INSTITUT PASTEUR

Research in Microbiology 161 (2010) 816–823

www.elsevier.com/locate/resmic

Ocimum sanctum essential oil and its active principles exert their antifungal activity by disrupting ergosterol biosynthesis and membrane integrity

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Received 15 March 2010; accepted 22 July 2010
Available online 22 September 2010

Abstract

The increasing incidence of drug-resistant pathogens and host toxicity of existing antifungals attracts attention toward the efficacy of natural products as antifungals in mucocutaneous infections and combinational therapies. The composition and antifungal activity of the essential oil obtained from *Ocimum sanctum* (OSEO) was studied. On GC–MS analysis, OSEO showed a high content of methyl chavicol (44.63%) and linalool (21.84%). Antifungal activity of OSEO and its two main constituents was determined against sixty clinical and five standard laboratory isolates of *Candida*. OSEO, methyl chavicol and linalool showed inhibitory activity toward all tested strains. The mechanism of their fungicidal action was assessed by studying their effect on the plasma membrane using flow cytometry, confocal imaging and determination of the levels of ergosterol, a fungal-specific sterol. Propidium iodide rapidly penetrated a majority of yeast cells when they were treated with OSEO concentrations just above MIC, implying that fungicidal activity resulted from extensive lesions of the plasma membrane. OSEO and its components also caused a considerable reduction in the amount of ergosterol. The present study indicates that OSEO, methyl chavicol and linalool have significant antifungal activity against *Candida*, including azole-resistant strains, advocating further investigation for clinical applications in the treatment of fungal infections.

Keywords: *Ocimum sanctum*; *Candida*; Ergosterol; Fluconazole; Propidium iodide

1. Introduction

Serious infections caused mostly by opportunistic fungal pathogens are increasingly common in immunocompromised patients. *Candida* species are now recognized as a major cause of hospital-acquired infections (Maschmeyer, 2006). *Candida albicans* is the predominant organism associated with candidiasis; but other *Candida* species, including *Candida glabrata*, *Candida parapsilosis* and *Candida krusei*, are now emerging as serious nosocomial threats to patient populations (Pfaller and Diekema, 2004). Existing antifungals can treat mucosal fungal infections, but very few treatments are available for invasive diseases. Polyenes cause serious host toxicity (Cohen, 1998) whereas azoles are fungistatic and their prolonged use contributes to the development of drug resistance in *C. albicans* and other species (Sanglard et al., 2003). Because of the 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 (Iwu et al., 1999; Ahmad et al., 2010). Essential oils possess a broad spectrum of antimicrobial activities attributed to the high content of phenolic derivatives, but a few have been reported to have significant antifungal activity (Bakkali et al., 2008; Kalemba and Kunicka, 2003; Rosato et al., 2008; Cavaleiro et al., 2006; Pina-Vaz et al., 2004; Eugenia et al., 2009). Limited information

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doi:10.1016/j.resmic.2010.09.008

exists about their activity toward human pathogens and the mechanism of their action is yet to explored.

Ocimum sanctum (L.)(OSEO), also known as Tulsi and 'Holy Basil', is widely known across South Asia as a medicinal plant and is distributed and cultivated worldwide (Sethi et al., 2003; Nweze and Eze, 2009). However its antimicrobial characteristics are used only in 'Ayurvedic medicines'. OSEO has been extensively studied for its antifungal activity against filamentous fungi such as *Aspergillus niger*, *Aspergillus fumigatus* (Dharmagadda et al., 2005; Bansod and Rai, 2008), *Aspergillus flavus* (Kumar et al., 2010) *Rhizopus stolinifera* and *Penicillium digitatum* (Grover and Rao, 1977). Other rare but clinically important filamentous fungi like *Fusarium solani*, *Penicillium funiculosum*, *Rhizomucor auricus* and *Trichoderma reesi* are also susceptible to OSEO (Dharmagadda et al., 2005). We have also shown previously that the combination of fluconazole (FLC) or ketoconazole with OSEO augments the efficiency of these azoles against *Candida* spp. (Khan et al., 2010).

The present work was an attempt to understand the mechanism of antifungal activity of OSEO against *Candida* species as a prerequisite for its application in the treatment of mucocutaneous infections or combinatorial therapies. Most therapies, designed to treat fungal infections, target the ergosterol biosynthesis pathway or its end product, ergosterol, a membrane sterol that is unique to fungi. Ergosterol is the main sterol of yeasts and other fungi, and thus is necessary for growth and normal membrane function of cells. Besides serving as a bioregulator of membrane fluidity, asymmetry and membrane integrity, ergosterol contributes to the proper function of membrane-bound enzymes (Lupetti et al., 2002). Therefore, our studies were conducted to test the effect of OSEO and its main constituents (methyl chavicol and linalool) on the structural and functional integrity of cytoplasmic membranes of a wide variety of *Candida* isolates.

2. Materials and methods

2.1. Strains and media

Fungal strains used in the present study are listed in Table 1. The clinical isolates were collected from the All India Institute of Medical Sciences and VMMC Safdarjung Hospital, New Delhi, India. All strains were grown on yeast extract (1%w/v) peptone (2%) dextrose (2%) (YPD) medium. Cultures were maintained on YPD agar plates. The aerial parts of *O. sanctum* (identified and dried) were subjected to hydrodistillation in a Clevenger apparatus for 3 h. The essential oil (volatile fraction) was separated and stored at 4 °C. Methyl chavicol and linalool were purchased from Sigma–Aldrich (USA), Fluconazole (FLC) and amphotericin B (AmB) from HiMedia. All inorganic chemicals were of analytical grade and procured from E. Merck (India).

2.2. Determination of minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The MIC was defined as the lowest concentration of the oil/test molecule that causes inhibition of visible growth of *Candida* cells. MIC_{90} was determined in vitro in liquid medium by the macrobroth dilution method as described by CLSI reference document M27-A3 (CLSI, 2008) for 65 *Candida* isolates (Table 1). To determine the MFC values after reading the corresponding MIC values, 20 µl samples from all optically clear tubes (complete growth inhibition) plus the last tube showing growth were subcultured on YEPD agar plates. The plates were incubated at 35 °C for a minimum of 3 days, until growth was clearly visible in the control samples, and MFC values were determined as the lowest concentration of the OSEO and its two major components for which there was no visible growth.

Table 1
Isolates used in the study.

Classification of isolates	Type of isolate
Sensitive (standard, n = 5)	
ATCC 90028	*C. albicans*
ATCC 44829	*C. albicans*
ATCC 10261	*C. albicans*
ATCC 750	*Candida tropicalis*
ATCC 90030	*C. glabrata*
Sensitive (clinical, n = 34)	
Invasive[a] (*n* = 17)	*C. albicans* (7), *C. tropicalis* (5), *C. glabrata* (4), *C. parapsilosis*
Cutaneous (*n* = 3)	*C. albicans*, *C. tropicalis*, *C. parapsilosis*
Hospital-acquired (post-operatory) (*n* = 7)	*C. albicans* (5), *C. tropicalis* (2)
Oropharyngeal (*n* = 5)	*C. albicans* (4), *C. tropicalis*
Esophageal (*n* = 2)	*C. albicans*, *C. tropicalis*
Resistant[b] (clinical, *n* = 26)	
Invasive[a] (*n* = 20)	*C. albicans* (5),[c] *C. tropicalis* (3), *C. glabrata* (6),[c] *C. parapsilosis* (3), *C. krusei* (3)
Cutaneous (*n* = 4)	*C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*
Hospital-acquired (catheter infection) (*n* = 2)	*C. tropicalis*, *C. glabrata*

[a] Includes cases of candidemia, vulvovaginal and urinary tract infections in HIV and non-HIV patients.
[b] FLC MIC $\geq$64 mg L^{-1} considered as resistant.
[c] AmB MIC $\geq$5 mg L^{-1} considered as resistant.

2.3. GC–MS analysis

OSEO was subjected to detailed GC-MS analysis using a Shimadzu 2010 gas chromatograph fitted with an AB wax column (Khan et al., 2010). Helium was used as the carrier gas. A 0.1 µL sample was injected with splitless mode. The chemical components from the essential oil, shown in Table 2, were identified by comparing the retention times of chromatographic peaks with those of authentic compounds using the WILEY8.LIB and NIST05s.LIB.

2.4. Flow cytometry analysis

Flow cytometry analysis of *Candida* cells exposed to OSEO and its two components was performed using propidium iodide (PI). Cells (10^6 mL^{-1}) were incubated at 35 °C up to mid-exponential phase and were then treated with different