Journal of Antimicrobial Chemotherapy (2002) **50**, 195–199
DOI: 10.1093/jac/dkf112

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In vitro activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi

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Received 4 November 2001; returned 4 February 2002; revised 1 April 2002; accepted 29 April 2002

The *in vitro* activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes (*n* = 106) and filamentous fungi (*n* = 78) was determined. Tea tree oil MICs for all fungi ranged from 0.004% to 0.25% and minimum fungicidal concentrations (MFCs) ranged from <0.03% to 8.0%. Time–kill experiments with 1–4 × MFC demonstrated that three of the four test organisms were still detected after 8 h of treatment, but not after 24 h. Comparison of the susceptibility to tea tree oil of germinated and non-germinated *Aspergillus niger* conidia showed germinated conidia to be more susceptible than non-germinated conidia. These data demonstrate that tea tree oil has both inhibitory and fungicidal activity.

Introduction

The essential oil of *Melaleuca alternifolia*, also called tea tree oil or melaleuca oil, is known in Australia, and increasingly overseas, as a natural topical antiseptic. Tea tree oil contains *c*. 100 components, which are largely monoterpenes, sesquiterpenes and related alcohols.[1] Anecdotally, tea tree oil is known as an excellent treatment for fungal infections, in particular vaginal candidiasis and dermatophytoses, and a recent publication suggests that it may be useful for treating oral candidiasis.[2] This has prompted several *in vitro* investigations into the anti-candidal properties of the oil.[3–5] In contrast, there have been few comprehensive *in vitro* studies of the effects of tea tree oil on filamentous fungi, including dematophytes. The aim of this study was to investigate the effects of tea tree oil on dermatophytes and other filamentous fungi by the use of *in vitro* susceptibility assays, time–kill methods and assays comparing the susceptibility of germinated and non-germinated conidia to tea tree oil.

Materials and methods

Antifungal agents

Tea tree oil (batch 971) was kindly supplied by Australian Plantations Pty Ltd (Wyrallah, NSW, Australia) and complied with ISO 4730 as described previously.[6] Griseofulvin (Sigma) was supplied as a powder and stock solutions were prepared in dimethylsulphoxide.

Fungal isolates

Recent clinical isolates (*n* = 184) were obtained from the Mycology Section of the Western Australian Centre for Pathology and Medical Research (Table 1). Isolates were maintained on potato dextrose agar (PDA) slopes stored at room temperature.

Preparation of fungal inoculum

Inocula were prepared by growing isolates on PDA slopes as described by the NCCLS[7] with the following exceptions: all dermatophytes, *Cladosporium* spp. and *Alternaria* spp. were incubated for 7 days at 30°C.[8] Slopes were flooded with 0.85% saline (dermatophytes) or phosphate-buffered saline (PBS) containing 0.05% Tween 80 (filamentous fungi).[9] Fungal growth was gently probed and the resulting suspension was removed and mixed thoroughly with the use of a vortex mixer. After the settling of the larger particles, suspensions were adjusted by nephelometry and diluted as necessary to correspond to final inoculum concentrations of *c*. 2.5×10^3–2.5×10^4 cfu/mL for dermatophytes[8] or 0.4×10^4–5.0×10^4 cfu/mL for the remaining filamentous fungi.[7]

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Broth microdilution method

Broth microdilution testing was based on reference method M38-P recommended by the NCCLS.[7] A series of doubling dilutions of tea tree oil ranging from 8% to 0.004% was prepared in a 96-well microdilution tray, with a final concentration of 0.001% (v/v) Tween 80 to enhance tea tree oil solubility. After the addition of inocula (prepared as described above), trays were incubated for 96 h at 30°C for dermatophytes,[8] 48 h at 35°C for *Aspergillus* spp. and *Fusarium* spp., and 48 and 72 h at 30°C for *Alternaria* spp. and *Cladosporium* spp., respectively. MICs were determined visually with the aid of a reading mirror, according to NCCLS guidelines.[7] Minimum fungicidal concentrations (MFCs) of tea tree oil were determined by subculturing 10 µL from wells not visibly turbid and spot inoculating on to Sabouraud dextrose agar (SDA) plates. MFCs were determined as the lowest concentration resulting in no growth on subculture. Isolates were tested on at least two separate occasions, and were retested if resultant MIC or MFC values differed. Modal values were then selected.

Time–kill curves

Time–kill studies were carried out against one isolate each of *Trichophyton rubrum*, *Trichophyton mentagrophytes* var. *interdigitale*, *Aspergillus niger* and *Aspergillus fumigatus*. Fungal inocula were prepared as described above except that dermatophyte inocula were suspended and diluted in PBS, and *Aspergillus* spp. were suspended and diluted in PBS with 0.02% (v/v) Tween 80. Starting inocula concentrations were *c*. 10^6 cfu/mL for dermatophytes and *A. fumigatus*, and *c*. 10^5 cfu/mL for *A. niger*. After preliminary experiments, tea tree oil concentrations were chosen that corresponded to 4 × MFC for dermatophytes and 1 × MFC for *Aspergillus* spp. Tea tree oil treatments were prepared in 1 mL volumes at twice the desired final concentrations in PBS, with final concentrations of 0.001% Tween 80 for dermatophytes and 0.02% Tween 80 for *Aspergillus* spp. Controls contained PBS with the relevant concentration of Tween 80. Test solutions and controls were inoculated with 1 mL volumes of inoculum and a 100 µL sample was taken immediately from the controls for viability counts. Test solutions were incubated at 35°C with shaking. Further samples were taken at 2, 4, 6, 8 and 24 h for viable counting. Viable counts were carried out by serially diluting samples 10-fold in sterile distilled water (SDW) and plating these dilutions on to SDA. Limits of detection were calculated based on a minimum of 30 cfu from the 10^{-1} dilution, and were 7.5×10^3 cfu/mL for dermatophytes and 3×10^3 for aspergilli. Assays were carried out two to six times. Colony count data for each experiment were converted into values relative to the colony count at time zero to normalize data and correct for slight variations in starting inocula concentrations between

Table 1. *In vitro* susceptibilities of fungi to tea tree oil and griseofulvin determined by the broth microdilution method

		Tea tree oil (% v/v)				Griseofulvin (mg/L)	
		MIC		MFC		MIC	
Fungus	No. of isolates	range	90%	range	90%	range	90%
Dermatophytes							
Epidermophyton floccosum	15	0.008–0.03	0.03	0.12–0.25	0.25	0.25–1	1
Microsporum canis	16	0.004–0.03	0.03	0.06–0.25	0.25	0.25–1	1
Microsporum gypseum	6	0.016–0.03		0.25–0.5		0.5–2	
T. interdigitale[a]	21	0.008–0.03	0.03	0.25–1	1	0.25–1	0.5
T. mentagrophytes[b]	14	0.008–0.06	0.03	0.25–1	0.5	0.5–2	1
T. rubrum	19	0.008–0.03	0.03	<0.03–0.25	0.25	0.25–2	1
Trichophyton tonsurans	15	0.004–0.016	0.016	0.12–0.5	0.5	0.5–1	1
Filamentous fungi							
Alternaria spp.	10	0.016–0.12	0.12	0.06–2	2		
Aspergillus flavus	12	0.06–0.12	0.12	2–4	4		
A. fumigatus	12	0.06–0.12	0.12	1–2	2		
A. niger	14	0.06–0.12	0.12	2–8	8		
Cladosporium spp.	10	0.008–0.12	0.12	0.12–4	2		
Fusarium spp.	10	0.008–0.25	0.12	0.25–2	2		
Penicillium spp.	10	0.03–0.06	0.06	0.5–2	2		

[a]*T. mentagrophytes* var. *interdigitale*.
[b]*T. mentagrophytes* var. *mentagrophytes*.

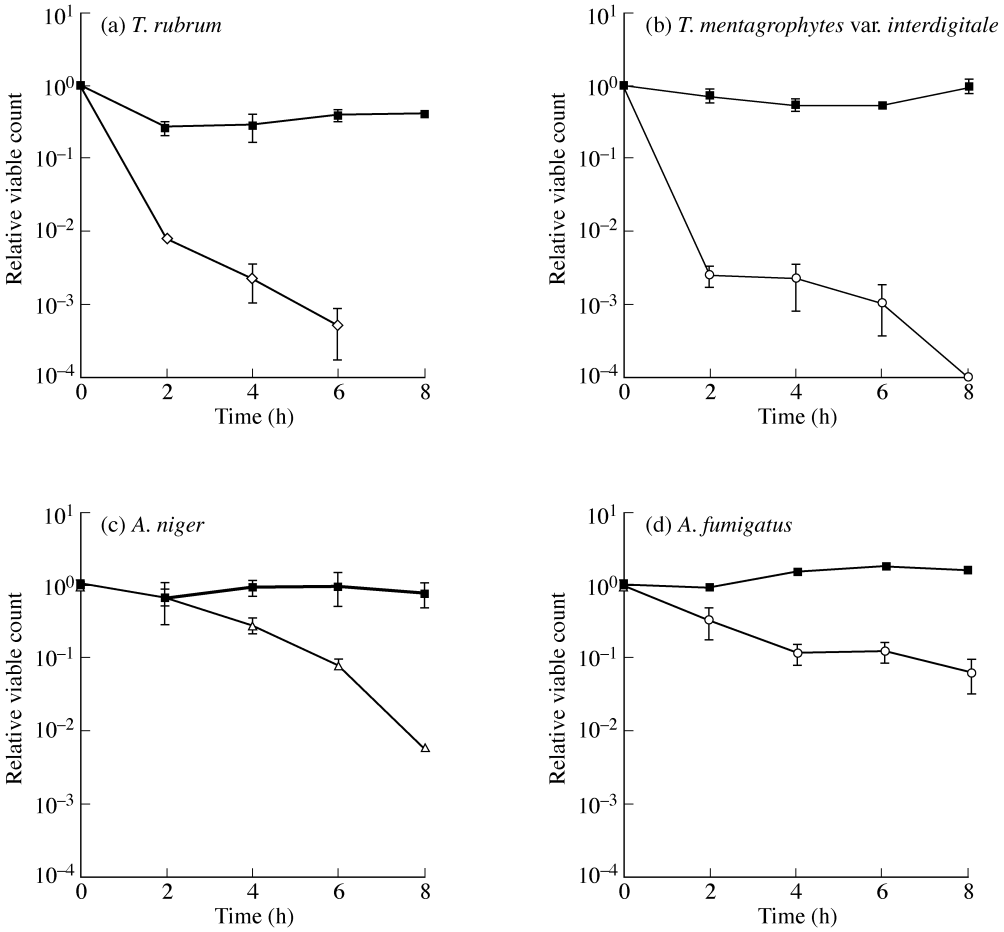

Figure 1. Time–kill curves for one isolate each of *T. rubrum* (a), *T. mentagrophytes* var. *interdigitale* (b), *A. niger* (c) and *A. fumigatus* (d). Relative viable count values were determined by dividing all viable count values by the cfu/mL count for the control at time zero. Mean ± S.E.M. plotted against time. Symbols: filled squares, control (a–d); diamonds, 0.5% tea tree oil (a); circles, 2% tea tree oil (b and d); triangles, 4% tea tree oil (c).

experiments. Mean and standard error values for each isolate at each time point were calculated and plotted against time on a log scale.

Antifungal activity against non-germinated and germinated conidia

The assay comparing the activity of tea tree oil against non-germinated and germinated conidia was carried out according to the method of De Lucca *et al.*,[10] with the following modifications. Tea tree oil test solutions ranging from 0.25% to 0.03% (final concentrations) were prepared in potato dextrose broth with Tween 80 at a final concentration of 0.001%. Colony counts were estimated from the control vials (0% tea tree oil) by spreading either 50 µL (non-germinated) or 100 µL (germinated) sample volumes on to each of four PDA plates. Colony counts from the test vials were determined by adding 0.45 mL of SDW to the test vial and spread plating either 100 µL (non-germinated) or 200 µL (germinated) volumes on to each of four PDA plates. The dilution step was used to counter the antimicrobial effects of the tea tree oil on the fungi. Assays were carried out two to four times per isolate per tea tree oil concentration. Data are expressed as proportions of the time zero non-germinated conidia viable count result. Data were compared by Student's two-tailed *t*-test assuming unequal variance.

Results

Broth microdilution assay

Tea tree oil MICs for all fungi ranged from 0.004% to 0.25%, and MFCs ranged from <0.03% to 8.0% (Table 1). Generally, MIC_{90} and MFC_{90} values were lower for dermatophytes compared with the other filamentous fungi.

Time–kill curves

Both of the dermatophytes showed a >1 $\log_{10}$ difference in viable count between treatment and control within the first hour, whereas the *Aspergillus* spp. did not (Figure 1). The viable counts for *Aspergillus* spp. did not differ from controls

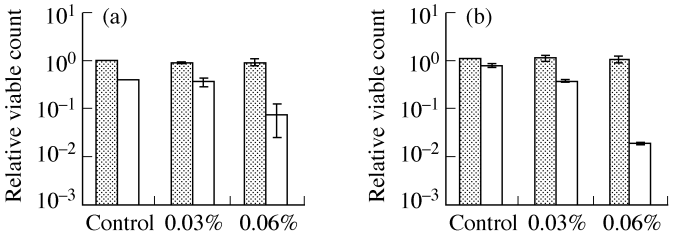

Figure 2. Susceptibilities of non-germinated (grey bars) and germinated (white bars) conidia of two clinical *A. niger* isolates (a and b) to several concentrations of tea tree oil. Relative viable count values were derived by dividing all viable count values by the cfu/mL count for the non-germinated conidia control at time zero. Mean ± S.E.M. plotted against tea tree oil concentration.

by >1 $\log_{10}$ until between 6 and 8 h of incubation with tea tree oil. By 24 h, none of the four test organisms was detected in the tea tree oil treatments.

Susceptibility of non-germinated and germinated conidia to tea tree oil

Germinated conidia treated with 0.03% tea tree oil differed significantly from control germinated conidia for isolate b only (P = 0.009) (Figure 2). However, germinated conidia treated with 0.06% tea tree oil differed significantly from control germinated conidia for both isolate a (P = 0.019) and isolate b (P = 0.005). No germinated conidia were recovered from tea tree oil treatments of 0.12% or 0.25%. The concentration of 0.12% corresponds to the MIC of tea tree oil for both organisms determined by the broth microdilution assay (Table 1). Non-germinated conidia treated with tea tree oil at 0.25% or less did not differ significantly from control non-germinated conidia ($P > 0.05$). Significantly less germinated control conidia were recovered than non-germinated control conidia ($P = 3.3 \times 10^{-5}$).

Discussion

Using several different methods, this study has illustrated that tea tree oil has fungicidal activity. Few previous studies have comprehensively investigated the activity of tea tree oil against dermatophytes and filamentous fungi, and limited data suggest MICs in the range of 0.1–1.0% for dermatophytes[4,5,11] and 0.02–>2% for the other filamentous fungi.[4,11–13] The MICs published previously have largely been obtained using the agar dilution method, and the differences between the agar dilution and broth microdilution assay may explain the difference between these results and those of the present study. Only one previous study has used NCCLS methods,[13] and although NCCLS methods are widely accepted for determining *in vitro* susceptibilities of micro-organisms to antimicrobial agents, how well essential oils, or indeed fungi, fit within these protocols is questionable.

It was important to establish whether tea tree oil was fungicidal as well as inhibitory, as an indication of the potential usefulness of the oil as an antifungal treatment. Most isolates showed a difference of several concentrations between inhibitory and cidal values, indicating that although tea tree oil does have fungicidal activity, at particular concentrations it is fungistatic only (Table 1). Despite the relatively low MICs seen in the broth microdilution assays, concentrations in excess of MFC amounts were required to produce fungicidal effects in the time–kill assays. This may be due, in part, to differences between the ways these assays measure antifungal activity. In the broth microdilution method, inocula are predominantly conidia and, during incubation of the assay (and where conditions allow), these conidia will germinate and grow into hyphae.[14] In the time–kill assay again the inocula are conidia; however, in contrast, this assay assesses the ability of the conidia to be penetrated and killed by tea tree oil. Since the time–kill assays showed that fungi are not killed rapidly, even at concentrations several times the MFC, the time of exposure may play a significant role in the fungicidal action of tea tree oil.

Several authors have recently investigated potential differences in the susceptibility of conidia, germinated conidia and hyphae to antifungal compounds. In general, the assays with comparatively long incubation times such as 24[14] or 48 h[15] showed no significant differences in susceptibility, whereas the studies using shorter incubation times have shown differences.[10] This suggests that the time of exposure is also a critical parameter for the outcome of these assays. In addition, the test agents assessed had different mechanisms of antifungal action, and the results of these assays may simply reflect these differences. Data from the present study suggest that the conidia of *A. niger* are comparatively less susceptible to tea tree oil than either germinated conidia or yeast cells.[3] As reported by Cheng & Levin,[16] the thickness and density of the conidial wall may be responsible for the reduced susceptibility of conidia to antifungal agents. However, since the thickness of the conidial wall is no greater than that of hyphae[15] or a yeast cell, the reduced susceptibility is more likely to be due to the composition and density of the conidial wall.

Based on both its inhibitory and fungicidal action, tea tree oil may be a useful agent for treating dermatophyte infections. However, exactly how this *in vitro* activity translates into *in vivo* effectiveness is unclear. In a trial investigating tea tree oil for treating onychomycosis, patients were treated twice daily with neat oil.[17] After 6 months of treatment, 18% of patients were culture negative, with a total of 60% of patients having full or partial resolution. In a second onychomycosis trial, 5% tea tree oil cream was applied three times a day and after 8 weeks of treatment the overall cure rate was 0%.[18] In a tinea pedis therapeutic trial patients were treated with a 10% tea tree oil product twice daily for 4 weeks.[19] This produced a mycological cure rate of 30% and clinical improvement in

65% of patients. Given that onychomycosis rarely responds to topical therapy and is therefore usually treated systemically,[20] it is perhaps not surprising that the topical application of tea tree oil was of limited effectiveness in these two clinical trials. This emphasizes the need for more clinical trial data, particularly in relation to tinea pedis, which can often be treated successfully topically.[20]

Although tea tree oil is still, to a large extent, grouped together with many other therapies as 'alternative' medicine, *in vitro* and *in vivo* studies are increasingly showing that some of the anecdotal claims made about the oil have a scientific basis. In particular, data from the present study have begun to illustrate the ways in which tea tree oil inhibits and kills fungi, which may ultimately be useful in developing tea tree oil therapies and in the search for novel antifungal agents.

Acknowledgements

The assistance of Dr A. J. De Lucca (assays with germinated conidia) is gratefully acknowledged. The Division of Microbiology and Infectious Diseases at The Western Australian Centre for Pathology and Medical Research, Nedlands and the Department of Microbiology at The University of Western Australia, provided isolates. This work was supported by grants from the Rural Industries Research and Development Corporation, and Australian Bodycare Pty, Ltd, Vissenbjerg, Denmark.

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