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Aliphatic fatty acids and esters: Inhibition of growth and exoenzyme production of *Candida*, and their cytotoxicity in vitro
Anti-*Candida* effect and cytotoxicity of fatty acids and esters

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ABSTRACT

The secretion of extracellular phospholipases and proteinases of *Candida* has been described as a relevant virulence factor in human infections. Aliphatic fatty acids have antimicrobial properties, but the mechanism by which they affect the virulence factors of microorganisms, such as *Candida*, is still unclear, and there are a few reports about their toxicity. The current study investigated the in vitro antifungal activity, exoenzyme production and cytotoxicity of some aliphatic fatty acids and their ester derivatives against the *Candida* species. The minimum inhibitory concentration and minimum fungicidal concentrations of aliphatic medium-chain fatty acids, methyl and ethyl esters were performed using the CLSI M27-A3 method and the cytotoxicity assay was performed according to ISO 10993-5. The influence of these compounds in the inhibition of the production of hydrolytic enzymes, phospholipases and proteinases by *Candida* was also investigated. Data analysis was performed using the one-way ANOVA method ($p \leq 0.05$). In relation to the MIC against *Candida* species, the fatty acid with the best result was Lauric acid, although its ester derivatives showed no activity. The inhibition of phospholipase production was more significant than the inhibition of proteinase production by *Candida*. Tested fatty acids revealed more than 80% cell viability in their MIC concentrations. Additionally, a cell viability of 100% was reported at concentrations of anti-enzymatic effect. Therefore, the potential use of these fatty acids could be the basis for more antimicrobial tests.

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1. Introduction

Great efforts have been made to develop new antimicrobial drugs in order to keep pace with the emergence of organisms that are resistant to current chemotherapeutics and to develop new chemical compounds that circumvent existing drug resistance mechanisms. The screening of natural product libraries may provide useful chemical entities for the development of new antimicrobial agents with low toxicity.[1]

The antimicrobial properties of some free fatty acids are well-recognised. Because they act through different mechanisms than most conventional antibiotics, they have a potential factor for commercial exploitation.[2] However, there are few reports about the mechanism by which they affect the virulence factors of *Candida* and few reports on their cytotoxicity.[3–5]

Candida species develop specific virulence mechanisms that confer them with the ability to colonise host epithelial cells, to invade deeper cells or to influence host defenses.[6] The ability of *Candida albicans* to persist within the host and cause infection has been attributed to many virulence properties, which include: adhesion, dimorphism, the ability to switch between different morphological types, interference with the immune system, synergism and the production of extracellular enzymes, such as proteinases and phospholipases.[7,8]

One of the most important virulence factors of *Candida* is the production of extracellular proteinases and phospholipases.[9] The secreted aspartic proteinases are responsible for adhesion, tissue damage and invasion of host immune responses. Their proteolytic activity has been associated with tissue invasion.[10] The secretion of extracellular phospholipases is considered a key attribute that aids in the invasion of the host mucosal epithelia. In general, phospholipases catalyse the hydrolysis of phospholipids, which are the major components of all cell membranes.[11]

Recent studies have suggested an effect at sub-minimal concentrations of antifungal agents on *Candida*' virulence factors.[6,7,12,13] This current study investigates in vitro antifungal activity, exoenzyme production and cytotoxicity of some aliphatic fatty acids and their ester derivatives against *Candida* species.

2. Materials and methods

2.1. Antifungal agents

The antifungal assay was carried out using medium-chain fatty acids (caprylic acid, capric acid, lauric acid), and a long-chain fatty acid (myristic acid) obtained from Aldrich (Steinheim, Germany) and their methyl and ethyl ester derivatives synthesised by Hobuss et al.[14] Ten dilutions of the compounds were prepared with concentrations ranging from 0.005 to 2.5 mg/mL in ethanol, with a fold dilution of 0.5. The general structure of each compound is shown in Table 1. Fluconazole was used as a reference compound.[15]

2.2. Isolates and culture conditions

Eight strains of *C. albicans* and six non-*albicans Candida* strains were used to determine the in vitro anti-fungal activity of fatty acids and their derivatives. The six species of oral yeasts were: *C. albicans* (8), *C. parapsilosis* (1), *C. lipolytica* (2), *C. tropicalis* (1), *C. famata* (1), and *C. glabrata* (1). One *C. albicans* strain was obtained from the American Type Culture Collection (ATCC 62342), and all other strains are clinical isolates.[16] The strains were isolated from cervical swabs and identified by a germ tube test. The strains were obtained from the Laboratory of Oral Microbiology, Pelotas Dental School of the Federal University of Pelotas, RS, Brazil. Samples were cultivated aerobically on SDA (Sabouraud dextrose agar) with chloramphenicol added and incubated at 36 ± 2 °C for 24 h.

2.3. Antifungal activity

The antifungal assay was carried out following CLSI M27-A3 method.[17] After a 24 h incubation period of *Candida* strains on fresh SDA, yeasts were harvested and suspended in sterile RPMI (Roswell Park Memorial Institute medium) culture medium at a turbidity equal to an optical density of 0.5 McFarland units. The final suspension was adjusted to 1.5×10^8 cell/mL. The solution containing each fungal inoculum was transferred in aliquots of 100 μL into each well of a sterile 96-well plate that already had 100 μL of the solution containing the dilution of the compound tested. The plates were incubated at 37 °C for 48 h. The readings were made visually. In order for the minimum fungicidal concentrations (MFC) to be evaluated, 20 μL of each solution, considered a minimum inhibitory concentration (MIC), was placed into a plate containing SDA. The MFCs were defined as the lowest drug dilutions that did not yield growth of yeast colonies (100% killing activity) after 24 h of aerobic incubation at 37 °C.[17]

2.4. Enzymatic activity determination by the agar plate method

The test medium for proteinases was BSA (bovine serum albumin) agar medium containing 2 g of BSA, 1.45 g of YNB (yeast nitrogen base – Difco Laboratories, Detroit), 20 g of glucose and 20 g of agar per litre of distilled water. The test medium for phospholipases consisted of SDA containing 57.3 g of sodium chloride, 0.55 g of calcium chloride, and 100 mL of 50% sterile egg yolk (egg yolk enrichment) per litre of distilled water. Test isolates were grown on SDA for 24 h and were suspended in 1% sterile PBS (phosphate buffered saline), with a refraction and visual turbidity equal to 0.5 McFarland units. Each fatty acid used was first diluted in ethanol at a particular concentration: the MIC and three following smaller concentrations. For each concentration, and starting with the greatest, 20 μL were emulsified in 1980 μL of sterile PBS.

Eight strains of *C. albicans* were prepared in a cell suspension in order to determine the effect of fatty acids on phospholipase and proteinase activity. From this cell suspension, 0.5 mL were added to tubes containing 2 mL of PBS (control) and 2 mL of PBS/fatty acid. This dilution resulted in a concentration of 10^6 and 10^7 cells/mL in each assay tube. The tubes were then incubated for 30 min at 37 °C. The agent was