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Antibiofilm activity of nanoemulsions of *Cymbopogon flexuosus* against rapidly growing mycobacteria

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ABSTRACT

Rapidly growing mycobacteria (RGM) are opportunistic microorganisms that can cause both local and disseminated infections. When in biofilm, these pathogens become highly resistant to antimicrobials used in clinical practice. Composed abundantly of polymeric substances, biofilms delay the diffusion of antimicrobials, preventing the drug from penetrating the deeper layers and having an effective action. Therefore, the search for new and alternative therapeutic options has become of fundamental importance. Natural products fall into these options, especially essential oils. However, these oils present problems, such as low miscibility in water (which decreases its bioavailability) and degradation by light and temperature. Thus, the objective of this work was to explore the action of free essential oil and nanoemulsions of *Cymbopogon flexuosus* on strains of RGM, in planktonic and sessile forms. In this work, standard strains of *Mycobacterium fortuitum* (ATCC 6841), *Mycobacterium massiliense* (ATCC 48898) and *Mycobacterium abscessus* (ATCC 19977) were used. The susceptibility of the microorganisms in planktonic form was obtained by conventional microdilution techniques and by cell viability curve. The analysis of the antibiofilm activity was performed by a semi-quantitative macrotechnique. The nanoemulsion exhibited significant antimicrobial activity, with minimum inhibitory concentration values lower than those presented by the free essential oil, against strains in the planktonic state. However, both were efficient in destroying the already formed biofilm, whereas only the free oil inhibited the formation of mycobacterial biofilm. This study demonstrated the therapeutic potential of *C. flexuosus* essential oil, especially in its nanostructured form, which can be demonstrated against infections caused by rapidly growing mycobacteria.

1. Introduction

Non-tuberculous mycobacteria (NTM) have been documented since 1950 as microorganisms capable of causing diseases in humans, receiving greater clinical recognition due to the control of tuberculosis and improvementsin culture techniques and the identification of NTM [1,2]. When responsible for pathological processes in humans, NTMs can cause both localized disease, like skin infections, and disseminated disease [3].

Among the NTM species, there is a group of non-pigmented mycobacteria, known as rapidly growing mycobacteria (RGM), which are highly resistant and capable of developing, even in hostile environments [4,5]. The frequent presence of these microorganisms in hospitals' tap water, their relative resistance to sterilizing agents, as well as their frequent involvement in the formation of biofilms, contribute to the species of RGM causing infections related to healthcare [6,7].

The diseases caused by these microorganisms are called mycobacteriosis and are characterized by a difficult and prolonged treatment. In addition, RGMs have a high ability to form biofilms, which makes the efficacy of these therapies even more difficult [8,9]. Biofilms are complex functional structures that present a variable distribution of cells and aggregates, which constitute a protected mode of growth, allowing their survival in a hostile environment [10]. It is a successful

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survival strategy developed by microorganisms, making them more resistant to conventional treatments, requiring higher doses and a longer treatment time. Thus, in addition to the intrinsic difficulty involved in the treatment of mycobacteriosis, biofilm poses a great challenge for therapeutics, since most of the available antimicrobials are directed only to microorganisms in the planktonic state [11,12].

Due to the resistance of these microorganisms to the antimicrobials used in therapeutics, it is necessary to use new approaches that can combat this problem. The use of secondary plant metabolites, such as essential oils (EOs), has already been studied as an alternative to antimicrobial therapy [13]. The *Cymbopogon flexuosus*species is a plant belonging to the Poaceae family, popularly known as lemongrass [14]. This oil is composed basically of monoterpenes, with citral (geranial and neral isomer) being the major component, representing about 61% of its composition. Lemongrass presents anticancer, antifungal and antibacterial activities, and citral is the element responsible for these properties, mainly in relation to the antimicrobial potential [15–18]. On the other hand, the use of these oils as a biopharmaceutical product is hampered by some of their own characteristics, such as low miscibility in water, which leads to a decrease in their bioavailability, photosensitivity and ease of degradation when exposed to temperature fluctuations, as well as their volatile nature [19,20].

The use of nanotechnology as a strategy to combat resistance mechanisms of pathogens has also been gaining importance and the focus of researchers [21]. Essential oils carried by nanostructured systems have been investigated as a potential strategy to improve their use, stability and effectiveness [20]. The literature presents some important reports on the use of nanostructures technology in order to increase the antimicrobial potential [22–24]. It has been shown that oil nanoparticles of *Melaleuca alternifolia* are efficient in reducing biofilm production by *Pseudomonas aeruginosa*PA01 and in bacterial adhesion in epithelial cells [25]. Likewise, it was demonstrated that the antibiofilm activity of glycerol monolaurate is increased when it is nanoencapsulated, being able to significantly reduce the biofilm biomass of *Candida albicans*, an activity not exerted by the free manolaurate [26].

Since anti-mycobacterial therapy for combating mycobacteriosis is becoming increasingly difficult and complex, due to the evolution and improvement of the mechanisms of resistance, this study has evaluated the anti-mycobacterial and antibiofilm activities of the lemongrass essential oil, in its free form and in nanoemulsions.

2. Materials and methods

2.1. Essential oils

The essential oil of *Cymbopogon flexuosus* was obtained commercially from the companyFERQUIMA Indústria e Comércio Ltda., Vargem Grande Paulista, São Paulo, Braziland was used in the free form and in nanoemulsions containing 5% EO (LGNE). The concentrations tested varied from 56.18 mg/mL to 0.1096 mg/mL for the oil and 11.23 mg/mL to 0.0438 mg/mL for the LGNE.

2.1.1. Characterization of essential oil

The characterization of the C. flexuosus essential oil was performed according to Godoi et al. [53] by gas chromatography using the Varian Star 3400CX equipment (CA, EUA) equipped with flame ionization detector (CG-FID). The retention times of the compounds were compared with a series of homologous n-alkanes which were analyzed under the same chromatographic conditions [27].

The qualitative analysis of the compounds was performed by the Shimadzu QP2010 Plus gas chromatograph coupled to a mass spectrometer (CG-MS, Shimadzu Corporation, Kyoto, Japan). The analytes were identified based on the comparison with the mass spectra available from the National Institute of Standards and Technology (NIST) and by comparing the calculated linear retention indices with those available in the scientific literature. From the peak area revealed in CG-FID, the relative percentage amount of each compound was calculated [27].

2.2. Nanoemulsions

2.2.1. Preparation of nanoemulsions

The nanoemulsions were prepared using the homogenization method under high stirring using the equipment Ultra-Turrax® (IKA, Germany). The formulation consists of two phases: an oil phase containing 5% lemongrass essential oil and the Span 80 surfactant (Sigma Aldrich, Brazil), and an aqueous phase containing Tween 80 (Synth, Brazil) and ultrapure water. After the solubilization of each phase separately, the oil phase is injected into the aqueous phase under a stirring rate of 10,000 rpm in the Ultra-Turrax®. Stirring was increased to 17,000 rpm and held for 1 h. Theblank nanoemulsion (BNE), nanoemulsion without active principle, was also assayed under the same conditions.

2.2.2. Characterization of nanoemulsions

The nanoemulsionswere characterized according to particle average diameter, polydispersity index (PDI) and zeta potential by means of the Zetasizer Nano-ZS ZEN 3600 (Malvern, United Kingdom). For the determination of particle size and PDI, the dynamic light scattering technique was employed, while the zeta potential was determined through the electrophoretic mobility technique. The pH values of the formulations were determined immediately after preparation using a potentiometer (DM-22, Digimed®), previously calibrated with standard solution.

The morphology of nanoemulsion was analyzed by Transmission Electron Microscopy (TEM). The water-diluted (1:10 v/v) nanoemulsions were deposited in a drop-sample grid. After 1 min, the samples were dried and stained with uranyl acetate (2%), for TEM evaluation at 80 KV (JEM-1200 EXII, JEOL, Japan) [28].

2.3. Microorganisms

For the realization of this study, 3 standard strains were used: *Mycobacterium fortuitum* (ATCC 6841), *Mycobacterium massiliense* (ATCC 48898) e *Mycobacteriumabscessus* (ATCC 19977). These strains were maintained at −80 °C and were grown on Löwenstein-Jensen Agar for use (HiMedia Laboratories Pvt. Ltd, India).

2.4. Determination of minimum inhibitory concentration (MIC)

The anti-mycobacterial activity of free EO, LGNE and BNE was evaluated by the broth microdilution method, following the guidelines of CLSI M24-A2 [29]. The oil and nanoemulsions were used in different concentrations from serial dilutionsin Mueller Hinton Broth (HiMedia Laboratories Pvt. Ltd, India). The inoculum density was standardized according to the MacFarland 0.5 scale and transferred to the 96-well microplates. The plates were incubated at 37 °C for 72 h, and the minimum inhibitory concentration (MIC) was determined using the indicator 2,3,5-triphenyltetrazolium chloride (TTC) (Vetec®, Rio de Janeiro, RJ, Brazil).

2.5. Time kill assay

Evaluation of the action of free EO and LGNE during different growth times of the RGM strains was performed through the time-kill curve. CIM and 2xCIM aliquots were used for plating in Mueller Hinton Agar (HiMedia Laboratories Pvt Ltd, India) after 24, 48, 72, 96 and 120 h of exposure to the compounds. The plates were incubated at 37 °C for 72 hoursand then the viable microorganismswere counted [8].