Journal of Antimicrobial Chemotherapy (2004) **53**, 1081–1085
DOI: 10.1093/jac/dkh243
Advance Access publication 12 May 2004

JAC

Antifungal effects of *Melaleuca alternifolia* (tea tree) oil and its components on *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*

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Received 23 December 2003; returned 7 February 2004; revised 5 March 2004; accepted 22 March 2004

***Objectives*: The aim of this study was to investigate the mechanism of action of tea tree oil and its components against *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*.**

***Methods*: Yeast cells were treated with tea tree oil or components, at one or more concentrations, for up to 6 h. During this time, alterations in permeability were assessed by measuring the leakage of 260 nm absorbing materials and by the uptake of Methylene Blue dye. Membrane fluidity was measured by 1,6-diphenyl-1,3,5-hexatriene fluorescence. The effects of tea tree oil on glucose-induced medium acidification were quantified by measuring the pH of cell suspensions in the presence of both tea tree oil and glucose.**

***Results*: The treatment of *C. albicans* with tea tree oil and components at concentrations of between 0.25 and 1.0% (v/v) altered both permeability and membrane fluidity. Membrane fluidity was also increased when *C. albicans* was cultured for 24 h with 0.016%–0.06% (v/v) tea tree oil, as compared with control cells. For all three organisms, glucose-induced acidification of the external medium was inhibited in a dose-dependent manner in the presence of 0.2%, 0.3% and 0.4% tea tree oil.**

***Conclusions*: Data from this study support the hypothesis that tea tree oil and components exert their antifungal actions by altering membrane properties and compromising membrane-associated functions.**

Keywords: essential oils, terpenes, membranes, permeability, fluidity

Introduction

Tea tree oil has been used medicinally in Australia for more than 80 years, with uses relating primarily to its antimicrobial[1,2] and anti-inflammatory[3] properties. The oil is obtained by steam distillation from the Australian native plant *Melaleuca alternifolia*, and contains ~100 components, which are mostly monoterpenes, sesquiterpenes and related alcohols. Compositional ranges for 14 of the major components are stipulated in the International Standard (ISO 4730) and as such, oils compliant with the standard vary little in chemical composition.

Tea tree oil shows promise as a topical antifungal agent, with recent clinical data indicating efficacy in the treatment of dandruff[4] and oral candidiasis.[5] Data from an animal model also indicate that it may be effective in the treatment of vaginal candidiasis.[1] These clinical uses are supported by a wealth of *in vitro* susceptibility data.[1,6] Further *in vitro* work has shown that tea tree oil and components cause the leakage of intracellular compounds[2] and inhibit respiration in bacteria.[7] Extensive studies specifically investigating the mechanisms of action of tea tree oil and components against *C. albicans* or other yeasts have not been conducted.

Materials and methods

Strains, growth media and culture conditions

Candida albicans ATCC 10231, *Candida glabrata* ATCC 15545 and *Saccharomyces cerevisiae* NCTC 10716 were obtained from the culture collection of the Microbiology Discipline at The University of Western Australia. Unless stated otherwise, all broth cultures were incubated with shaking at 35°C for *Candida* spp. and at 30°C for *S. cerevisiae*. Where necessary, the concentrations of viable cells in suspensions were confirmed by viable counts. Minimum inhibitory and fungicidal concentrations of tea tree oil and components were determined previously for these strains.[6]

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Tea tree oil and components

Tea tree oil (batch 971) was kindly donated by Australian Plantations Pty Ltd, Wyrallah, NSW, Australia, and complied with the International Standard ISO 4730, as described previously.[8] The following seven components, listed with their percentage level in tea tree oil batch 971, were also investigated; (+)-terpinen-4-ol (41.5%) (Fluka Chemie AG, Buchs, Switzerland), γ-terpinene (21.2%) (Aldrich Chemical Company Inc., Milwaukee, WI, USA), α-terpinene (10.2%) (Sigma Chemical Co., St Louis, MO, USA), terpinolene (3.5%) (Fluka), α-terpineol (2.9%) (Aldrich), 1,8-cineole (2.1%) (Sigma) and ρ-cymene (1.5%) (Aldrich). All components were of ≥97% purity, except for terpinolene and α-terpinene which were ~90% pure. Dilutions of oil/components were prepared as % v/v solutions in the relevant buffer or medium.

Leakage of 260 nm absorbing material

Cells of *C. albicans* or *C. glabrata* were grown in Sabouraud dextrose broth for 18 h, collected, washed three times and resuspended to ~10^9 cfu/mL in PBS, pH 7.4. Treatments containing tea tree oil/components were prepared in PBS with final concentrations of 0.001% Tween 80 (PBSTw). Preliminary studies showed that results for terpinolene, α-terpinene and γ-terpinene were not reproducible with only 0.001% Tween 80, and a final concentration of 0.1% Tween 80 was therefore used for these treatments. Controls were prepared with both Tween 80 concentrations. After inoculation, treatments were mixed for ~10 s, and samples were taken after a total of 20 s. Treatments and controls were incubated at 35°C with shaking and were sampled again at 1, 2, 4 and 6 h. Samples were diluted 1 in 10 in PBSTw and filtered with a 0.45 μm filter. The absorbance of filtrates (in quadruplicate) was read against the appropriate blank (prepared as described above but without *C. albicans*) at 260 nm and averages were determined. Additional assays with tea tree oil were performed in succinate buffer (pH 6.0), in the presence of either 50 mM Ca^{2+} or Mg^{2+}, to assess whether the presence of these cations affected leakage.

Methylene Blue dye exclusion assay

C. albicans was grown in Sabouraud dextrose broth for 18 h, collected, washed twice and resuspended in sterile distilled water (SDW) to ~10^7 cfu/mL. Treatments containing tea tree oil/component were prepared in PBSTw, inoculated at 1 min intervals and mixed for 20 s before time zero samples were taken. Treatments were incubated at 35°C with shaking and additional samples were taken at 30 min, 1, 2, 3, 4 and 6 h. Each sample of 80 μL was added to 20 μL of 0.05% Methylene Blue (w/v, prepared in SDW), mixed well and left for 5 min at room temperature. Cells were examined microscopically using a final magnification of ×400. A minimum of 100 cells in consecutive visual fields was examined and the percentage staining of cells calculated.

Measurement of medium acidification

Cells of *S. cerevisiae*, *C. albicans* and *C. glabrata* were prepared as described previously[9] but were resuspended in cold SDW with 0.001% Tween 80. Tea tree oil was added to cell suspensions to result in final concentrations of 0.1, 0.2, 0.3 and 0.4 (% v/v). Controls contained no tea tree oil. The mixtures were pre-incubated for 5 min at 30°C, then at timed intervals a glucose solution was added to a final concentration of 2%. Treatments were mixed for ~20 s and time zero pH readings were taken within 30 s of the addition of glucose. Mixtures were incubated at room temperature and the pH was determined at 0, 5, 10, 20, 30, 40, 50 and 60 min. Since the addition of tea tree oil alone caused a slight decrease in pH, the net pH decrease for each treatment was determined by subtracting the pH measurements taken at time zero from the readings taken at, and after, 5 min.

Effects of pre-treatment with CCCP, DES or calcium on susceptibility to tea tree oil

Cells of *C. albicans* were prepared as described for the Methylene Blue assay but were resuspended and adjusted to ~10^7 cfu/mL in either PBS [for assays with carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) and diethylstilbestrol (DES)] or succinate buffer (SB) (for assays with Ca^{2+}). Cells were pre-treated by incubating at 35°C with shaking for 2 h with final concentrations of 200 μM CCCP, 100 μM and 125 μM DES (all prepared in methanol w/v), or 100 mM Ca^{2+}.[10] PBS control cells were pre-treated with vehicle only (4% v/v methanol). Cells were then post-treated with tea tree oil for 1 h, incubated at 35°C with shaking. Concentrations of each pre-treatment agent (CCCP, DES or Ca^{2+}) were maintained at pre-treatment concentrations in the post-treatment phase. Viable counts were then performed using Sabouraud dextrose agar spread and pour plates.

Relative fluorescence intensity of cells grown in the presence of tea tree oil

C. albicans was grown in 1% yeast extract, 2% peptone and 2% glucose (YEPG) broth for 18 h and then diluted 1 in 100 into fresh YEPG containing 0, 0.016, 0.03 and 0.06% tea tree oil. After 24 h of incubation, cells were collected, washed twice and resuspended in PBS to an OD_{540} of between 0.40 and 0.42, corresponding to ~3 × 10^6 cfu/mL. To label cells, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Sigma) was added at a final concentration of 2 μM[11] and cells were incubated for 30 min at 35°C in the dark. Fluorescence intensity was determined with 350 nm as the excitation wavelength and 430 nm as the emission wavelength, using unlabelled control cells as blanks. The relative fluorescence intensity of treated cells was determined by dividing the fluorescent intensity measurements for treated cells by that of control cells.

Immediate changes in fluorescence intensity after treatment with tea tree oil or components

C. albicans cells were prepared by inoculating 1–2 colonies into YEPG and incubating for 24 h. Cells were collected, washed twice and resuspended in PBSTw to ~10^8 cfu/mL. Tea tree oil or component was added to each cell suspension at a final concentration of 0.25% (v/v), mixed thoroughly and incubated at 35°C with shaking. Samples were taken at 0 (control cells only), 10 and 30 min. Cells were collected by centrifugation, washed twice in PBS with 0.5% Tween 80 and washed twice in PBS without Tween. Cells were finally resuspended in PBS to an OD_{540} of between 0.40 and 0.42, labelled and fluorescence intensity was determined as described above.

Statistical analyses

All assays were repeated at least twice. Data were compared using Student's two-tailed *t*-test, assuming unequal variance. *P* values of <0.05 were considered significant.

Results

Permeability assays

Treatments resulting in significant increases in OD_{260} or Methylene Blue staining are shown in Table 1. In addition, results for 1.0% 1,8-cineole were very similar to those shown for 0.5% 1,8-cineole, by both assays. Treatments not resulting in significant permeability changes by either assay were 0.12% tea tree oil, terpinen-4-ol and α-terpineol, 0.25% 1,8-cineole, and 1.0% γ-terpinene and α-terpinene. Also, no significant increases in OD_{260} were seen after treatment with

Table 1. Effects of tea tree oil and components (% v/v) on *C. albicans* ATCC 10231, measured by loss of 260 nm absorbing materials, Methylene Blue staining, medium acidification and changes in DPH fluorescence

	Time (min)			
Treatment (% v/v)	0	30	60	360
Filtrate absorbance at 260 nm[a]				
control	0.01 ± 0.01		0.02 ± 0.01	0.04 ± 0.01
tea tree oil (0.25)	0.00 ± 0.01		0.03 ± 0.02	**0.17 ± 0.09**
tea tree oil (0.5)	0.00 ± 0.01		0.02 ± 0.01	**0.36 ± 0.04**
tea tree oil (1.0)	–0.01 ± 0.00		0.00 ± 0.01	**0.51 ± 0.06**
terpinen-4-ol (0.5)	0.01 ± 0.00		**0.46 ± 0.07**	**0.72 ± 0.14**
1,8-cineole (0.5)	0.01 ± 0.01		**0.12 ± 0.02**	**0.41 ± 0.06**
terpinolene (0.5)	0.00 ± 0.01		0.03 ± 0.01	0.27 ± 0.04
Methylene Blue staining[b]				
control	4.6 ± 1.3	5.0 ± 1.4	7.3 ± 2.1	5.8 ± 4.9
tea tree oil (0.25)	2.1 ± 2.5	5.7 ± 5.3	11.1 ± 1.6	**94.5 ± 5.9**
tea tree oil (0.5)	1.6 ± 1.6	21.2 ± 5.0	41.2 ± 9.3	**100**
tea tree oil (1.0)	1.6 ± 1.6	**63.2 ± 11.3**	**84.0 ± 11.3**	**100**
terpinen-4-ol (0.25)	5.6 ± 2.7	**100**	**100**	**100**
1,8-cineole (0.5)	7.1 ± 2.1	32.1 ± 12.4	**58.1 ± 12.4**	**99.3 ± 0.0**
α-terpineol (0.25)	8.2 ± 1.0	**82.4 ± 14.8**	**99.2 ± 0.8**	**100**
Medium acidification[c]				
control	0	–1.58 ± 0.21	–1.63 ± 0.21	
tea tree oil (0.1)	0	–1.63 ± 0.26	–1.51 ± 0.28	
tea tree oil (0.2)	0	**–1.14 ± 0.18**	**–0.95 ± 0.10**	
tea tree oil (0.3)	0	**–0.90 ± 0.21**	**–0.76 ± 0.20**	
tea tree oil (0.4)	0	**–0.53 ± 0.08**	**–0.35 ± 0.21**	
Fluorescence intensity[d]				
control	1	1.02 ± 0.08		
tea tree oil (0.25)	1	**2.44 ± 0.44**		
terpinen-4-ol (0.25)	1	**1.61 ± 0.02**		
1,8-cineole (0.25)	1	**3.75 ± 1.12**		
γ-terpinene (0.25)	1	1.66 ± 0.56		
α-terpinene (0.25)	1	**1.29 ± 0.09**		
α-terpineol (0.25)	1	**0.67 ± 0.10**		
terpinolene (0.25)	1	**2.08 ± 0.15**		

Values in bold differ significantly from controls.
[a]Mean optical density (±S.D.) of filtrates at 260 nm.
[b]Mean percentage staining (±S.D.) of cells with Methylene Blue.
[c]Net decrease in pH (±S.D.)
[d]Fluorescence intensity relative to control (±S.D.)

0.25% α-terpineol, and treatment with 1% terpinolene or ρ-cymene did not result in increased Methylene Blue staining. The treatment of *C. glabrata* with 0.25%, 0.5% and 1.0% tea tree oil (data not shown) produced increases in OD_{260} similar to those shown for *C. albicans.* No significant increases in OD_{260} or Methylene Blue staining were seen for control cells during either assay. Furthermore, OD_{260} data for *C. albicans* control cells tested with 0.001% Tween 80 did not differ significantly from those tested with 0.1% Tween 80. The presence of 50 mM Ca^{2+} or Mg^{2+} did not significantly alter the loss of 260 nm absorbing materials from tea tree oil-treated cells.

Medium acidification

Acidification by *C. albicans* (Table 1) and *C. glabrata* was not altered in the presence of 0.1% tea tree oil, whereas acidification by *S. cerevisiae* was significantly inhibited after 40 min (data not shown). The presence of 0.2%, 0.3% and 0.4% tea tree oil each significantly inhibited acidification after 20 min for *C. albicans* and *S. cerevisiae*, and after 40, 20 and 10 min, respectively, for *C. glabrata.*

Pre-treatment and tea tree oil susceptibility

Pre-treatment with CCCP or DES significantly increased subsequent susceptibility to all concentrations of tea tree oil (Table 2). The pre-treatment of cells with 200 μM CCCP alone did not cause a significant decrease in cell viability (compared with vehicle-treated control cells) but the pre-treatment of cells with either 100 μM or 125 μM DES alone did. Cells pre-treated with Ca^{2+} did not have significantly altered susceptibility to tea tree oil.

Table 2. Mean viable counts (cfu/mL) of *C. albicans* ATCC 10231 pre-treated with CCCP, DES or calcium and post-treated with tea tree oil

	Post-treatment (tea tree oil % v/v)			
Pre-treatment	0	0.2	0.3	0.4
PBS control[a]	3.1×10^6	2.3×10^6	3.4×10^6	1.1×10^4
100 μM DES	1.2×10^6	$\mathbf{4.0\times10^5}$	–[b]	–
125 μM DES	5.6×10^5	$\mathbf{9.1\times10^4}$	–	–
200 μM CCCP	3.0×10^6	$\mathbf{3.8\times10^5}$	–	–
SB control	2.5×10^6	8.1×10^5	4.6×10^3	–
100 μM Ca^{2+}	2.4×10^6	7.0×10^5	9.3×10^4	–

Values in bold differ significantly from the relevant control.
[a]Pre-treated with vehicle only.
[b]<300 cfu/mL.

Membrane fluidity of cells grown in the presence of tea tree oil

The mean relative increases in fluorescence intensity for cells grown with 0.016, 0.03 and 0.06% tea tree oil were 1.16 (±0.05 S.E.M.), 1.68 (±0.06) and 2.16 (±0.18). Relative increases for cells grown with 0.03% and 0.06% tea tree oil differed significantly from control cells ($P = 0.002$ and 0.024, respectively).

Immediate changes in membrane fluidity

Significant increases in relative fluorescence intensity occurred in cells treated with 1,8-cineole for 10 min ($P = 0.032$), and in cells treated for 30 min with tea tree oil, terpinen-4-ol, 1,8-cineole, α-terpinene and terpinolene (Table 1). Treatment with γ-terpinene did not cause significant changes whereas treatment with α-terpineol resulted in a significant decrease ($P = 0.012$). Between 10 and 30 min, relative fluorescence intensity increased significantly for cells treated with tea tree oil, terpinen-4-ol, 1,8-cineole, α-terpinene and terpinolene only. No significant changes occurred in control cells over time.

Discussion

This study showed that tea tree oil and/or components increased yeast cell permeability and membrane fluidity, and inhibited medium acidification. Terpenes are thought to induce alterations in cell permeability by inserting between the fatty acyl chains that make up the membrane lipid bilayers,[12] disrupting lipid packing and causing changes to membrane properties and functions.[12] This theory is strongly supported by data from this and previous studies demonstrating changes in permeability and increases in membrane fluidity after treatment with terpenes.[13,14] The components of tea tree oil were also shown to induce changes in fluidity to varying degrees, which may correspond to the position of each terpene within the membrane lipid bilayer. This position is thought to depend on the hydrophobicity of the compound; however, no obvious correlation between changes in membrane fluidity and the water solubility or octanol-water partition coefficient of each compound was evident. Membrane fluidity was also increased in cells grown for 24 h with sub-inhibitory tea tree oil. Changes such as these are usually due to alterations in membrane lipid composition[12] and are thought to be a compensatory mechanism to counter the lipid disordering effects of the treatment agent. However, another compensatory or stress mechanism, the accumulation of intracellular trehalose, was not shown by *C. albicans* or *S. cerevisiae* in response to tea tree oil (data not shown). Further research into the adaptive and stress responses of yeasts to tea tree oil is clearly required.

The pre-treatment of cells with both CCCP and DES resulted in increased susceptibility to tea tree oil, suggesting that the cell functions inhibited by these two compounds are critical in preventing the damage caused by tea tree oil. In particular, the plasma membrane ATPase, which is inhibited by DES, may protect cells by maintaining cell homeostasis and by countering the permeabilizing effects of tea tree oil. On the other hand, tea tree oil appeared to impair the functioning of the plasma membrane ATPase, as suggested by the inhibition of medium acidification. Although enzyme functioning may have been impaired by direct effects, indirect effects appear to be more likely, based on previous studies. Tea tree oil and terpenes have been shown to inhibit respiration in *Candida*, suggesting adverse effects on mitochondria.[7,14] Also, the sesquiterpene dialdehyde polygodial has been shown to inhibit the yeast mitochondrial ATPase, affecting medium acidification indirectly by reducing or eliminating the large amounts of cellular ATP required to fuel the plasma membrane ATPase.[9]

In conclusion, tea tree oil and components appear to affect membrane properties and integrity in a manner consistent with other lipophilic, membrane-active agents such as the terpenes thymol[15] and geraniol.[13] However, several inconsistent observations were also found in the present work. For example, 1,8-cineole and terpinolene both caused large changes in membrane fluidity, but did not greatly increase Methylene Blue permeability. Conversely, 0.25% terpinen-4-ol caused a dramatic increase in Methylene Blue permeability but only a modest increase in membrane fluidity. These discrepancies are not yet fully understood, but they suggest that the different components of tea tree oil vary in their modes of action against yeasts and that tea tree oil has several mechanisms of antifungal action. Further work is required to explain these differences.

Acknowledgments

The assistance of the Microbiology Discipline of The University of Western Australia in obtaining isolates is appreciated. This work was supported by grants UWA-57A and 58A from the Rural Industries Research and Development Corporation, Australia, and Australian Bodycare Pty Ltd, Vissenbjerg, Denmark.

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