characterized as well as the potential toxicity, antioxidant action, and antifungal effect were assessed using *in vitro* techniques. The NCs were prepared employing Eudragit® RS 100 as polymeric wall and medium chain triglycerides or virgin coconut oil (CO) as core. All NC suspensions had pH around acid range, compound content close to theoretical value (1 mg/mL/drug), average diameter in nanometric range, positive values of zeta potential as well as high encapsulation efficacy and mucoadhesive property. Physicochemical stability was performed over a 30-day period and showed no modification in the aforementioned parameters to all samples.

Preliminary screening of toxicological potential performed by the hen's egg test chorioallantoic membrane technique classified the formulations as non-irritant. The DPPH radical assay revealed that nanoencapsulated compounds had superior antioxidant action in comparison to their free forms (concentration range tested 1.0-25.0 µg/mL). Importantly, the formulation composed of CO and containing C and $(PhSe)_2$ showed the highest antioxidant potential and was selected for further investigation regarding antifungal effect against some *Candida spp* strains. Results of *in vitro* antifungal assay demonstrated that the C and $(PhSe)_2$ co-encapsulation had a minimum inhibitory concentration (MIC) values around 60. Thus, our study supplies additional data about advantages achieved by encapsulating active compounds.

Keywords: nanoparticles; selenium; inflammation; *Candida spp*; antioxidant property.

INTRODUCTION

Every human has fungi as part of their microbiota [1]. In healthy hosts, they do not present any disturb, but when our immune system weakens, these opportunistic organisms cause fungal infections. The incidence and prevalence of such diseases have raised in last years, especially in immunocompromised patients [2]. Among the most common infections are those caused by yeasts of the genus *Candida spp* in the female genitourinary tract, which are known as vulvovaginal candidiasis [3]. This pathology is considered a recurrent gynecological problem, because around up to 75% of women will be affected by vulvovaginal candidiasis at least once in their life and another 40 to 50% will have relapses [4]. Invasive Candida infection is another emerging problem in critically ill patients. *Candida albicans* alone represents 13% of all infections acquired in an intensive care unit [5] and associated with an attributable mortality of up to 49% [6].

The clotrimazole is one of the most prescribed drugs for the treatment of vulvovaginal infections [7]. It is also used to treat oropharyngeal candidiasis in patients with HIV/AIDS and cancer [8]. Its action mechanism is based on the inhibition of ergosterol synthesis, which causes structural and functional damage to the fungal cytoplasmic membrane [9]. The clotrimazole is usually administered by topical application of vaginal cream, however, its use is associated with some unpleasant sensations such as burning, irritation and rash [4]. In addition, considering the poor water solubility of clotrimazole and the reduce retention time of the formulation in the applicated site, the amount of permeated clotrimazole could not be satisfactory [10]. Then, the facility of extravasation from the applied region generates an insufficient residence time, affecting the drug bioavailability due to the natural cleansing process that occurs in the vaginal region [9].

In this context, several studies in scientific literature explore alternatives to overcome these issues, among them, the nanocarrier systems presented promising results, especially the polymeric nanostructures [11]. These structures promote a general improvement in the physicochemical and pharmacological properties of drugs in comparison to conventional forms [12]. By design, nanocapsules are composed by an oily nucleus surrounded by a polymeric shell, where the drug is usually dispersed or dissolved in or even adsorbed onto the surface [13]. Nanocarrier systems can provide drug controlled release, which increases the residence [11]. In addition, nanoparticles also confer greater protection to the active as well as decrease the adverse effects and consequently improve the therapeutic efficacy of drugs [13]. Importantly, the association of antifungal agents into nanocarriers was already described in the scientific literature [14,15]. A previous study of our research group investigated the potentialities of polymeric nanocapsules soughing to improve the efficacy of candidiasis pharmacological treatment [16,17]. In these studies, distinct oils, medium chain triglycerides (MCT) and virgin coconut oil (CO), were used to compose the oil core of nanocapsules. In this regard, extracted from *Cocos nucifera* Linn (Palmae) and used as emollient by the pharmaceutical industry [18], the CO has antifungal and antioxidant actions already described for its major components: lauric (40-50%) and myristic acids (15-20%) [19,20]. It is important to mention that the development of formulations by applying vegetable oils could provide an improvement in the global pharmacological action due to the composition rich in biologically active molecules [21,22].

Besides the infectious process initiated by the fungus action, there is also the fact that the pathology triggers an inflammatory process at the affected site, which worsens the condition and causes great discomfort for the patient [23]. Such scenario brings out the absence of effects exerted by clotrimazole onto this pharmacological aspect. Thus, diphenyl diselenide [$(PhSe)_2$], an organoselenium compound, appears as a promising drug at this matter. Several scientific reports showed a broad spectrum of biological effects, which includes the antioxidant and anti-inflammatory actions [24] as well as fungistatic and fungicidal actions [25–27]. Despite these biological potentialities, $(PhSe)_2$ has some physicochemical issues, such as poor aqueous solubility and toxic effects. Recently, our research group demonstrated the development of $(PhSe)_2$ nanocapsules. The formulations showed lower toxicity and improved therapeutic effect against cancer cells [28–30].

Therefore, the aim of this study was to develop clotrimazole and $(PhSe)_2$ polymeric co-loaded Eudragit® RS 100 nanocapsules formulations using different oils to compose the nanocarriers (MCT and CO). The stability, scavenger and toxicological potential as well as *in vitro* antifungal activity evaluation against *Candida* species were also carried out for the formulations.

MATERIAL AND METHODS

Drugs, reagents and materials

Clotrimazole (99.22%, w/w) was purchased from Pharma Nostra (São Paulo, Brazil). Diphenyl diselenide [$(PhSe)_2$] was synthesized following the method described by Paulmier (1986) [31]. The ^{1}H and ^{13}C nuclear magnetic resonance, as well as gas chromatography, were employed to confirm the compound chemical structure and to determine its purity (99.9%), respectively. Eudragit® RS100 (Röhm Pharma, Germany) was a gift from Almapal (São Paulo, Brazil). Span 80® (sorbitan monooleate) was purchased from Sigma Aldrich (São Paulo, Brazil) and Tween 80® (polysorbate 80) from Delaware (Porto Alegre, Brazil). Thera Herb (Niterói, Brazil) supplied the virgin CO. HPLC-grade methanol was acquired from Tedia (Rio de Janeiro, Brazil). All other solvents and reagents were analytical grade and used as received.

Analytical procedures

Using a previously validated method [16], the clotrimazole and $(PhSe)_2$ content was revalidated for a simultaneous determination by HPLC. The quantification was performed on a LC-10A HPLC system (Shimadzu, Japan) equipped with a LC-20AT pump, an UV-VIS SPD-M20A detector, a CBM-20A system controller and a SIL-20A HT valve sample automatic injector. The separation was achieved at room temperature (25 ± 2 ºC) using a C_{18} column (Phenomenex Gemini reversed phase, 5 µm, 110 Å, 150 mm × 4.60 mm) coupled to a C_{18} guard column. The isocratic mobile phase consisted of methanol and ultrapure water (90:10, v/v), a flow rate at 1 mL/min and an injection volume of 20 µL. The clotrimazole and $(PhSe)_2$ were both detected at 229 nm in a retention time of 3.2 and 5.1 minutes, respectively. The method was validated in agreement with the ICH guidelines to determine, simultaneously, clotrimazole and $(PhSe)_2$ in nanocapsules, demonstrating a linear response in a concentration range 3.0 – 15.0 µg/mL ($r = 0.9989$ and $r = 0.9988$), respectively. The method was considered specific, accurate (95 – 96.2%, relative standard deviation ≤ 2.51%) and robust.

Clotrimazole and $(PhSe)_2$ solubility in the oils

The clotrimazole and $(PhSe)_2$ solubility was performed to evaluate the compatibility with the oil cores used to compose the nanocapsules. The solubility of the molecules in oils was estimated separately or together in the same system in order to simulate a condition of co-loading in the nanostructures. The experiment was carried out by adding an excess amount of the compounds in 2 mL of each oil, MCT and virgin CO. The systems were kept under magnetic stirring overnight. Following, the samples were centrifuged at 2500 g for 10 min and an aliquot of the supernatant was diluted in acetonitrile and acetone (50:50) to a 10 mL of final volume. The quantity of each compound was determined using the chromatographic methodology described in the analytical procedures section.

Nanocapsules suspensions preparation

Nanocapsules suspensions were prepared by interfacial deposition of preformed polymer method. An organic phase containing acetone (27 mL), virgin CO or MCT (0.3 g), Span® 80 (0.077 g), Eudragit® RS100 (0.1 g), clotrimazole (0.01 g) and $(PhSe)_2$ (0.01 g), was kept under magnetic stirring at 40 °C for 30 min. The drugs were added after the solubilization of the structural components to avoid any interference. The organic phase was injected into the Tween® 80 (0.077 g) aqueous phase (53 mL) and the mixture was maintained under magnetic stirring for 10 min. In the sequence, the organic solvent and part of the water were eliminated by evaporation under reduced pressure to achieve a final volume of 10 mL, which corresponds to a clotrimazole and $(PhSe)_2$ concentration of 1.0 mg/mL to each drug. For comparison purposes, formulations containing only clotrimazole or $(PhSe)_2$ loaded nanocapsules and without the drugs (blank) were also prepared. In addition, the nanocapsule structural core was modified by altering the virgin CO by MCT.

Characterization of the nanocapsules suspensions

pH

The pH of nanocapsules suspensions was verified by directly immersing the electrode of a calibrated potentiometer (Model pH 21, Hanna Instruments, Brazil) in the formulations. Measurements were made at room temperature (25 ± 2 °C).

Average diameter, polydispersity index and zeta potential

The average diameter and polydispersity index were determined by photon correlation spectroscopy (Zetasizer Nanoseries®, Malvern Instruments, UK) after diluting an aliquot of the samples in ultrapure water (1:500). Zeta potential analyses were evaluated by the microelectrophoresis technique using the same instrument after the dilution of samples in 10 mM NaCl (1:500).

Granulometric distribution

Granulometric distribution was performed by laser diffraction (Mastersizer® 3000E, Malvern Instruments, UK) after diluting the samples in distilled water (200 mL) until reaching a laser obscuration of 10 - 15%. A refractive index of 1.31 was used to perform the measurement.

Drug content and encapsulation efficiency

The total clotrimazole and $(PhSe)_2$ content in nanocapsules suspensions were determined by diluting 90 µL of the formulations in 10 mL of acetonitrile and acetone (50:50, v/v) and submitting it to sonication for 10 min to extract the drugs. Before injecting into the HPLC system, the samples were filtered through a 0.45 µm membrane. To determine encapsulation efficiency, an aliquot of the samples was placed in a 10,000 MW centrifugal filter device (Amicon® Ultra, Millipore) and the free drug was separated from the nanostructures using the ultrafiltration/centrifugation technique (2200 g for 10 min). The encapsulation efficiency (%) was calculated as the difference between total and free concentrations of clotrimazole and $(PhSe)_2$, determined in the nanostructures and ultrafiltrate, respectively (Equation 1).

$$\mathrm{EE\%} = \frac{(\text{total content - free content})}{\text{total content}} \times 100 \quad (1)$$

Mucoadhesion evaluation in vitro

The nanocapsules mucoadhesivity was evaluated based on a mucin-particle method described by Takeuchi and coauthors [32]. Mucin porcine Type II was suspended in 0.1% ultrapure water (w/v). The nanocapsules suspension were diluted into the mucin solution (1:500, v/v) and the mean particle size as well the zeta potential was determined in Zetasizer®.

Stability studies

To evaluate the stability of the formulations, nanocapsules suspensions were packaged in amber glass flasks and stored at room temperature. After 30 days, the parameters of average diameter, PDI, zeta potential, pH and drug content in the formulations were evaluated.

Evaluation of irritant potential of the formulations

The hen's egg test chorioallantoic membrane (HET-CAM) was used to estimate the irritant potential of the developed formulations. For the test, fertilized hen's eggs, with 10 days of incubation (37 °C and 65% relative humidity), were used. During the test, the most external shell and the white membrane were removed without injuring the inner membrane. An amount of formulations (300 µL) was directly instilled onto distinct chorioallantoic membranes (CAM) (n=3/formulation). After 20 s, the samples were removed with saline solution and the CAM was monitored over 300 s. During this period, the onset of the vasoconstriction phenomena, hemorrhage and coagulation was recorded. The formulations (NC-T-CD and NC-CO-CD) were compared with the free compounds (dispersion in aqueous solution with 10% of Tween® 80 and 10% of dimethyl sulphoxide) and blank nanocapsules suspensions (NC-T-B, NC-CO-B) were evaluated to verify if the constituents of the formulations may cause any irritant effects. The positive (0.1 M sodium hydroxide - NaOH) and negative controls (saline solution) were also measured. Each test was performed in triplicate and the mean score of three eggs was determined. The irritation scores (IS) were calculated using the following equation (Equation 2):

$$IS = \frac{(301 - h)}{300} \times 5 + \frac{(301 - v)}{300} \times 7 + \frac{(301 - c)}{300} \times 9 \quad (2)$$

Where, h = hemorrhage time; v = vasoconstriction time, and c = coagulation time. From the IS values obtained, the lesions were classified non-irritant (0 - 0.9); slightly (1 - 4.9); moderate (5 - 8.9) and severe irritant (9 - 21).

Determination of antioxidant potential

The antioxidant potential was assessed using the DPPH radical scavenging assay as described by Sharma & Bhat (2009) [33]. The samples were evaluated at concentrations 1.0, 2.5, 5.0, 10.0 and 25.0 µg/mL. All nanocapsules were diluted in distilled water to achieve the desired concentrations for testing. Unloaded C, D, CO, MCT and the combinations (CO-CD and MCT-CD) were diluted in DMSO. The DPPH was dissolved in methanol and used as obtained (50 µM). The samples were incubated with DPPH solution for 30 min under light protection. Following, each sample had the absorbance measured using a UV/Vis spectrophotometer (Shimadzu, Japan) at 517 nm (DPPH assay). Pure DPPH and ascorbic acid solutions were used as negative and positive controls, respectively. The radical scavenging activity was expressed as percentage of scavenging capacity, as follows (Equation 3):

$$SC\% = 100 - \frac{(Abs - Abb) \times 100}{Abc} \quad (3)$$

Where SC% is the scavenging capacity in percentage, Abs is the absorbance of the incubated sample with DPPH, Abb is the blank sample absorbance, and Abc is the negative control absorbance.

Inoculum preparation

Candida albicans (ATCC 24433) and *C. glabrata* fluconazole resistant (clinical isolate) (MIC > 64 µg/mL) inoculum was prepared with after growth (48 h/35 °C) on Sabouraud dextrose agar. The colonies were suspended in sterile saline (0.85%) and the suspension was mixed in a vortex for 15 s; following, the cell density was defined in a spectrophotometer and the transmittance (λ=530 nm) was adjusted to match standard 0.5 on the McFarland scale (1×10^6 to 5×10^6 cells/mL). Suspensions were diluted at 1:50 in water, followed by a 1:20 dilution in the Roswell Park Memorial Institute (RPMI 1640) medium (Sigma Chemical Co. St Louis, MO, USA) and adjusted to pH 7.0 with morpholinepropanesulfonic acid buffer (MOPS; Sigma Chemical Co. St Louis, MO, USA), providing final cells concentration at $1.5 \pm 1.0\times10^3$ cells/mL.

In vitro susceptibility tests

Antifungal susceptibility testing

Among the samples tested, the NC-CO-CD showed the most promising profile in the DPPH test and it was used in the further steps of the study. The NC-CO-CD as well as the free components (clotrimazole, $(PhSe)_2$ and CO) had its antifungal activity evaluated by the broth microdilution method against *Candida albicans* (ATCC 24433) and *C. glabrata* fluconazole resistant (clinical isolate) (MIC > 64 µg/mL). The test was performed based on standardized protocol M27-A3, CLSI. The assay was carried out using 96-well flat disposable polystyrene sterile plates that were covered with 100 µL of samples diluted in RPMI medium (twice more concentrated than the desired final concentrations) in wells 1–10. The tested samples were as follow: the formulation NC-CO-CD and solutions of C, D, CO and a solution of both actives and CO (CD-CO) that were prepared using DMSO (1%). The 11 and 12 rows were used to the negative (medium only) and positive controls (medium + inocullum), respectively. To each well, 100 µL of the standardized inoculums was added, except for the negative control. In sequence, the plates were incubated at 37 °C for 48 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the sample resulted from an inhibition of visible fungal growth.

Statistical analysis

All the analyses were performed at room temperature in triplicate of batch. The results are expressed as the mean (s) ± standard deviation. Data normality was evaluated by the D'Agostino and Pearson omnibus normality test and probability values less than 0.05 ($p < 0.05$) were considered as statistically significant and able to be submitted to post hoc tests. The statistically significant difference was calculated by means of unpaired and paired Student's *t* test or using One-way ANOVA of ordinary or repeated measures followed by the Newman-Keuls' multiple range test. The GraphPad Prism software version 7 (San Diego, CA, USA) was used to perform these analyses.

RESULTS

Clotrimazole and $(PhSe)_2$ solubility in the oils

Clotrimazole solubilities were 9.70 ± 0.58 mg/mL and 5.78 ± 1.08 mg/mL in MCT and CO, respectively. In its turn, $(PhSe)_2$ solubilities were 18.50 ± 1.22 mg/mL and 24.08 ± 4.06 mg/mL in MCT and CO, respectively. The solubility of both compounds associated in the MCT oil was 12.79 ± 0.48 mg/mL and 19.77 ± 1.49 mg/mL, for clotrimazole and $(PhSe)_2$, respectively. In relation to CO, the solubility of both compounds associated was 5.34 ± 0.52 mg/mL and 17.02 ± 3.80 mg/mL, for clotrimazole and $(PhSe)_2$, respectively.

Clotrimazole and $(PhSe)_2$ loaded polymeric nanocapsules characterization

All formulations showed a macroscopic turbid aspect and opalescent bluish reflection were also observed, which are characteristic of the chaotic motion presented by the colloidal particles. The results of the characterization are expressed in Table 1. The nanocapsules suspensions had an average diameter in the nanometric range (≤ 200 nm) and PDI values lower than 0.2, indicating a narrow distribution of the particle population. The laser diffractometry technique demonstrated an absence of microparticles in all tested formulations (volume weighted mean diameters (D (4,3)) and SPAN values lower than 2.0. Positive values of zeta potential were obtained and pH values were in the acid range. The statistical analysis performed using Student's t test showed that there is a significant difference between the nanocapsules suspensions of virgin coconut oil and MCT regarding the mean diameter and the zeta potential ($p < 0.05$). However, the other parameters analyzed did not show any significant difference ($p > 0.05$). The total clotrimazole and $(PhSe)_2$ content in nanocapsules suspension was near to 100% for each active ingredient, which was close to the theoretical value (1.0 mg/mL). The EE achieved 100% in all formulations.

Concerning the other formulations, NC-CO-B and NC-T-B, the particle size was about 170 nm and 142 nm to CO NCs and MCT NCs, respectively. Thus, the results obtained agree with those already reported in the scientific literature [16,17].

Table 1. Nanocapsules suspensions characteristics

Samples	NC-CO-CD	NC-T-CD
Mean diameter (nm)	199 ± 12*	156 ± 3
PDI	0.18 ± 0.05	0.11 ± 0.01
Zeta Potential (mV)	+10.9 ± 0.9*	+14.7 ± 0.9
pH	5.04 ± 0.23	5.08 ± 0.14
D [4:3] (µm)	0.43 ± 0.03	0.43 ± 0.01
Span	1.8 ± 0.1	1.9 ± 0.1
C Content (%)	98.6 ± 3.6	98.9 ± 3.3
$(PhSe)_2$ Content (%)	97.5 ± 1.8	98.2 ± 2.4

Asterisks denote the significant difference (*) $p < 0.05$ by unpaired Student's t test between NC-CO-CD x NC-T-CD or NC-CO-D x NC-T-D. Abbreviations: PDI, polydipersity index; C, clotrimazole.

Mucoadhesion evaluation

After the interaction with mucin, all the formulations had an increase in the mean diameter in comparison to the original values (Figure 1A). Regarding zeta potential, the results showed an inversion in the polymer electric charge for all samples tested when compared to the initial values (Figure 1B), confirming the occurrence of interactions between the particles and mucin. The statistical evaluation performed by paired Student's t test revealed a significant difference among the groups ($p < 0.05$).

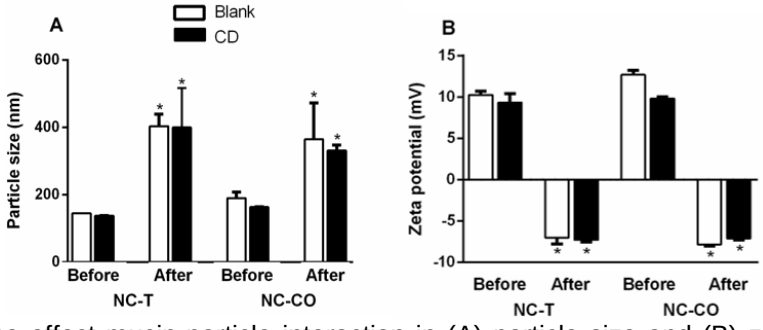

Figure 1. Evaluation of the effect mucin-particle interaction in (A) particle size and (B) zeta potential. Each column represents the mean ± S.E.M of three experiments performed in triplicate. Data were analyzed by paired Students' t test ($p > 0.05$). Asterisks denote the significant difference in comparison to the respective initial value (*) $p < 0.05$.

Stability studies

After 30 days of storage the formulations showed the same macroscopic appearance without any visible alterations. However, there was an increase in pH values for NC-T-CD (Figure 2A; $p < 0.05$), as can be seen in Figure 2. No statistical difference was observed in relation to mean diameter (Figure 2B), PDI (Figure 2C), zeta potential (Figure 2D) and drug content in comparison to the initial time ($p > 0.05$).

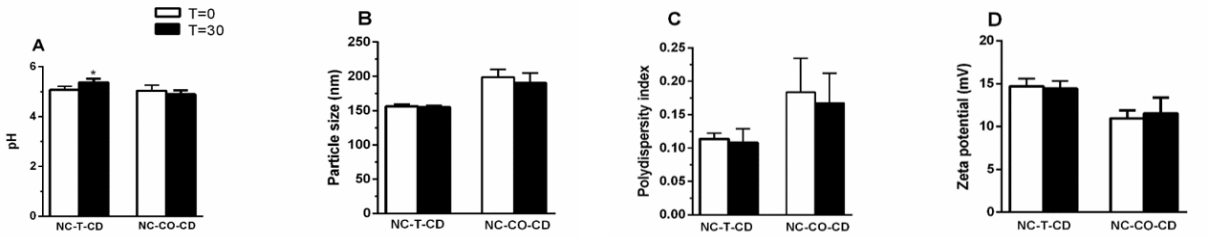

Figure 2. Evaluation of NCs suspension parameters throughout the stability studies. (A) pH values, (B) particle size, (C) PDI values and (D) zeta potential. Each column represents the mean ± S.E.M of three experiments performed in triplicate. Data were analyzed by One-way ANOVA of repeated measures ($p > 0.05$). Asterisks denote the significant difference in comparison to the respective initial time (T0) analyzed by One-way ANOVA, followed by Newman-Keuls' test (*) $p < 0.05$.

Evaluation of irritant potential by HET-CAM assay

The IS values obtained for the control groups were 0 for the negative control (saline solution) and 16.07 ± 1.02 for the positive control (0.1 M NaOH). The score obtained for all the tested polymeric nanocapsules suspension was 0, meaning non-irritant, which are illustrated in Figure 3. The results of the HET-CAM assay indicated that all nanoparticles suspensions developed were classified as non-irritant.

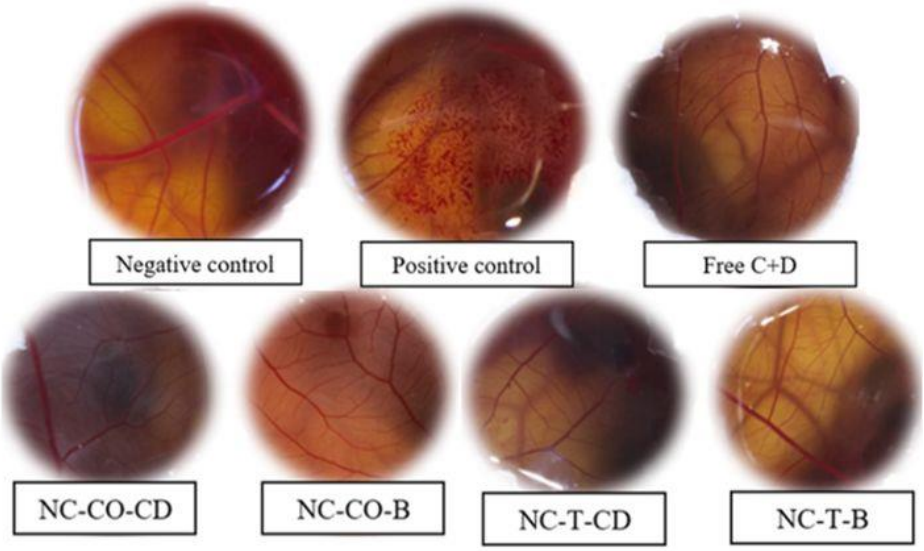

Figure 3. Results of the HET-CAM tested eggs, after applying the positive controls (0.1 M sodium hydroxide - NaOH), negative control (saline solution) and samples (MCT NCs, CO NCs and free association (C+D).

Determination of DPPH scavenging capacity

Figure 4 shows the results of the antioxidant potential assessed by the DPPH assay. The C, D, CO and MCT were not included in the Figure once the results found were similar to CO-CD and MCT-CD. At 2.5 and 5 µg/mL, the NC-CO-CD presented a DPPH scavenging capacity of 22.46 ± 12.79% and 26.25 ± 16.43%, respectively, which was significantly higher than the free form CO-CD (4.19 ± 5.93% and 3.32 ± 4.70%, respectively) ($p < 0.05$). At the highest tested concentration (25.0 µg/mL), all the formulations containing coconut oil demonstrate a significant capacity to neutralize DPPH radicals in comparison to the previously tested concentrations. The NC-CO-CD also exhibited a difference in comparison to the free form (67.75 ± 10.87% and 4.19 ± 5.93%, respectively) and presented a significant difference in relation to the NC-T-CD (67.75 ± 10.87% and 23.87 ± 8.75%, respectively), as the NC-CO-B towards the NC-T-B (36.04 ± 32.42% and 20.24 ± 4.90%, respectively) ($p < 0.05$). Concerning MCT nanocapsules, only the NC-T-CD exhibited a variation in comparison to the free form (23.87 ± 8.75% and 2.17 ± 3.07%, respectively) ($p < 0.05$). In contrast, all the other formulations showed no significant scavenger capacity ($p > 0.05$). The ascorbic acid, which was

used as the positive control, exhibited a significant difference in all concentrations tested, except at the lowest concentration (1.0 µg/mL).

In vitro antifungal activity

The evaluation of the antifungal activity was performed comparing the formulation (NC-CO-CD) to the compounds in DMSO solution (C, D, CO and CO-CD) against C. albicans and C. glabrata resistant to fluconazole strains. The MIC results are described in Table 2. DMSO used to solubilize the compounds did not interfere in fungal growth (>250 µg/mL). For both yeasts tested, the nanoencapsulation of the compounds increased the MIC when compared to all free compounds (C, D, CO and CO-CD), decreasing the antifungal effect of the actives. The free clotrimazole solution (C) compared to CO-CD has presented a better growth inhibition against C. albicans, while free $(PhSe)_2$ solution (D) showed opposite results against the strains, once it was more effective versus C. glabrata FR when compared to CO-CD. The CO solution presented a discrete antifungal action against the strains when compared to the clotrimazole, $(PhSe)_2$ and association solution, but showed a comparable inhibition with NC-CO-CD.

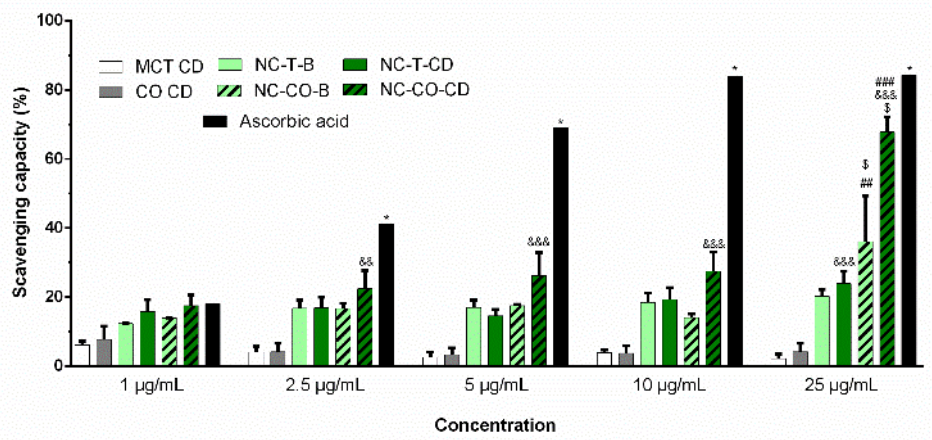

Figure 4. DPPH radical scavenging capacity. Each bar represents the mean ± SEM of three independent experiments (ordinary one-way ANOVA, followed by Tukey's test). *$p < 0.05$. Significant difference between ascorbic acid (positive control) and samples. #$p < 0.05$. Significant difference between the samples but considering only one concentration. &$p < 0.05$. Significant difference between the free form and the NCs. \$$p < 0.05$. Significant difference between NCs with the same oil core (MCT or CO).

Table 2. Antifungal activity of the formulation and bulk materials assessed by MIC.
Formulations (µg/mL)

MICROORGANISMS	NC-CO-CD	COCD	C	D	CO
C. *ALBICANS* (ATCC24433)	62.5	3.906	0.015	15.625	62.5
C. *GLABRATA* FR	125	7.812	15.625	3.906	62.5

Abbreviations – ATCC: American Type Culture Collection; FR: fluconazole-resistant; C: clotrimazole; D: $(PhSe)_2$; CO: virgin coconut oil; COCD: association of clotrimazole and $(PhSe)_2$ in CO oil core; NC-CO-CD: clotrimazole and $(PhSe)_2$-loaded nanocapsule at 1 mg/mL.

DISCUSSION

The co-loading of two active molecules in a nanocapsules formulation aimed at improving the treatment of fungal infections is until now unprecedented in the scientific literature. The results of characterization and stability evaluation suggested that all formulations prepared had suitable physicochemical properties. The HET-CAM assay and mucoadhesiveness test indicated that the formulations are promising candidates for further studies aiming at the preparation of a final pharmaceutical dosage form for topical application. Besides, the antioxidant effect found in the DPPH test also reinforces the complementary effect proposed.

Among the pre-formulation studies, the solubility of the compounds in the oil that would be used to compose the nanostructures is an important feature. Such information predicts the amount of active substance necessary to saturate the oil and provide an idea of compatibility and encapsulation efficiency. Both evaluated oils, MCT and CO, had a great ability to solubilize the compounds, suggesting that they are suitable to compose the formulation. The MCT oil is widely used in polymer nanocapsules preparation since it is considered a biocompatible material and pharmacologically inert [34]. While CO arises as an interesting material because of its already reported biological properties, such as antioxidant and antifungal actions [19,20]. The nanocapsules showed an adequate mean diameter, around 200 nm, and an appropriate

nanoparticulate granulometric distribution (PDI < 0.2), indicating a narrowed particle size profile and system homogeneity. An absence of a micrometric population was also observed, suggesting a unimodal particle distribution [35]. These results are in agreement with other reports that showed the incorporation of organoselenium compounds into nanostructures [29], including the same approach to assess antifungal potential [36].

The zeta potential values were positive because of the positive charge of the quaternary ammonium groups present in the acrylic copolymer, Eudragit® RS 100 [16,17]. The pH values were slightly acid (5.0), which is in accordance to previous reports that employed the same constituents to prepare the formulation [16,17,29,37], and also compatible with the pH of the vaginal tract (about 4.5). In all formulations, the content of the active ingredients showed values close to the theoretical reference, indicating few losses during the preparation process. Besides, a high efficiency of encapsulation was achieved, which could be attributed to the lipophilicity of both compounds and the high solubility of them in the oils. Moreover, we emphasize that the co-encapsulation of drugs is not an ordinary approach and are rarely addressed due to the unpredictability of physicochemical phenomena and compatibility among the constituents.

An important feature of formulations is physicochemical stability after preparation. Such data provide an idea of how long a pharmaceutical product remains stable and suitable for usage. For this, the chemical and physical stability of the formulations was assessed 30 days after the development. The results demonstrated that no changes were detected in the nanocapsules NC-CO-D and NC-T-CD concerning the macroscopic appearance, drug content, average size, PDI, zeta potential and pH values. Meanwhile, the NC-T-CD presented a significant difference in the pH values in comparison to the initial values, which could be attributed to a relaxation of polymeric chains, leading to exposure of some chemical groups that altered the pH without compromising any other parameter. No degradation occurred over the period studied, reinforcing some advantages of incorporating molecules into nanocarriers [38].

In an attempt to estimate the mucoadhesive property of the formulations, the electric charge theory was used by applying the mucin-particle method [32]. Our results demonstrated that mucin exposure caused a modification in the mean particle size and zeta potential in comparison to the initial values. The change in electrical charge, where particles positively charged when in contact with mucin (negatively charged) transfer electrons, creating a double layer at the interface [37]. The mucoadhesive property occurs due to the polymer Eudragit® RS100, which creates attraction forces at the interface, motivated by the charges difference, increasing the mean size and inverting the zeta potential [37]. It was expected for the formulations due to the polymeric bioadhesive material used to compose the nanocapsules, which may increase the residence time of the pharmaceutical form in the vaginal mucosa.

Despite attractive functions and a bright outlook for nanoformulations, there is an increasing concern regarding their safety. In the worldwide trend of animal replacement, the search for alternative methods for pharmacological and toxicological testing has grown in recent years [39]. In addition to being more ethical, they are part of the three Rs: reduction, refinement and replacement, which aim to reduce harmful effects, the number of animals and substitute them by alternative methodologies. In the current study, we applied as a preliminary tool for toxicological screening of the HET-CAM method. After exposing the membrane to the samples, it is possible to monitor the occurrence of any physiological response. Based on the results, all formulations and free materials were classified as non-irritant because they triggered no toxic effect, such as coagulation and hemorrhage, indicating a low toxic potential.

Following, the next step of the study was to determine if the co-loading of active ingredient would provide any positive impact in the antioxidant action. In this sense, the DPPH assay is a very useful methodology for assessing the antioxidant potential of substances. It is based on a reduction reaction of the DPPH radical for diphenyl-picrylhydrazine by changing the reaction medium from violet to light yellow which will be measured in a UV/visible light spectrophotometer at 517 nm [40]. In the higher concentration tested (25 µg/mL), the nanocapsules composed of CO exhibited superior scavenging action in comparison to the formulations containing MCT oil, pointing out the antioxidant action already described to CO [19,20]. Furthermore, all nanocapsules suspensions were more effective than the free forms (MCT CD and CO CD). This result can be explained due to the superior contact provided by the nanometer scale of the particles in suspension, facilitating the hydrogen donation to the radical site [41]. Besides, our results corroborate with other studies that showed the improved radical scavenging activity provided by the nanoparticles [21,22]. Among the nanostructures, the NC-CO-CD presented the best results in the DPPH assay (67.75 ± 10.87%), which is interesting, since nanoparticles prepared with the same oil, but with only one compound or none, had a lower effect than the formulation containing the co-loading of both active ingredients. It is important to mention that, although the results of DPPH assay did not demonstrate the antioxidant action of $(PhSe)_2$, several scientific reports have already showed such biological property of the compound [24].

The hypothesis that driven our study was that the co-encapsulation of clotrimazole and $(PhSe)_2$ into a nanocapsules formulation would potentiate the antioxidant and antifungal actions. We observed that the encapsulation of clotrimazole and $(PhSe)_2$ caused an increase in the MIC values in comparison to their free form. A possible explanation may be the highly controlled rate of drug release that could be provided by the association of both compounds into the nanocarrier system. The molecules must overcome the partition barrier between the oil core and polymeric membrane to be released to external medium, which is aqueous (RPMI) and do not provide an adequate condition to clotrimazole and $(PhSe)_2$ release. The polymeric shell barrier would hinder instant drugs release from the structures and, consequently, the instant action of the encapsulated drugs on the Candida spp strains compared to unencapsulated actives. In this way, the release profile is likely to be too slow that hinders conducting some in vitro evaluations. Additionally, DMSO was used to aid the poor water solubility of free clotrimazole and $(PhSe)_2$ in the assay while NC-CO-CD was used as water suspension with no organic solvent. Therefore, the improvement in water dispersibility by encapsulation could not be observed by this test. The test, however, confirmed that the drug was still active after being encapsulated and the formulation still had acceptable activity levels. Therefore, for proper data interpretation, we assume that our study has some limitations regarding the absence of the determination of drugs release profile. Such data may help to justify the difficulties faced to assess the antifungal action of the formulation hindering carrying.

CONCLUSION

The present results point to the feasibility in preparing nanostructured formulations containing clotrimazole and $(PhSe)_2$ co-loaded using two different oil cores. The formulations had adequate physicochemical characteristics, an appropriate mucoadhesive potential, high chemical stability and no irritant reactions to the biological membrane (HET-CAM test). Furthermore, nanoencapsulation improved the in vitro antioxidant action in comparison to the free form of the active ingredients, highlighting the NC-CO-CD as the best formulation concerning such property. The in vitro antifungal assay revealed that the incorporation of clotrimazole and $(PhSe)_2$ into the nanocapsules increased MIC values. Therefore, our study supplies additional information about the potential advantages achieved by encapsulating active compounds. Future studies will enable a better understanding of the real impact of the physicochemical properties of these formulations in their biological effects.

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