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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.

Palavras-chave: *Candida albicans. Nanoemulsões. Nanocapsulas. óleo de melaleuca.*

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.*

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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[3]. 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[4, 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 injured, 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[4]. 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 (TTO, *Melaleuca alternifolia* essential oil), shows antibacterial, antifungal[20] and anti-inflammatory[21] properties and is commonly used in topical formulations[20]. This essential oil is composed of a wide range of constituents, mainly monoterpene 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 grater 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 nanoemulsion 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[26] and activity against *Trichophyton rubrum* in an *in vitro* onychomycosis model[28], especially for nanocapsule suspension. Thus, this study was outlined with the purpouse of study the *in vitro* antifungal activity of nanoemulsion 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-workers[26], by spontaneous emulsification[29] and interfacial deposition of the preformed polymer[29] 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 ºC for 48 h. The cells were harvested into a saline solution and their volumes were adjusted to yield suspensions of 1.6 x 10^7 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 10^3-10^4 and 10^5 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 (10^3-10^4 and 10^5 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 ºC. 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 volatilisation[26], and more control on the growth of *T. rubrum* in an *in vitro* model of nail infection[28]. In the present work, we evaluated the effect of the nanostructured systems on the growth of the *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

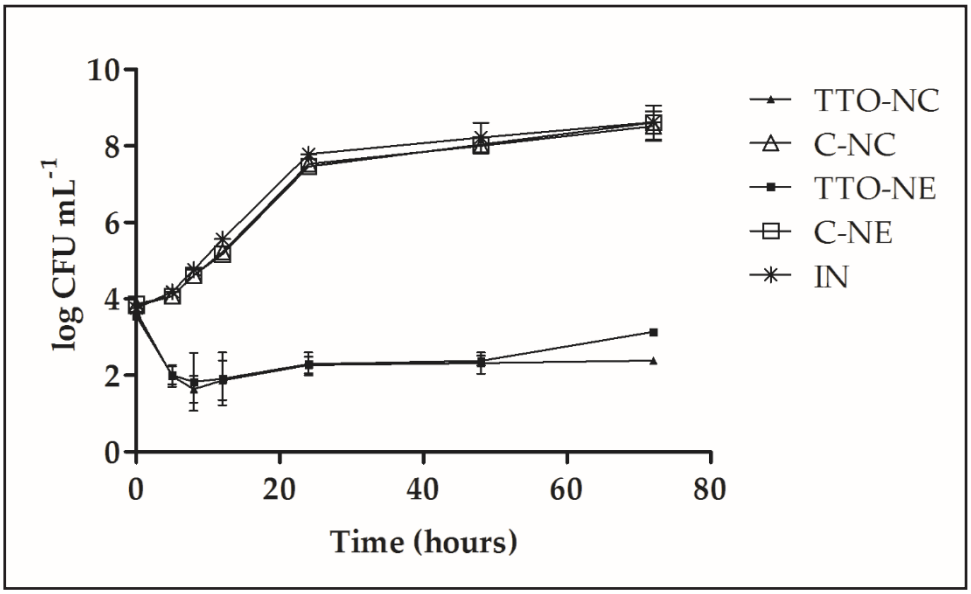

Fig 1 - Microbiological efficacy of formulations against *C. albicans* (yeast suspension at 10^3 - 10^4 CFU mL^{-1}) (nanocapsules containing TTO – TTO-NC; nanoemulsions containing TTO – TTO-NE; blank nanocapsules – C-NC; blank nanoemulsions – C-NE; and medium without the samples – IN)

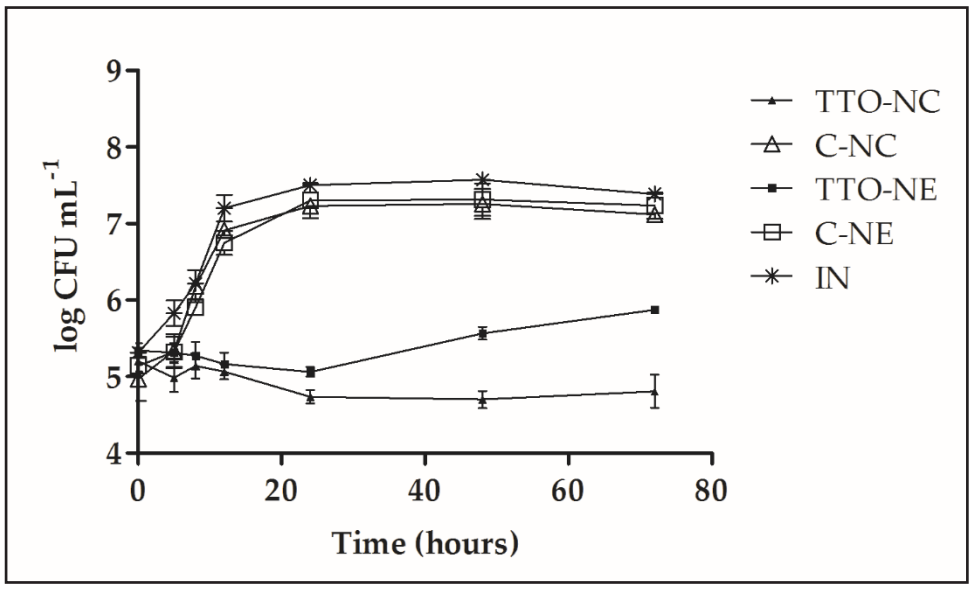

Fig 2 - Microbiological efficacy of formulations against *C. albicans* (yeast suspension at 10^5 CFU mL^{-1}) (nanocapsules containing TTO – TTO-NC; nanoemulsions containing TTO – TTO-NE; blank nanocapsules – C-NC; blank nanoemulsions – C-NE; and medium without the samples – IN)

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. albicans* growth. After 24 h of incubation, a significant increase on *C. albicans* concentration (approximately 1.0 unit log) was observed ($p<0.05$). Conversely, a reduction in *C. albicans* growth and maintenance values was observed for TTO-NC through all experiments. Moreover, this sample was able to reduce *C. 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. 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. 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 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. 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].

*Melaleuca alternifoli*a essential oil has a broad anti-

microbial spectrum, including yeasts and filamentous fungi, such as *C. albicans* and *T. rubrum*, respectively[22]. Our research group have developed aqueous nanocapsule suspensions containing TTO and the presence of polymeric wall showed protection of oil volatilization[26] and increased activity against filamentous fungi in an *in vitro* onychomycosis model[28]. 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 trating skin infections, especially those caused by *Candida* spp.

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References

1 Cevc G, Vierl U. Nanotechnology and the transdermal route. A state of the art review and critical appraisal. J. Control. Release. 2010;141:277–299.

2 Noskin GA, Rubin RR, Schentag JJ, Kluytmans J, Hedblom EC, Smulders M et al. The burden of *Staphylococcus aureus* Infections on Holpitals in the United States. Arch. Intern. Med. 2005;165(15):1756-1761.

3 Jayatilake JAMS, Tilakaratne WM, Panagoda GJ. Candidal onychomycosis: A Mini-Review. Mycopathologia. 2009;168:165-173.

4 Sidrim JJC, Rocha MFG. Micologia médica à luz de autores contemporâneos. Rio de Janeiro: Guanabara Koogan S.A., Copyright; 2004.

5 Degreef H. Clinical Forms of dermatophytosis (Ringworm Infection). Mycopathologia. 2008;166:257-265.

6 Martinez-Rossi NM, Peres NTA, Rossi A. Antifungal Resistance Mechanisms in Dermatophytes. Mycopathologia;2008;166:369-383.

7 Mignon B, Tabart J, Baldo A, Mathy A, Losson B, Vermout S. Immunization and dermatophytes. Curr. Opin. Infect. Dis. 2008;21:134–140.

8 Vermout S, Tabart J, Baldo B, Mathy A, Losson B, Mignon B. Pathogenesis of Dermatophytosis. Mycopathologia. 2008;166:267–275

9 Khengar RH, Jones SA, Turner RB, Forbes B, Brown MB. Nail swelling as a Pre-formulation Screen for the Selection an Optimisation of Ungual Penetration Enhancers. Pharm. Research. 2007;24(12):2207-2212.

10 Repka MA, Mididoddi PK, Stodghill SP. Influence of human nail etching for the assessment of topical onychomicosis therapies. Int. J. Pharm. 2004;282:95-106.

11 Kanbe T. Molecular Approaches in the Diagnosis of Dermatophytosis. Mycopathologia. 2008;166:307–317.

12 Gupta AK, Chow M, Daniel CR, Aly R. Treatments of tinea pedis. Dermatol. Clin. 2003;21:431- 462.

13 Murdan S. Drug delivery to the nail following topical application. Int. J. Pharm. 2002;236:1- 26.

14 Gupta AK, Tu LQ. Therapies for Onychomycosis: A Review. Dermatol. Clin. 2006;24:375–379.

15 Kaur IP, Kakkar S. Topical delivery of antifungal agents. Expert Opin. Drug Del. 2010:1-25.

16 Güngör S, Erdal MS, Aksu B. New Formulation Strategies in Topical Antifungal Therapy. Journal of Cosmetics, Dermatological Sciences and Applications. 2013;3:56-65.

17 Buck SD, Nidore DM, Addino JG. Comparison of two topical preparations for the treatment of onychomycosis: *Melaleuca alternifolia* (tea tree) oil and clotrimazole. J. Pham. Practice. 1994;38(6):601-05.

18 Syed TA, Qureshi ZA, Ali SM, Ahmad SA. Treatment of toenail onychomycosis with 2% butenafine and 5% *Melaleuca alternifolia* (tea tree) oil in cream. Trop. Med. Int. Health. 1999;4(4):284-287.

19 Nascimento PFC, Nascimento AC, Rodrigues CS, Antoniolli AR, Santos PO, Júnior AMB et al. Atividade antimicrobiana dos óleos essenciais: uma abordagem multifatorial de métodos. Rev. Bras. Farmacog. 2007;17(10):108-113.

20 Bakkali F, Averbeck S, Averbeck D, Idaormar M. Biological effects of essential oils- A review. Food Chem. Toxicol. 2008; 46:446-475.

21 Hart PH, Brand C, Carson CF, Riley TV, Prager RH, Finlay-Jones JJ. Terpinen-4-ol, the main component of the

essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. Inflamm. Res. 2000;49:619-626.

22 Carson CF, Hammer KA, Riley TV. *Melaleuca alternifolia* (Tea Tree) Oil: a Review of Antimicrobial and Other Medicinal Properties. Clin. Microbiol. Rev. 2006;19(1):50-62.

23 Hammer KA, Carson CF, Riley TV, Nielsen JB. A review of the toxicity of *Melaleuca alternifolia* (tea tree) oil. Food Chem. Toxicol. 2006;44:616-625.

24 Guterres SS, Alves MP, Pohlmann AR. Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications. Drug Target Insights. 2007;2:147-157.

25 Mishra B, Patel BB, Tiwari S. Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery. Nanomedicine: Nanotechnology, Biology and Medicine. 2010;6:9-24.

26 Flores FC, Ribeiro RF, Ourique AF, Pohlmann AR, Beck RCR, Guterres SS et al. Nanostructurated systems containing an essential oil: protection against volatilization. Quim. Nova. 2011(34):968-972.

27 Mora-Huertas CE, Fessi H, Elaissari A. Polymer-based nanocapsules for drug delivery. Int. J. Pharm. 2010(385):113-142.

28 Flores FC, Lima JA, Ribeiro RF, Rolim CMB, Beck RCR, Silva CB. Antifungal Activity of Nanocapsule Suspensions Containing Tea Tree Oil on the Growth of Trichophyton rubrum. Mycopathologia. 2013;175(3-4):281.

29 Bouchemal K, Briançon S, Perrier E, Fessi H. Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimization", Int. J. Pharm. 2004;280:241-251.

30 Fessi H, Puisieux F, Devissaguet J, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm. 1989 ;55(1) :R1-4.

31 Anton N, Benoit J-P, Saulnier P. Design and production of nanoparticles formulated from nano-emulsion templates – A review. J. Control. Release. 2008;128:185-199.

32 Fontana MC, Coradini K, Guterres SS, Pohlmann AR, Beck RCR. Nanoencapsulation as a Way to Control the Release and to Increase the Photostability of Clobetasol Propionate: Influence of the Nanostructured System. Journal of Biomedical and Nanotechnology. 2009;5:1-10.

33 Poletto FS, Jäger E, Cruz L, Pohlmann AR, Guterres SS. The effect of polymeric wall on the permeability of drug-loaded nanocapsules. Mat. Sci. Eng. 2008;28:472-478.

34 Ranjita S, Loaye AS, Khalil M. Present status of Nanoparticle Research for Treatment of Tuberculosis. J. Pharm. Sci. 2011;4(1):100-116.

35 Gunt H, Kasting GB. Effect of hydration on the permeation of ketonazole through human nail plate *in vitro.* Eur. J. Pharm. Sci. 2007;32:254-60.