Microbial Pathogenesis 107 (2017) 390–396

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Synergistic anticandidal activity of menthol in combination with itraconazole and nystatin against clinical *Candida glabrata* and *Candida krusei* isolates

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ARTICLE INFO

Article history:
Received 25 February 2017
Received in revised form
17 April 2017
Accepted 17 April 2017
Available online 19 April 2017

Keywords:
Anticandidal effect
Candida glabrata
Candida krusei
Menthol
Drug combination
Synergy

ABSTRACT

Background: *Candida glabrata* (*C. glabrata*) and *C. krusei* are now emerging as serious hospital acquired infections in immunocompromised patients. Menthol, a terpenic compound, has been reported to have antifungal activity.
Objectives: The aim of this study was to investigate the effect of menthol in combination with itraconazole or nystatin against *C. glabrata* and *C. krusei* isolates.
Methods: The effects of menthol along with itraconazole and nystatin, were evaluated by the Clinical Laboratory Standards Institute (CLSI) M44-A and CLSI M27-A3 methods. The fractional inhibitory concentration index (FICI) was determined for menthol plus itraconazole and nystatin combinations using the checkerboard method.
Results: The mean of minimum inhibitory concentration (MIC) values of menthol, nystatin and itraconazole were 53.2, 2.30 and 1.50 μg/ml for *C. glabrata* isolates and 121, 1.08 and 0.38 μg/ml for *C. krusei* isolates, respectively. Menthol in combination with itraconazole or nystatin exhibited the synergistic effects against all species of *Candida* tested. FICI values for menthol plus itraconazole and nystatin combinations ranged from 0.250 to 0.561 and 0.139 to 0.623 for *C. glabrata* isolates, and 0.182 to 0.750 and 0.188 to 0.760 for *C. krusei*, respectively.
Conclusions: These results support the potential use of menthol as an anticandidal agent, and it can be used complementarily with other conventional antifungal agents.

1. Introduction

Candida, which includes around 200 yeast species, is an opportunistic commensal of the human cutaneous, oral, gastrointestinal, vaginal and other mucosal surfaces [1]. *Candida albicans* (*C. albicans*) is the predominant causative organism of virtually all types of candidiasis, but other emerging *Candida* species, including *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis*, are now posing serious nosocomial threats to patient populations [2]. During the last three decades the number of fungal infections caused by yeasts of *Candida* species has increased dramatically, mainly due to the rise in the number of immunocompromised patients [3]. Currently, candidiasis represents the fourth leading cause of nosocomial infections, at 8–10%, and mortality due to systemic candidiasis remains high, ranging from 15 to 35% depending on the infecting *Candida* species [4].

Available antifungal drugs can treat superficial fungal infections, but only a few treatments are available for invasive diseases. Polyenes cause serious host toxicity, whereas azoles are fungistatic and their prolonged use leads to drug resistance [5]. Moreover, some species of the genus *Candida,* including *C. glabrata* and *C. krusei*, are intrinsically resistant to fluconazole and other azoles, and frequency of isolations of these two species has significantly increased recently [6]. Due to a dramatic rise in fungal infections and the current trend toward increasing awareness in traditional medicine, plant-derived antifungal compounds are attracting much interest as natural alternatives owing to their versatile applications [7]. Many studies have been conducted on the antifungal activity of natural products against *Candida* species involved in fungal

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http://dx.doi.org/10.1016/j.micpath.2017.04.021
0882-4010/

infections of the oral and vaginal cavities [8,9]. Natural products include essential oils and their constituents, which may belong to several classes of compounds, but terpenes are the most common [10]. The interest in isolated monoterpenes has grown over the past years as a result of their pharmacological use. This increase in interest is particularly true for menthol, which is a known antimicrobial agent [11].

Menthol ($C_{10}H_{20}O$) is a terpenoid, found in the essential oils of the mint family such as peppermint, horse mint and others. Several isomers of menthol exist, some with a menthol smell, others without. In nature it only occurs as (−) menthol, which has the strongest smell and its formal name is (1R,2S,5R)-2-isopropyl-5-methylcyclohexanol. The other isomers are known as isomenthol, neomenthol and neoisomenthol [12]. The use of individual components provides greater predictability, allowing the minimization of adverse effects. The aim of the current study was to investigate the *in vitro* activity of menthol against clinical isolates of *C. glabrata* and *C. krusei* from patients suffering from candidiasis and synergistic effect in combination with the synthetic antifungal nystatin and itraconazole.

2. Materials and methods

2.1. Fungal isolates and identification

A total of 22 oral and vaginal *Candida* isolates (10 *C. glabrata* isolates, 10 *C. krusei* isolates, one *C. glabrata* ATCC 90030 and one *C. krusei* ATCC 6258) were used in this study (Table 1). All yeast isolates were grown onto sabouraud dextrose agar (Merck Co., Darmstadt, Germany) containing 5% chloramphenicol. The cultures were incubated at 35 °C and examined daily for one week. Clinical *Candida* strains were identified using conventional methods, including CHROM agar, urease test, sugar fermentation and assimilation tests by RAPID yeast plus system (remel Inc., USA), and were confirmed by polymerase chain reaction-restriction fragment length poly-morphism.

2.2. Test products

The phytoconstituent menthol provided by Biobasic Canada Inc. (C.N:NBQJ30BR1) and the synthetic antifungal nystatin (Biobasic Canada Inc., C.N:1400-61-9) and itraconazole (Sigma-Aldrich, St. Louis, USA) were used in the *in vitro* assays. Nyststin, itraconazole and menthol powders were solubilized in distilled water, dimethyl sulfoxide (DMSO) and 70°GL isopropanol, respectively.

2.3. Disc diffusion assay

A disc diffusion test was used to determine the anticandidal activity as described in the document M44-A from the Clinical Laboratory Standards Institute (CLSI) for yeasts [13]. Mueller-Hinton agar containing 2% glucose was inoculated with yeast cell suspension (10^6 cells/ml) and poured into sterile petri dishes. Sterile filter paper discs 6 mm in diameter were impregnated with 20 μl of menthol and placed on the inoculated agar surface. Standard 6 mm discs containing nystatin 50 μg/disc and itraconazole 10 μg/disc (Bioanalyse) were used as synthetic drugs. The plates were incubated overnight at 35 °C for 24–48 h. The diameter of any resulting zones of growth inhibition was measured (mm). For quality control, *C. albicans* ATCC 10241 and *C. krusei* ATCC 6258, were used. Each experiment was tested in duplicate.

2.4. Broth microdilution assay

The anticandidal activities of menthol, nystatin and itraconazole were determined by broth microdilution method using two fold serial dilutions in RPMI 1640 medium, as described in the document M27-A3 from the CLSI for yeasts [14]. Briefly, test compounds were dissolved in RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid. After shaking, 100 μl aliquots were added to the wells of 96-well microtiter plates with final concentrations ranging from 4.8 to 2500 μg/ml for menthol, 0.12–125 μg/ml for nystatin and 0.125–64 μg/ml for itraconazole. Yeast inocula were prepared by growing the isolates on sabouraud dextrose agar plates at 35 °C for 24 h and adjusting to a final concentration between 0.5–2.5 × 10^3 cells/ml in sterile saline. A 100 μl suspension of each of the *Candida* strains was added to individual wells and cultivated at 35 °C for 24–48 h. Chemical-free (positive) and yeast-free (negative) controls were included. The minimum inhibitory concentration (MIC) of the test compounds for each isolate was defined as the lowest concentration that produced a prominent decrease of fungal growth (inhibition ≥ 50%) compared with growth control. Classification of isolates in terms of their susceptibilities to these antifungal agents was based on the MIC breakpoints recommended in the M27-A3 protocol. So that, for itraconazole the MIC for susceptibility was ≤0.125 μg/ml, the MIC for susceptible-dose dependent was 0.25–0.5 μg/ml, and the MIC for resistance was ≥1 μg/ml. Minimum fungicidal concentration (MFC) was defined as a ≥99.9% reduction in the number of colony-forming units from the starting inoculum count, whereas fungistatic activity was defined as ≤ 99.9% reduction. For quality control, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019, were used. Each experiment was tested in duplicate.

2.5. Checkerboard assay (FIC index, FICI)

Checkerboard synergy testing was performed to determine the fractional inhibitory concentration index (FICI) as reported earlier

Table 1
The characteristics of various *Candida* isolates.

Candida glabrata isolates[a]	Infection site	*Candida krusei* isolates	Infection site
Cg1	Vagina	Ck1	Mouth (HIV^+)
Cg2	Mouth (HIV^+)	Ck2	Vagina
Cg3	Mouth (HIV^+)	Ck3	Vagina
Cg4	Mouth (HIV^+)	Ck4	Vagina
Cg5	Mouth (HIV^+)	Ck5	Vagina
Cg6	Mouth (HIV^+)	Ck6	Vagina
Cg7	Mouth (HIV^+)	Ck7	Mouth (HIV^+)
Cg8	Vagina	Ck8	Mouth (HIV^+)
Cg9	Mouth (HIV^+)	Ck9	Mouth (HIV^+)
Cg10	Mouth (HIV^+)	Ck10	Vagina
Cgr	ATCC 90030	Ckr	ATCC 6258

[a] *Candida glabrata* isolates: Cg1-10, *Candida glabrata* reference strain: Cgr; *Candida krusei* isolates: Ck1-10, *Candida krusei* reference strain: Ckr.