Atividade antifúngica de nanocarreadores contendo óleo de melaleuca no crescimento de *C. albicans*: um estudo do perfil de inibição

Antifungal activity of nanocarriers containing tea tree oil on the growth of *C. albicans*: an inhibition profile study

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Resumo

Objetivos: O objetivo deste trabalho foi avaliar, pela primeira vez, a eficácia antifúngica de suspensões de nanocápsulas e nanoemulsões contendo óleo essencial de Melaleuca alternifolia (TTO) segundo avaliação do perfil de inibição de crescimento in vitro de Candida albicans. Métodos e resultados: A atividade antimicrobiana das formulações foi verificada frente a *C. albicans* a fim de se obter um perfil de inibição do crescimento após 0, 5, 8, 12, 24, 48 e 72 horas, utilizando duas concentrações da levedura. A atividade do óleo associado às nanoestruturas na curva de crescimento da levedura demonstrou diferenças entre as formulações. Para menores concentrações do microrganismo, as formulações diminuíram significantemente a carga microbiana por 48 horas. Além disso, o controle do crescimento exercido pelas nanocápsulas permaneceu similar durante todos os períodos de análise. Conclusões: A associação do óleo de melaleuca em nanocápsulas poliméricas demonstrou ser mais eficiente em reduzir e controlar o crescimento de *C. albicans* por até 72 horas.


Abstract

Aim: The aim of this study was to evaluate, for the first time, the antifungal efficacy of nanocapsules suspension and nanoemulsions containing Melaleuca alternifolia essential oil (tea tree oil). Methods and results: An in vitro assay measuring antimicrobial activity against Candida albicans was performed in order to obtain an inhibition profile after 0, 5, 8, 12, 24, 48 and 72 h growth, using two different yeast concentration. The activity of oil-loaded nanostructures on C. albicans growth curve showed differences between formulations. For small concentrations of microorganism, the formulations significantly decreased yeast charge for 48 h. Moreover, the control of growth provided by the nanocapsules remained similar at all analysis times. Conclusions: The inclusion of the oil in nanocapsules proved to be more efficient in reducing and controlling C. albicans growth.

Keywords: Candida albicans. Nanoemulsions. Nanocapsules. Tea tree oil.
1. Introduction

The skin is the interface between the body and the external environment and is a biological barrier, essential in protecting the body against trauma, dehydration and external agents such as chemicals and microorganisms\(^1\). The occurrence of lesions in this organ makes the body susceptible to invasion by microbial agents, leading to formation of skin abscesses and, in severe cases, the emergence of generalized infections\(^2\).

Superficial mycosis, are among the diseases that most affect the world population and are caused mainly by dermatophyte fungi of the genera *Epidermophyton*, *Microsporum* and *Trichophyton*, as well as yeast, such as *Candida* spp. Candida species usually inhabit the skin and mucous membranes innocuous and commensally. Occasionally these yeasts might incite opportunistic infections that may range from superficial lesions, less severe conditions for systemic concern, depending on the weakness of the host’s immune system\(^1\). Because of proteolytic enzymes secreted by these fungi, they settle in keratinized tissues like skin, hair and nails, resulting in lesions arising from the destruction of keratin added to a host inflammatory response\(^3\)\(^-\)\(^5\). The inflammatory responses generated against etiologic agents are related to differential expression of genes encoding the secretion of enzymes\(^6\)\(^-\)\(^7\)\(^-\)\(^8\) and vary according to the fungal species and pathophysiologic state of the host\(^8\). These infections require attention, mainly in cases where the immune state is impaired, like pregnant women, people who use immunosuppressive treatments (eg cancer patients), patients with the acquired immunodeficiency syndrome\(^5\)\(^-\)\(^9\) and when there is impairment of the peripheral circulation\(^10\). In these cases, superficial infection tends to progress to cutaneous and deeper subcutaneous infections\(^11\), and depending on the extent of the injury and microbial load may also, achieve the blood circulation, leading to generalized infections\(^2\).

The therapy used in cases of superficial mycoses, mostly is topical, usually with azoles and allylamines. Nevertheless, when there is involvement of large areas, chronicity or recurrence, the use of systemic therapy is required\(^12\). On the other hand, systemic antifungal therapy is the risk of drug interactions, contraindications, and adverse effects\(^12\)\(^-\)\(^13\). These factors may cause treatment interruption, and, therefore, incomplete recovery\(^1\). Another reason for the treatment failure is the development of antimicrobial resistance by the fungi or yeast\(^6\). Moreover, conventional topical formulations are easily removed in friction with clothes or cleaning procedures\(^14\). In order to obtain successful topical therapy of superficial mycosis, it is necessary that the antifungal agent reach the site of infection and the fungal elements in sufficient and effective concentrations\(^15\). In this respect, the efficiency of topical therapy depends on the penetration of the active substance at the site of infection in order to reach the required effective concentrations\(^16\).

Essential oils obtained from aromatic plants have been widely used alone or in pharmaceutical formulations, with or without the use of adjuvants, to complement conventional medicine or even replace it, especially in antifungal and antibacterial therapies\(^17\)\(^-\)\(^19\). Tea tree oil (*Melaleuca alternifolia* essential oil), shows antibacterial, antifungal\(^20\) and anti-inflammatory\(^21\) properties and is commonly used in topical formulations\(^22\). This essential oil is composed of a wide range of constituents, mainly monoprene and sesquiterpene hydrocarbons. This complex mixture avoids the development of antimicrobial resistance\(^22\) and its use is related to treatment of skin infections. However, its compounds can undergo oxidations reactions during storage\(^22\) and, in its pure form or when incorporated in topical formulations, may cause irritation and allergic reactions when applied to the skin\(^23\).

Different nanocarrier systems, such as liposomes, nanoemulsions, lipid nanoparticles and polymeric nanoparticles, have been created to encapsulate different active substances in order to increase their properties\(^24\)\(^-\)\(^27\). Polymeric nanoparticles are solid colloidal particles that are classified as nanospheres and nanocapsules according to their composition. These systems are made of polymers; in the case of nanocapsules, oil is included in order to obtain a vesicular structure\(^27\). They confer advantages like improve drug efficacy, bioavailability and stability, as well as providing a reduction of toxicity and irritation\(^24\)\(^-\)\(^25\). Furthermore, its submicrometric particle size enables more intimate contact of the active substance with skin, giving greater adhesion and longer stay on this site, which are an advantageous tool for sites with clothing friction and subjected to routine activities\(^24\).

In this context, we have evaluated the feasibility of the development of nanoeumulsion and polymeric nanocapsules containing TTO. The incorporation of the oil into nanostructurated systems showed structures with adequate phisico-chemical properties, conferred TTO protection against volatilisation\(^28\) and activity against *Trichophyton rubrum* in an *in vitro* onychomycosis model\(^29\), especially for nanocapsule suspension. Thus, this study was outlined with the purpose of study the *in vitro* antifungal activity of nanoeumulsion and nanocapsule suspension containing TTO, and the influence of each type of structure in the growth profile of *Candida albicans*.

2. Materials and method

2.1. Materials

The *Melaleuca alternifolia* essential oil was commercially obtained (Laszlo Aromaterapia, Belo Horizonte, Brazil). *Poly(ε-caprolactone)* (MW = 80,000), and sorbitan monooleate were acquired from Sigma (São Paulo, Brazil) and polysorbate 80 from Delaware (Porto Alegre, Brazil). All other chemicals and solvents were of pharmaceutical
grade and were used as received.

*Candida albicans* ATCC 10231 was generously supplied by Instituto Nacional de Controle de Qualidade em Saúde (INCQS – FIOCRUZ, Rio de Janeiro, Brazil).

2.2. Preparation of nanoemulsions and nanocapsules suspensions

Nanoemulsions (TTO-NE) and nanocapsules (TTO-NC) containing TTO (10.0 mg mL⁻¹) were prepared in triplicate, according to Flores and co-workers²⁶, by spontaneous emulsification²⁹ and interfacial deposition of the preformed polymer²⁹ methods, respectively. In addition, control formulations (containing only the components of the nanoemulsions and nanocapsules, without the essential oil) were prepared and named C-NE and C-NC, respectively, in order to compare the oil effectiveness.

2.3. Activity of nanostructures on the growth of *Candida albicans*

A strain of *C. albicans* was cultured on a Sabouraud dextrose agar (SDA, Merck) medium at 25 °C for 48 h. The cells were harvested into a saline solution and their volumes were adjusted to yield suspensions of 1.6 x 10⁷ CFU mL⁻¹ (colony forming unit mL⁻¹). Two different concentrations of the yeast were used; subsequent dilutions, in Sabouraud dextrose broth (SDB, Merck), were performed in order to obtain a yeast concentration of approximately 10³-10⁴ and 10⁵ CFU mL⁻¹. A 2.5 mL aliquot of each formulation (TTO-NE, TTO-NC, C-NE and C-NC) was placed into sterile tubes and separately inoculated with the yeast suspension (10³-10⁴ and 10⁵ CFU mL⁻¹). The quantity of TTO-NE and TTO-NC formulations corresponded to 2.5 mg mL⁻¹ of oil. The samples were incubated at 37 °C, and after a contact time of 0, 5, 8, 12, 24, 48 and 72 h, serial dilutions of each sample (200 µL) were prepared in saline solution. Cell viability at different times was determined by the count plate method in SDA after 72 h at 25 °C. Plates with 10-100 colonies were used for CFU counts. Results were expressed in log CFU mL⁻¹.

2.4. Statistical analysis

Results are expressed as means ± SD (standard deviation). One-way analysis of variance (ANOVA) was employed for comparison of the experimental data at a significance level of 5%.

3. Results

In earlier studies, we developed nanoemulsions and polymeric nanocapsules containing TTO (TTO-NE and TTO-NC, respectively) and showed the feasibility in prepare sub-micrometric formulations with essential oil. The formulations presented nanometric mean size (around 200 nm) with an adequate polydispersity index (below 0.25). The inclusion of TTO in nanocapsules showed higher protection against volatilisation²⁶, and more control on the growth of *T. rubrum* in an in vitro model of nail infection²⁸. In the present work, we evaluated the effect of the nanostructured systems on the growth of *C. albicans*, a microorganism of clinical importance.

Results obtained regarding the *C. albicans* antifungal activity of TTO-NE and TTO-NC are shown in Fig 1 and 2. When the initial *C. albicans* concentration was close to 3.7 log CFU mL⁻¹, the nanostructured formulations...
were able to reduce the concentration approximately 1.5 log units and maintain this value for 48 h. No statistical difference was obtained between the formulations (p>0.01). After 72-h incubation, an increase in yeast growth approaching the initial concentration was observed for TTO-NE (2.8 ± 0.1 log CFU mL\(^{-1}\) at 48 h to 3.1 ± 0.2 log CFU mL\(^{-1}\)). The same was not observed for TTO-NC. At an initial concentration of 5 log CFU mL\(^{-1}\) (Fig 2), TTO-NE did not demonstrate the same efficacy, as it was not able to reduce \(C. \text{albicans}\) growth. After 24 h of incubation, a significant increase on \(C. \text{albicans}\) concentration (approximately 1.0 unit log) was observed (p<0.05). Conversely, a reduction in \(C. \text{albicans}\) growth and maintenance values was observed for TTO-NC through all experiments. Moreover, this sample was able to reduce \(C. \text{albicans}\) concentration 0.5 log units as compared to the initial concentration, while TTO-NE demonstrated an increase of 0.5 log units. The components present in C-NE and C-NC formulations had no effect on the microorganism growth in the concentrations tested, and the values obtained were similar to untreated yeast growth (culture medium without the samples).

4. Discussion

The activity of the nanostructures on \(C. \text{albicans}\)' growth curve showed that the incorporation of oil into nanostructured systems did not cause a loss in its antifungal activity as a function of time. This study clearly demonstrated that the two developed formulations have a different performance. At low initial concentrations of yeast, TTO-NE and TTO-NC significantly decreased the yeast growth for 48 h. Moreover, the control of growth provided by TTO-NC remained similar at all analysis times, which result was not observed for TTO-NE, due to a marked difference at the last time point. In another test, we increased the \(C. \text{albicans}\) initial concentration, and differences between the colloidal systems were observed in the first 24 h of incubation. TTO-NC significantly reduced the initial yeast concentration, showing control of yeast growth throughout the experiment.

Differences presented by the formulations studied are related to their composition and structure type. Polymeric nanocapsules are composed of an oil core surrounded by a polymeric wall\(^{24}\), while nanoemulsions, which are non-polymeric carriers, are fine oil-in-water dispersions (o/w)\(^{31}\). More recently, we reported that the inclusion of oil in polymeric nanocapsules allowed increased oil protection against evaporation, probably due to the presence of the polymeric wall\(^{26}\). Thus, the oil could not be entirely available to exert its inhibitory effect, due to its slow release into the environment. This observation corroborates with several release studies. Fontana and co-workers\(^{32}\) evaluated the release profiles of clobetasol propionate-loaded nanocapsules, nanospheres and nanoemulsions, and concluded that the type of system influences drug release and that the polymer presence plays an important role controlling such release. In another study, the same effect was obtained for indomethacin-loaded nanocapsules in comparison with nanoemulsions\(^{33}\). Differences between TTO-NC and TTO-NE in controlling \(C. \text{albicans}\) growth may be due to differences in the release profile of the essential oil from these formulations, and in case of TTO-NC, the presence of the polymeric wall was able to control its inhibitory effect for a longer time. In addition, nanostructured systems are able to improve bioavailability and reduce the frequency of drug administration\(^{34}\).

\textit{Melaleuca alternifolia} essential oil has a broad anti-
microbial spectrum, including yeasts and filamentous fungi, such as C. albicans and T. rubrum, respectively. Our research group have developed aqueous nanocapsule suspensions containing TTO and the presence of polymeric wall showed protection of oil volatilization and increased activity against filamentous fungi in an in vitro onychomycosis model. In this study, nanocapsules also increased TTO effectiveness, probably by the oil protection and the differences of the release profiles of TTO conferred by the presence of the polymeric wall.

5. Conclusion

According to the results obtained in this work, nanocapsules and nanoemulsions containing M. alternifolia essential oil were able in reducing the growth of C. albicans. Nanocapsules suspension, probably to the presence of the polymeric wall, were able to control its inhibitory effect for longer time. The developed nanostructured systems show potential in treating skin infections, especially those caused by Candida spp.

Acknowledgements

The authors thank to CNPq-Brasilia/Brazil by financial support.

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