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Melaleuca alternifolia nanoparticles against *Candida* species biofilms

M.E. Souza [a, b], L.Q.S. Lopes [a, b], P.C. Bonez [g], A. Gündel [d], D.S.T. Martinez [e], M.R. Sagrillo [b], J.L. Giongo [f], R.A. Vaucher [a, b], R.P. Raffin [a], A.A. Boligon [c], R.C.V. Santos [g, *]

[a] Laboratory of Microbiological Research, Centro Universitário Franciscano, Santa Maria, Brazil
[b] Laboratory of Nanotechnology, Post Graduate Program of Nanosciences, Centro Universitário Franciscano, Santa Maria, Brazil
[c] Laboratory of Phytochemical, Industrial Pharmacy Department, Universidade Federal de Santa Maria, Santa Maria, Brazil
[d] Universidade Federal do Pampa, Bagé, Brazil
[e] Laboratory of Chemical Solid State, Chemical Institute, Universidade Estadual de Campinas, Campinas, Brazil
[f] Laboratory of Pharmacotechnique, Universidade Regional Integrada, URI-Santiago, Santiago, Brazil
[g] Department of Microbiology and Parasitology, Universidade Federal de Santa Maria, Santa Maria, Brazil

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ABSTRACT

Candida infection is an important cause of morbidity and mortality on immunosuppressed patients. This growing trend has been associated with resistance to the antimicrobial therapy and the ability of microorganism to form biofilms. TTO oil is used as antimicrobial which shows antibiofilm activity against *Candida* species. However, it presents problems due to its poor solubility and high volatility. The present study aimed to evaluate *in vitro* antibiofilm activity of TTO nanoparticles against many *Candida* species. It was performed the characterization of the oil and nanoparticles. The levels of exopolysaccharides, proteins, and the biomass of biofilms were measured. The chromatographic profile demonstrated that the TTO oil is in accordance with ISO 4730 with major constituents of 41.9% Terpinen-4-ol, 20.1% of γ-Terpinene, 9,8% of α-Terpinene, and 6,0% of 1,8-Cineole. The TTO nanoparticles showed pH of 6.3, mean diameter of 158.2 ± 2 nm, polydispersion index of 0.213 ± 0.017, and zeta potential of −8.69 ± 0.80 mV. The addition of TTO and its nanoparticles represented a significant reduction of biofilm formed by all *Candida* species, as well as a reduction of proteins and exopolysaccharides levels. It was possible to visualize the reduction of biofilm in presence of TTO nanoparticles by Calcofluor White method.

1. Introduction

The adhesion of *Candida* species is the initial and fundamental step on biofilm formation, contributing significantly to high morbidity and hospital mortality [1,2]. The biofilms are complex microbial communities where the microorganisms are linked on a surface, wrapped by a polymeric matrix of exopolysaccharides (EPS). The biofilm structure, as well as the communication mechanisms between microorganisms and phenotypic alterations are factors that inhibit the penetration of antimicrobial agents making the treatment more difficult.

Before the need of new therapeutic forms, the plants are excellent options to obtain a wide variety of drugs and they have been used throughout the evolutionary process. In this context, the *Melaleuca alternifolia* oil (TTO) is an essential oil used as anti-inflammatory, antimicrobial, and antifungal agent [3,4]. Previous studies demonstrated that the TTO decreases the biofilm formation and the adhesion of different isolates of *C. albicans* on biotic and abiotic surface. However, the TTO has presented some problems when associated to its physical properties, such as the low water miscibility and high volatilization rates, resulting in low stability [5,6]. Moreover, the TTO is able to induce allergic reactions when applied topically, due to the oxidation of constituents, according to storage conditions [3,7].

The nanostructuration of some natural products has shown promising results, with superior therapeutic efficiency, controlled release, significant decrease of toxicity, more time in circulation, protection of the compound against instability and decomposition [8–11]. Studies performed by our research group demonstrated that TTO nanoparticles were capable of solving problems such as instability of oil and also the enhance of the antimicrobial effects

Abbreviations: EPS, exopolysaccharides; TTO, *Melaleuca alternifolia*; NTA, Nanoparticle tracking analysis; ATCC, American type culture collection; BHI, Brain Heart Infusion; OD, optical density; AFM, Atomic force microscopy.

* Corresponding author. Rua dos Andradas 1614, Sala 115, 97010-032 Santa Maria, RS, Brazil.
E-mail address: robertochrist@gmail.com (R.C.V. Santos).

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against many microorganisms including *Candida* species [12].

Taking into account the high antimicrobial effect of TTO nanoparticles, the aim of this study was to evaluate, for the first time, the antibiofilm activity of such nanoparticles. It has been tested the action of the TTO and TTO nanoparticles, through the determination of the biofilm extent, measurement of exopolysaccharides and proteins levels.

2. Materials and methods

2.1. TTO and TTO nanoparticles

The TTO was purchased from *Delaware* (Brazil). The TTO nanoparticles were obtained from Inventiva® (Porto Alegre, Brazil). Nanostructured lipid carriers were prepared with 7.5% (w/v) of tea tree oil using a proprietary method from Inventiva®, based on high pressure homogenization. Cetyl palmitate was used as solid lipid and polysorbate 80 as surfactant. Total solid content was 18.6% (w/v) [12].

2.2. TTO and TTO nanoparticles characterization

After the preparation, the nanoparticles were characterized in particle size, polydispersion index, and zeta potential using Zetasizer Nano-ZS ZEN 3600 (Malvern, England) and determination of pH using potentiometer (Digimed®). Each measurement was performed into 3 replicates.

The oil composition was performed in a 6890N model chromatograph, coupled with a 5975B model mass detector, both from Agilent Technologies. Chromatographic conditions were as follows: oven initial temperature was 50 °C, for one minute, followed by heating at a rate of 5 °C/min reaching 300 °C, keeping this temperature for 9 min, of a total of 60 min; separation was achieved in a DB-5MS column (30 m × 320 μm × 0.25 μm), at a constant rate of 1.5 ml/min of helium; detection was performed in the quadruple equipment using electron ionization [13]. The peak identification was carried by comparison of the experimental mass spectrum with those of the NIST standard library and published papers.

2.3. Nanoparticle tracking analysis (NTA)

NTA experiments were performed applying a digital microscope LM10 System (NanoSight, Salisbury, UK). A small amount of the diluted sample was introduced into the chamber by a syringe. The particles in the sample were observed using the digital microscope. The video images of the movement of particles under Brownian motion were analyzed by the NTA, version 1.5 (B196) image analysis software (NanoSight). Each video clip was captured over 30 s. The detection threshold was fixed at 100, whereas the maximum particle jump and minimum track length were both set at 10 in the NTA software.

2.4. Microorganisms

The fungal isolates used in the study included: *C. albicans* (ATCC 14053), *C. glabrata* (ATCC 66032), *C. parapisilosis* (ATCC 220190), *C. tropicalis* (ATCC 66029), and *C. membranafaciens* (ATCC 2013770).

2.5. Biofilm formation and treatment

The assay for biofilm formation was conducted according to the method described by Merritt et al. [14] with some modifications. *Candida* species were inoculated in Brain Heart Infusion (BHI) broth on 1 Mcfarland scale. Later, 200 μL of fungal suspension was added into 96 well-plates. The BHI broth without microorganism was considered the negative control. The plate was incubated for 24 h at 37 °C. After the incubation, different concentrations of TTO or TTO nanoparticles (31.2; 15.6; 7.8; and 3.9%) were added into wells and the plate was re-submitted to incubation for 24 h.

2.6. Quantification of biofilm biomass

After the biofilm formation, cells weakly adhered on the surface were removed by washing with saline, and the biofilm was set at 60 °C for 60 min. A 200 μL suspension of 0.1% crystal violet was added to each well and maintained at rest for 10 min, followed by washing with saline to remove planktonic cells and the remaining excess. Then, 200 μL of ethanol was added to each well, kept for 15 min and transferred to another plate for subsequent reading at an optical density (OD) of 492 nm on a plate reader (TP-Reader; ThermoPlate, Goiás, Brazil). Biofilm formation was determined by the difference between the average readings of OD obtained in the positive control (culture medium and bacteria) and the negative control (culture medium only). The assay was performed in 3 replicates.

2.7. Protein determination

The protein concentration of samples was measured by the method described previously by Bradford [16] modified by Ref. [17]. For this assay, the Bradford reagent was applied and a standard curve with bovine albumin (BA) (Sigma) was performed. The concentration of samples was determined by the comparison with the standard curve of BA, plotted and analyzed by linear regression on GraphPad Prism version 6.0.

2.8. Exopolysaccharide assay

The determination of amount of EPS was performed by the method previously described by Shetlar et al. [18], adapted by Dall & Herndon [19] with some modifications. A suspension (1 mL) containing the microorganism was added into a tube with rabbit serum (3 mL) and the treatment (TTO or TTO nanoparticles) on 2 concentrations (15.6% or 31.2%). The tubes were incubated at 37 °C for 48 h. After incubation, the samples were submitted to boiling water bath and sonication. The samples were centrifuged (950 g for 15 min) and 1 mL of supernatant (which containing EPS) was transferred to other tube and was precipitated with add of drops (10 mL) of absolute ethanol and after re-centrifuged (2400 g for 15 min). The pellet was dissolved on 1 mL of distilled water, digested in 7 mL of sulfuric acid (77%) and put into ice bath for 10 min. Afterwards, 1 mL of tryptophan (1%) was added in each tube and mixed. Immediately the tubes were submitted to boiling water bath for 20 min and colds on ice the absorbance of samples were measured at 500 nm. The assay was performed into 3 replicates.

2.9. Calcofluor white stain

For the visualization of the biofilm formed, it was used the Calcofluor White Stain technique. For this, microscope slides were used (26 × 76 mm and thickness 1.0–1.2 mm). The microorganisms were inoculated on BHI broth and incubated at 37 °C for 24 h. From this growth, suspensions corresponding to the 0.5 McFarland scale were performed. It was added 2 mL of suspension in petri dishes containing 20 mL of Mueller Hinton broth and microscope slide. The plate was incubated for 24 h at 37 °C for the adherence of microorganisms. The assay was performed into 2 replicates. After incubation period, it was added the TTO nanoparticles (15.6 and 31.2%) and the plate was re-incubated for 24 h. The revelation of the test was performed according Gonçalves et al. [15]. After