In vitro susceptibilities of *Candida* and *Aspergillus* species to *Melaleuca alternafolia* (tea tree) oil

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Summary

Candida species are an important cause of opportunistic infection in the oral cavity of immunocompromised patients, especially HIV infected patients. Melaleuca oil obtained commercially was investigated since it is known to have broad antifungal properties. The in-vitro susceptibilities of *Aspergillus* and susceptible and resistant *Candida* species were performed utilizing serial dilutions in microtiter plates with Sabouraud dextrose agar and the commercial preparation of *Melaleuca*. As a comparator, *in vitro* susceptibilities to amphotericin B and fluconazole were also determined using the broth microdilution technique. The results demonstrate that *Melaleuca* inhibited the *Candida* species. However, the growth of *Aspergillus* was not inhibited at the concentrations tested. Thus, preparations containing *Melaleuca alternafolia* may be a useful alternative for superficial candidal infections. In fact, it may be a useful alternative regimen for advanced HIV-positive patients with oropharyngeal candidiasis refractory to fluconazole. However, controlled clinical studies to evaluate its efficacy are still needed.

Key words *Melaleuca alternafolia*, *Candida*, *In vitro* susceptibility

Susceptibilidad *in vitro* de *Candida* y *Aspergillus* al aceite de *Melaleuca alternafolia* (Tea Tree)

Resumen

La candidiasis oral es una de las causas más importantes de infección en pacientes inmunocomprometidos, especialmente con VIH. Las propiedades antimicóticas del aceite de melaleuca han sido descritas El aceite de melaleuca, obtenido comercialmente, fue investigado *in vitro*. Las susceptibilidades *in vitro* de *Candida* y *Aspergillus* fueron estudiadas usando el método de dilución serial y placas de microtítulo en agar de Sabouraud. Como comparación, las susceptibilidades de anfotericina B y fluconazol fueron determinadas simultáneamente usando la técnica del NCCLS. Los resultados demuestran que melaleuca inhibe las especies de *Candida* que fueron analizadas. Sin embargo, las especies de *Aspergillus* no fueron inhibidas. En conclusión, las preparaciones que contienen *Melaleuca alternafolia* posiblemente se podrían usar en infecciones superficiales por *Candida*. Es también posible que sea un agente alternativo en pacientes con VIH y candidiasis oral resistente a fluconazol o agentes antimicóticos. Se necesitan estudios controlados para establecer su valor clínico.

Palabras clave *Melaleuca alternafolia*, *Candida*, Susceptibilidad *in vitro*

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Aceptado para publicación el 31 de marzo de 2000

1130-1406/99/5.00 Euros

Oropharyngeal candidiasis develops in 80-95% of patients with AIDS [1,2]. The pathogenesis of this seemingly innocuous disease is very complex. Until now, *Candida albicans* has accounted for virtually all mucosal candidiasis. Recently, however, other species such as *Candida glabrata, Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* have caused serious symptomatic oropharyngeal candidiasis (OPC), and on occasion it may also be associated with esophageal candidiasis. The oral azole antifungals clotrimazole, ketoconazole, fluconazole and itraconazole are frequently used in patients who are HIV-positive as initial or suppressive therapy for oropharyngeal and esophageal candidiasis. Unfortunately, the incidence of fluconazole-refractory OPC is becoming increasingly more common and frequently may emerge during therapy in advanced HIV-positive patients [3,4]. Many of these patients they may suffer from frequent clinical relapses despite high doses of fluconazole and require parenteral amphotericin B. These overwhelming infections frequently impair the quality of life and may result in a reduction of fluid or food intake.

In searching for newer and less toxic compounds, we have evaluated the oil of melaleuca. The oil was originally obtained from the leaves of a paperbark tea tree grown in the central coastal region of eastern Australia. Penford initially discovered the therapeutic value in 1922, when he discovered antibacterial and antifungal properties related to *Melaleuca.* Several of the active ingredients of the tea tree oil include terpinen-4-ol and alpha-terpineol [5-7]. Several investigators have recently evaluated its in-vitro activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Malassezia furfur, Fusobacterium* spp*, Bacteroides* spp*, Prevotella* spp, and *C.albicans* [6-15].

In this study, we evaluate the in-vitro activity of Melaleuca oil against *Aspergillus* species and known resistant *Candida* species that have been isolated from either advanced HIV-positive patients suffering from fluconazole and amphotericin B refractory OPC or from immunocompromised patients with disseminated fungal infections.

MATERIALS AND METHODS

Fungal strains. The organisms included clinical specimens recovered from patients with candidemia, OPC, esophageal candidiasis, or asymptomatic colonization. The distribution of species included 50 *C. albicans* isolates, 21 *C. glabrata* isolates, 10 *C. tropicalis* isolates, seven *C. parapsilosis* isolates and five isolates each of *C. krusei, Candida lusitaniae, Candida kefyr,* and *Candida guilliermondii*. The quality control strains included *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 90018, and *C. glabrata* ATCC 90030. In addition, five isolates of *Aspergillus fumigatus* and five isolates of *Aspergillus nidulans* were also evaluated.

In-vitro susceptibility analysis. Amphotericin B and fluconazole were obtained from their respective manufacturers. Oil of melaleuca T36-C7 (Tea-tree oil) was obtained from Melaleuca Inc., Idaho Falls, Idaho, U.S.A. This formulation contains 36% Terpinen 4-ol and less than 10% 1,8 cineole as determined by gas liquid chromatography. The MICs of all of the antifungal agents for all of the isolates were determined in accordance with the National Committee for Clinical Laboratory Standards M27-A by a microdilution method [16].

A standard inoculum of *Candida* was diluted to a final concentration of $1x10^2$ to $5x10^2$ CFU/well in microtiter plates. As previously published by other authors, the *Aspergillus* inoculum was prepared by suspending *Aspergillus* conidia in buffered-saline. The conidia were counted by a hemocytometer and then diluted to a concentration of 10^6 conidia/ml [17]. Controls were grown on drug-free and one drug containing media.

Candida and *Aspergillus* species were tested against doubling dilutions of the oil of melaleuca [range, 2 %-0.03 % (v/v)] as previously published by Hammer *et al.* [11,12] prepared in RPMI in a 96-well microtiter plate. Tween 80 (Sigma, St Louis, Mo.) was added at a final concentration of 0.001% (v/v).

The MICs for amphotericin B and melaleuca were defined as the lowest concentration that inhibited 100% of the visible growth. The MICs of fluconazole was defined as the lowest concentration that inhibited 80% of visible growth when compared with the growth control. The data are reported as the concentrations of each antifungal agent necessary to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates evaluated. All assays were done in duplicate to verify the results. Since there are no definitive MIC breakpoints that separate resistant from susceptible strains, we used an MIC of $\geq$ 16 mg/ml to define fluconazole resistance.

The MFC (mean fungicidal concentration) were determined by subculturing 0.1 ml from the first microtiter well demonstrating complete growth inhibition and from all wells with no visible growth onto Sabouraud dextrose agar plates that were incubated at 30°C for 72 hours. Afterwards, colonies were counted, and the MFC was defined as the lowest concentration at which 99% of the initial inoculum was killed.

RESULTS

Melaleuca oil had the lowest MIC_{S50} and the lowest ranges against *C. albicans, C. parapsilosis* and *C. kefyr* with a range of 0.06 - 0.25 % (Table 1). The most susceptible of all of the *Candida* species is still *C. albicans*, with an MIC range of 0.06 – 0.25%, an MIC_{50} of 0.12%, an MIC_{90} of 0.25%, and an MIC_{50} of 0.50% (Table 1). The *C. albicans* strains included 10 isolates for which the MIC_{50} of fluconazole was 32 μg/ml and the MIC_{90} was 64 mg/ml. The MIC_{S50} of melaleuca for *C. albicans* isolates for which fluconazole MICs were $\geq$ 16 μg/ml or $\leq$ 8 μg/ml were the same (0.12 %) (Table 2). The second most susceptible group of yeast isolates includes the five *C. lusitaniae* and five *C. guilliermondii* that have an MIC_{50} of 0.25% to melaleuca, with a similar MIC range of 0.12 – 0.25%. *C. krusei* and *C. tropicalis*

Table 1. In-vitro susceptibilities of *Candida albicans,* non-*albicans Candida* species and *Aspergillus* species to Melaleuca oil using broth microdilution assays.

	MIC (% vol/vol)			MFC (%vol/vol)
Organisms (No. tested)	Range	50%	90%	50%
C. albicans (50)	0.06 – 0.25	0.12	0.25	0.50
*C. glabrata (*21)	0.25 – 0.50	0.25	0.50	0.50
C. tropicalis (10)	0.12 – 0.50	0.25	0.50	0.50
C. parapsilosis (7)	0.06 – 0.25	0.25	-	0.50
C. kefyr (5)	0.06 – 0.25	0.25	-	0.50
*C. krusei (*5)	0.12 – 0.50	0.5	-	0.50
*C. lusitaniae (*5)	0.12 – 0.25	0.25	-	0.50
C. guilliermondii (5)	0.12 – 0.25	0.25	-	0.50
Aspergillus fumigatus (5)	NI	> 2.0	-	NI
Aspergillus nidulans (5)	NI	> 2.0	-	NI

NI = non-inhibitory

Table 2. Comparison of the in-vitro susceptibility of azole-susceptible and -resistant strains of *Candida* to melaleuca oil, amphotericin B and fluconazole.

Organisms (no. tested)	Antifungal agent	MIC Range	MIC 50%	MIC 90%	MFC 50%
C. albicans -S [a] (40)					
	Melaleuca oil (% vol/vol)	0.06 - 0.25	0.12	0.25	0.50
	Amphotericin (μg/ml)	0.01 - 0.8	0.10	0.20	
	Fluconazole (μg/ml)	0.062 - 8	0.12	2	
C. albicans-R [a] (10)					
	Melaleuca oil (% vol/vol)	0.06 - 0.25	0.12	0.25	0.50
	Amphotericin (μg/ml)	0.02 - 0.40	0.05	0.20	
	Fluconazole (μg/ml)	16 - 64	32	64	
C. glabrata-S [b] (10)					
	Melaleuca oil (% vol/vol)	0.12 - 0.50	0.12	0.25	0.50
	Amphotericin (μg/ml)	0.05 - 0.80	0.20	0.80	
	Fluconazole (μg/ml)	0.50 - 4	1	8	
C. glabrata-R [b] (7)					
	Melaleuca oil (% vol/vol)	0.25 - 0.50	0.25	-	0.50
	Amphotericin (μg/ml)	0.20 - 0.40	0.40	-	
	Fluconazole (μg/ml)	16 - 64	32	-	

[a] *C. albicans* susceptible (S) and resistant (R) isolates, the MIC_{S90} of fluconazole were 2 and 64 μg/ml, respectively. [b] *C. glabrata* susceptible and resistant isolates, the MIC_{S50} of fluconazole were 1 and 32 μg/ml, respectively.

have very similar MIC_{S50} of 0.5 and 0.25%, respectively, and an MIC range of 0.12 – 0.5% for both species. The least susceptible of the *Candida* species to melaleuca were the *C. glabrata*, with an MIC range of 0.25 – 0.50 %, an MIC_{50} of 0.25%, and an MIC_{90} of 0.50% (Table 1). As was the case with *C. albicans*, the MIC_{S50} of melaleuca for the strains of *C. glabrata* for which fluconazole MICs were ≥ 16 μg/ml or ≤ 8 μg/ml were similar at 0.25% and 0.12% for both groups, respectively (Table 2).

Unlike, the activity detected against the *Candida* species; the melaleuca oil had essentially no effect against any of the *Aspergillus* isolates we tested.

DISCUSSION

The results of this study confirm the excellent *in vitro* efficacy of the tea tree oil Melaleuca, against the more common *Candida* species. Melaleuca oil (tea tree oil) is an old over the counter remedy with that possesses potent in-vitro antifungal activity against a broad spectrum of *Candida* species.

Melaleuca demonstrates the lowest MICs and is the most active against *C. albicans*, *C. kefyr*, and *C. parapsilosis*, with similar MIC_{S50} and narrow MIC ranges. Melaleuca also has similar activity against *C. lusitaniae*, *C. guilliermondii and C. tropicalis*. On the other hand, melaleuca demonstrates less activity against *C. glabrata* although still within the efficacy range, and not much higher than the MICs for the very susceptible strains of *Candida*. Moreover, the MIC and MFC results indicate that melaleuca is fungicidal for all of the *Candida* species evaluated, including those *Candida* species that were fluconazole resistant.

In addition to the broad anti-candidal activity of melaleuca oil, the most exciting observation was the remarkably good activity it demonstrated against the strains of C. *albicans* and *C. glabrata* for which fluconazole MICs were high. These putatively resistant strains were collected from patients with clinical failure to respond to high dose fluconazole (1.2- 1.5 g/day). Essentially the same melaleuca concentration was demonstrated for both the putatively fluconazole-susceptible and fluconazole-resistant strains of *C. albicans* and *C. glabrata*. Moreover, melaleuca also demonstrated good activity with low MICs against several *Candida* species for which the MICs of fluconazole were high.

Unfortunately, melaleuca oil demonstrated poor *in vitro* activity against the two filamentous fungi, *A. fumigatus* and *A. nidulans*.

In summary, the oil of melaleuca demonstrates great potential as a novel antifungal compound with potent in-vitro fungicidal activity against *C. albicans*, *C. glabrata, C. tropicalis* and *C. parapsilosis*, the four most commonly isolated species causing disseminated and mucocutaneous candidiasis in the United States [1,3]. Melaleuca compounds may be a valuable addition to the management of bacterial and fungal infections in the future [13,15,18,19]. In addition, because of its excellent *in vitro* activity against azole-resistant strains of *C. albicans*, *C. glabrata*, and *C. krusei* including the multi-azole resistant strains which have been recovered from AIDS patients, melaleuca should be particularly useful for the management of these clinically resistant candidal infections.

Recently, we published a small prospective study evaluating a melaleuca based oral solution in patients with AIDS and fluconazole-refractory oropharyngeal candidiasis [20]. At the 4-week evaluation, eight of the 13 patients enrolled showed a significant response. Additionally, seven out of 12 patients also demonstrated a significant mycological response rate with a decrease in the colony counts of *Candida* species recovered during follow-up.

In addition, comparison of our data with previously published data by Hammer *et al.* and Concha *et al.* demonstrate very similar *in vitro* susceptibility results [10,11]. Unfortunately, as previously stated by Hammer *et al.*, it is difficult to compare data from different investigators since the chemical composition of the oils may be different, as well as the methodology of the studies [11].

We feel that the results of the in-vitro assays and the small clinical study are extremely promising and that further large, comparative prospective clinical studies are warranted to determine the efficacy of the melaleuca compounds for multi-drug resistant thrush. Especially in the population of HIV-positive patients with advanced disease who suffer from repeated episodes of mucosal candidiasis.

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