Melaleuca alternifolia (tea tree) oil inhibits germ tube formation by *Candida albicans*

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The effect of tea tree oil (TTO) on the formation of germ tubes by *Candida albicans* was examined. Two isolates were tested for germ tube formation (GTF) in the presence of TTO concentrations (% v/v) ranging from 0·25% ($\frac{1}{2}$ minimum inhibitory concentration [MIC]) to 0·004% (1/128 MIC). GTF at 4 h in the presence of 0·004 and 0·008% (both isolates) and 0·016% (one isolate) TTO did not differ significantly ($P > 0·05$) from controls. At all other concentrations at 4 h, GTF differed significantly from controls ($P < 0·01$). A further eight isolates were tested for GTF in the presence of 0·031% TTO, and at 4 h the mean GTF for all 10 isolates ranged 10·0–68·5%. Two isolates were examined for their ability to form germ tubes after 1 h of pre-exposure to several concentrations of TTO, prior to induction of germ tubes in horse serum. Cells pre-exposed to 0·125 and 0·25% TTO formed significantly fewer germ tubes than control cells at 1 h ($P < 0·05$), but only those cells pre-exposed to 0·25% differed significantly from control cells at later time points ($P < 0·01$). GTF by *C. albicans* is affected by the presence of, or pre-exposure to, sub-inhibitory concentrations of TTO. This may have therapeutic implications.

Keywords   antifungal, *Candida*, *Melaleuca*, tea tree

Introduction

The essential oil of *Melaleuca alternifolia* (tea tree oil) contains more than 100 components, largely monoterpenes, sesquiterpenes and related alcohols [1]. The oil is reported to have a range of medicinal properties, including antimicrobial, anti-inflammatory and analgesic effects, and has been used as a topical agent in the treatment of a variety of minor ailments [2,3]. In addition to recent reports on the *in vitro* antibacterial and antifungal properties of tea tree oil (TTO) [4–8], the oil has also been clinically evaluated for the treatment of several superficial fungal infections, including refractory oral candidiasis, onychomycosis and tinea [9–12].

Candida albicans is the most important yeast pathogen of humans [13]. *C. albicans* has several morphological forms; yeast cells (blastoconidia) which divide by budding, germ tubes, true hyphae and pseudohyphae [14,15]. The formation of germ tubes from blastoconidia is the first stage in the development of true hyphae [14] and this ability to morphologically transform has been suggested as a potential virulence factor [16,17]. Compared to the blastoconidial form, *C. albicans* hyphae have an increased ability to adhere to and penetrate human epithelial cells [14]. In addition, hyphae (commonly combined with blastoconidia and/or pseudohyphae) are almost always seen in smears from cases of vaginal and oral candidiasis, and human tissue infected with *C. albicans* [14,18]. Although the relationship between morphological transformation and virulence is still equivocal [17,19], these observations suggest that germ tubes and hyphae play a role in the pathogenesis of these conditions.

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Previous studies of the effects of TTO on *C. albicans* have demonstrated that the oil inhibits and kills *C. albicans* [4,6]; however, no studies have examined the effects of TTO on the morphological transition from blastoconidium to hypha. Given that TTO may be of use in the treatment of superficial *Candida* infections, the aim of this study was to investigate the effects of TTO on the formation of germ tubes by *C. albicans*.

Methods

Melaleuca alternifolia *(tea tree) oil*

TTO (batch 971) was kindly supplied by Australian Plantations Pty. Ltd. (Wyrallah, NSW, Australia). The oil complied with the International Standard ISO 4730 as described previously [8,20].

Organisms and culture conditions

The reference isolate *C. albicans* ATCC 10231 was obtained from the Department of Microbiology at The University of Western Australia. Nine further isolates were recent clinical isolates recovered from vaginal swabs submitted to the Western Australian Centre for Pathology and Medical Research. Isolates were identified as described previously [6], maintained on Sabouraud glucose agar (SGA) and stored at 4 °C. Isolates were selected based on the ability of approximately 100% of blastoconidia to form germ tubes after 4 h incubation at 37 °C in horse serum (HS).

Determination of minimum inhibitory and fungicidal concentrations

The susceptibility of *C. albicans* to TTO was determined using a modification of the National Committee for Clinical Laboratory Standards (NCCLS) method for broth dilution antifungal susceptibility testing of yeasts [21]. A series of doubling dilutions of TTO was prepared in 100 µl volumes in a 96-well microtitre tray, in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) with L-glutamine, without sodium bicarbonate, buffered with 0·165 M MOPS (pH 7·0) with a final concentration of 0·001% Tween 80 included to facilitate oil solubility. Inocula were prepared by growing each isolate overnight at 35 °C on SGA and then suspending colonies in 0·85% saline. This suspension was then adjusted to match the turbidity of a 1·0 McFarland standard using a nephelometer, corresponding to approximately 5×10^6 cfu ml^{-1}, as determined previously by viable counts. The standardized suspension was diluted as necessary to obtain a final inoculum concentration of $0·5–2·5 \times$ 10^3 cfu ml^{-1}, which was confirmed by viable counts. Microtitre trays were incubated at 35 °C for 48 h, then 10 µl subcultures were taken from each well and spot inoculated onto SGA. Minimum inhibitory and fungicidal concentrations (MIC and MFC, respectively) were determined as described previously [6]. Isolates were tested on at least two separate occasions and were re-tested if resultant MIC or MFC values differed. Modal MIC and MFC values were then selected.

Preparation of cells for the induction of germ tube assays

Isolates were subcultured onto SGA and incubated overnight at 35 °C. One colony was then used to inoculate approximately 6 ml of Sabouraud glucose broth (SGB). Broths were incubated for 16–24 h at 35 °C with shaking. Cells were collected by centrifugation for 3 min at 1300 *g*, washed twice and then resuspended in phosphate buffered saline (PBS) pH 7·4. Cell suspensions were adjusted to match the turbidity of a 1·0 McFarland standard. For the assay where germ tubes were induced in the presence of TTO, suspensions were diluted 1 in 10 in HS.

Induction of germ tubes in the presence of tea tree oil

A range of TTO concentrations were prepared in HS in 0·5 ml volumes in glass Bijou bottles at twice the desired final concentration as follows (%TTO v/v): 0·5, 0·25, 0·125, 0·062, 0·031, 0·016, 0·008, 0·004 and 0. Equal volumes of the prepared cell suspensions were added to each TTO treatment and mixed thoroughly. A sample was removed immediately from each control (0% TTO) for viable counts and microscopy. A sample was also removed from the 0·25% TTO treatment for a viable count at time zero. All treatments were then incubated at 37 °C, without shaking, and were sampled at 1, 2, 3 and 4 h. Prior to each sampling, bottles were mixed thoroughly. For some experiments, additional aliquots were removed from the 0·125 and 0·25% TTO treatments at each time point for viable counts. Viable counts were performed by diluting the sample 10-fold in PBS and spot inoculating 10 µl aliquots onto SGA. After overnight incubation at 35 °C, colonies were counted and the viable count determined. Two isolates (ATCC 10231 and 88E) were tested against the entire range of TTO concentrations sampled at each time point. These experiments were repeated between two and four times per isolate. Based on these results, a further eight isolates were tested with the 0·031% TTO treatment only and sampled at 4 h only. These experiments were repeated four times per isolate.

Germ tube induction after exposure to subinhibitory concentrations of TTO

Overnight broth cultures of *C. albicans* ATCC 10231 and isolate 88E were prepared as above except that after washing, cells were resuspended in RPMI 1640. A series of TTO dilutions in RPMI 1640 with 0·002% Tween 80 was prepared in 2 ml volumes at twice the desired final concentration (% v/v) as follows: 0·5, 0·25, 0·125, 0·031 and 0. Equal volumes of the cell suspensions were then added to the TTO treatments. These were incubated for 1 h at 35 °C with shaking. Cells were collected by centrifugation for 3 min at 1300 g, and washed twice with PBS with 0·5% Tween 80. Cells were finally resuspended in PBS without Tween 80. Each cell suspension was adjusted to match the turbidity of a 2·0 McFarland standard, corresponding to approximately 8×10^6 cfu ml^{-1}. An aliquot of 50 µl of each adjusted cell suspension was added to 0·95 ml HS, mixed thoroughly and incubated at 37 °C, without shaking. A time zero sample was taken from each 0% TTO treatment for microscopy. Additional samples were removed for microscopy at 1, 2, 3 and 4 h, and at 5 and 6 h for selected treatments. For some experiments, samples were also removed from the 0·125% and 0·25% treatments at each time point for viable counts. These experiments were repeated two to four times per isolate.

Microscopy

Cells were prepared for microscopy by adding each 50 µl sample to an equal volume of 1% glutaraldehyde in PBS (pH 7·4). After mixing well with the pipette tip, a portion was removed immediately and spread onto a glass slide. Slides were air-dried, fixed with methanol and stained with Loeffler's methylene blue, as described elsewhere [22]. Cells were examined using bright field microscopy, under oil immersion (final magnification × 1000). Fifty sequential cells from each slide were examined [23] and scored morphologically according to the following definitions. A germ tube was defined as a cell bearing a rounded outgrowth with a length greater than or equal to the diameter of the parent cell, not constricted at the base [24,25]. The presence of septa was noted. A protuberance was defined as a cell bearing a rounded outgrowth less than or equal to the parent cell diameter, not constricted at the base [26]. A bud was defined as being as large as the parent cell and/or fully delimited by a septum [25]. Cells bearing pseudohyphae (a marked constriction at the site of emergence) were disregarded [15]. The percentage of cells bearing each morphology type was then calculated.

Statistical analysis

Statistical analyses were performed using the computer program GraphPad Prism 2.01 from GraphPad Software Inc. (GraphPad Prism Software Inc., San Diego, CA, USA). Arithmetic means, standard deviations and standard errors were determined. Germ tube formation (GTF) in the presence of TTO, and after pre-exposure to TTO, was compared to controls using analysis of variance (ANOVA). Differences between the treatment groups were compared using Dunnett's multiple comparison test. GTF in the 10 isolates exposed to 0·031% TTO was compared to controls using a Student's t-test (two-tailed, two sample assuming unequal variance). P values $< 0·05$ were considered significant. Viable counts were converted to log values and then divided by their relevant time zero count value. These values were compared to time zero using ANOVA and Dunnett's multiple comparison test. P values $< 0·05$ were considered significant.

Results

MIC and MFC data are shown in Table 1. Inhibitory and fungicidal concentrations for each isolate were either identical or differed by one concentration only.

Mean percentage GTF in the presence of TTO for *C. albicans* ATCC 10231 and 88E is shown in Figures 1 and 2, respectively. Data for 0·004 and 0·008% were very similar; therefore, data for 0·004% are not shown. The highest concentration of TTO used in these assays (0·25%) represents $\frac{1}{2}$ MIC for both organisms. No GTF was seen in the presence of 0·125 and 0·25% TTO (both isolates). For all other concentrations of TTO at all other time points, mean percentage GTF was less than the controls, except isolate 88E at 1 h where this was greater than the control in the presence of 0·004, 0·008 and 0·016%. However, these differences were not statistically significant ($P > 0·05$).

The morphology of cells after 4 h in the presence of TTO in HS is shown in Table 2. For both isolates, cells exposed to 0·25% TTO showed no change in the proportions of cells of each morphology type, compared to time zero. Cells exposed to 0·062 and 0·125% TTO showed an increase in cells bearing multiple buds and single buds, and a decrease in single cells, compared to time zero controls. Cells exposed to 0·031% showed mean percentage GTFs of 44·0 and 51·3%, for strains ATCC 10231 and 88E, respectively, and the next largest proportion of cells was those showing a multibudded morphology. Analysis of viable count results from cells exposed to 0·25% TTO showed no difference in numbers of viable cells at any time point, for both isolates.

Table 1 *In vitro* susceptibility data (% TTO v/v) and mean ($\pm$ SE) percentage GTF at 4 h in the presence of 0·031% TTO for 10 *C. albicans* isolates

| Isolate | MIC | MFC | Mean percentage GTF | | *P*-value* |
			0% TTO	0·031% TTO	
10231	0·25	0·25	91·0 $\pm$ 5·3	10·0 $\pm$ 5·6	0·00004
204	0·25	0·25	98·0 $\pm$ 1·2	19·5 $\pm$ 10·8	0·0051
245	0·25	0·25	98·5 $\pm$ 0·5	23·0 $\pm$ 12·2	0·0084
39M	0·5	0·5	98·5 $\pm$ 1·5	26·0 $\pm$ 6·7	0·0012
202	0·25	0·5	100 $\pm$ 0	29·5 $\pm$ 10·4	0·0065
45S	0·25	0·5	99·5 $\pm$ 0·5	30·0 $\pm$ 7·4	0·0024
10231	0·5	0·5	99·5 $\pm$ 0·5	42·5 $\pm$ 7·2	0·0041
27U	0·25	0·5	98·5 $\pm$ 0·5	57·5 $\pm$ 17·6	0·1025
88E	0·5	1·0	99·5 $\pm$ 0·5	57·5 $\pm$ 9·3	0·0200
64T	0·5	1·0	96·0 $\pm$ 1·6	68·5 $\pm$ 12·3	0·1106

*, Student's two-tailed *t*-test.

The mean percentage GTF for all 10 isolates tested in the presence of 0·031% TTO is shown in Table 1. Mean percentage GTF in controls ranged 91–100% and, in the presence of 0·031% TTO, mean percentage GTF ranged 10·0–68·5%. Comparison of treatment groups with controls showed that treatments differed significantly from controls for eight of the 10 isolates ($P < 0.05$). Correlation of MICs and MFCs with mean percentage GTF in the presence of 0·031% TTO gave correlation coefficients of 0·548 and 0·858, respectively.

The results of the induction of germ tubes after 1 h of pre-exposure to subinhibitory concentrations of TTO are shown in Figures 3 and 4. Data for 0 and 0·016% were very similar; therefore, data for 0·016% are not shown. For both isolates, mean GTF in cells pre-exposed to 0·125 and 0·25% TTO differed significantly from controls at 1 h ($P < 0.05$); however, at all later time points only cells exposed to 0·25% differed significantly from controls

($P < 0.01$). Mean percentage GTF in control cells was always greater than in cells pre-exposed to TTO, except for *C. albicans* ATCC 10231 at 1 h, where mean percentage GTF was greater in cells that had been exposed to 0·016% TTO than in control cells. This difference was not statistically significant ($P > 0.05$). Analysis of viable count results for *C. albicans* ATCC 10231 pre-exposed to 0·25% TTO showed no difference in viability, as determined by ANOVA; however, Dunnett's multiple comparison test showed that at 5 and 6 h, viable counts were significantly lower than time zero values ($P < 0.05$).

Discussion

This study showed that the formation of germ tubes by *C. albicans* was affected by the presence of, or pre-exposure to, TTO. In the presence of 0·25% TTO, no germ tubes were observed, and the proportions of cells bearing
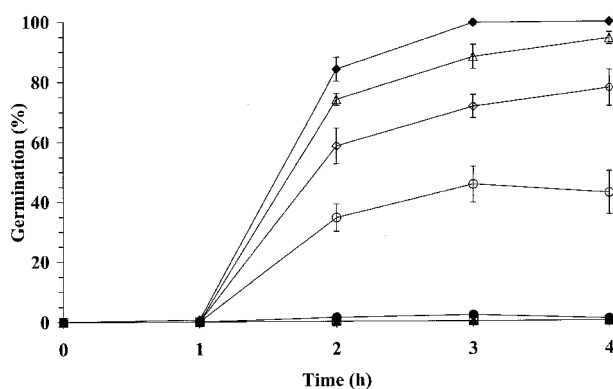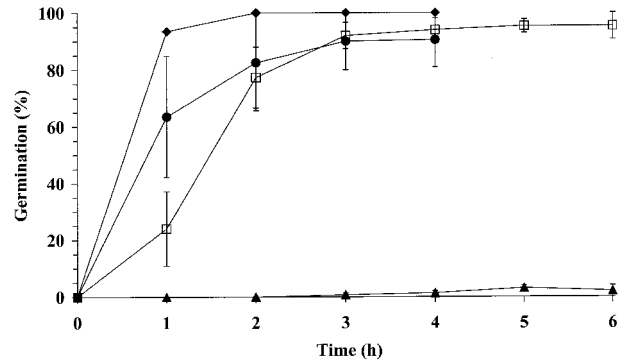

Fig. 1 Mean percentage GTF by *C. albicans* ATCC 10231 in the presence of TTO (% v/v). Bars represent standard errors. ◆, 0%; △, 0·008%; ◇, 0·016%; ○, 0·031%; ●, 0·062%; □, 0·125%; ▲, 0·25%.
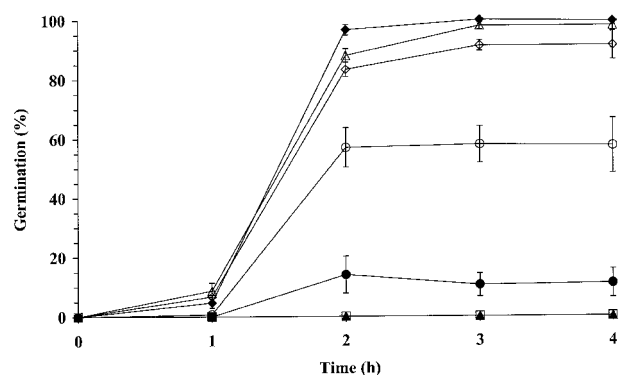
Fig. 2 Mean percentage GTF by *C. albicans* 88E in the presence of TTO (% v/v). Bars represent standard errors. ◆, 0%; △, 0·008%; ◇, 0·016%; ○, 0·031%; ●, 0·062%; □, 0·125%; ▲, 0·25%.
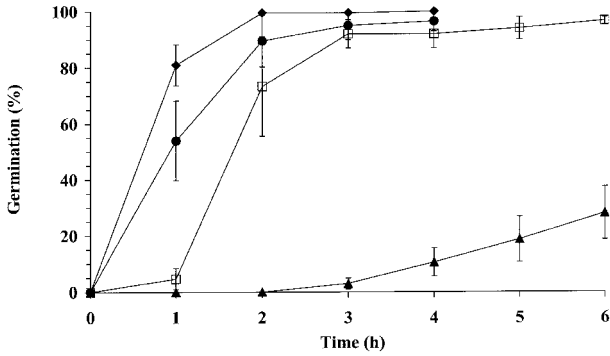
Fig. 3 Mean percentage GTF by *C. albicans* ATCC 10231 after 1 h pre-exposure to TTO (% v/v). Bars represent standard errors. ◆, 0%; ●, 0·062%; □, 0·125%; ▲, 0·25%.

Fig. 4 Mean percentage GTF by *C. albicans* 88E after 1 h pre-exposure to TTO (% v/v). Bars represent standard errors. ◆, 0%; ●, 0·062%; □, 0·125%; ▲, 0·25%.

each morphology did not change from 0 to 4 h, suggesting that no growth was occurring. Results of viable counts showed that viability was not affected, indicating that cells were neither multiplying nor being killed. This suggests that the presence of TTO was causing a generalized inhibition of growth. In the presence of 0·125%

TTO, no germ tubes were seen (both isolates) and there was a general trend of blastoconidia changing from single or singly budding morphologies to multiply budding morphologies over the 4 h test period. These changes in morphology suggest that these cells were actively growing, although still unable to transform to germ tubes.

Table 2 Morphology (mean percentage ± SE) of *C. albicans* 10231 and 88E at 0 and 4 h in the presence of TTO (% v/v)

		C. albicans 10231		*C. albicans* 88E	
TTO (%)	Morphology*	0 h	4 h	0 h	4 h
0	SC	68·0 ± 5·1	0 ± 0	88·0 ± 5·5	0 ± 0
	SB	24·0 ± 5·3	1·0 ± 0·6	15·0 ± 5·5	0·5 ± 0·5
	MB	9·3 ± 1·8	0 ± 0	0 ± 0	0 ± 0
	GT	0 ± 0	99·5 ± 0·5	0 ± 0	99·5 ± 0·5
0·016	SC		0·5 ± 0·5		0 ± 0
	SB		1·3 ± 0·7		0 ± 0
	MB		23·3 ± 7·0		8·7 ± 4·8
	GT		77·5 ± 6·0		91·3 ± 4·8
0·031	SC		1·5 ± 1·0		1·0 ± 1·0
	SB		8·7 ± 5·7		1·5 ± 0·5
	MB		54·0 ± 4·6		40·0 ± 8·0
	GT		42·5 ± 7·2		57·5 ± 9·3
0·062	SC		1·3 ± 1·3		1·0 ± 0·6
	SB		53·0 ± 33·0		12·0 ± 2·6
	MB		44·0 ± 30·0		76·0 ± 3·6
	GT		0·7 ± 0·7		11·0 ± 4·8
0·125	SC		4·0 ± 3·0		17·3 ± 4·0
	SB		32·0 ± 1·1		42·0 ± 5·0
	MB		3·3 ± 1·8		40·7 ± 5·0
	GT		0 ± 0		0 ± 0
0·25	SC		64·7 ± 2·9		80·0 ± 2·3
	SB		32·0 ± 1·1		17·3 ± 1·3
	MB		3·3 ± 1·8		2·7 ± 1·3
	GT		0 ± 0		0 ± 0

SC, single cells; SB, single bud; MB, multiple bud; GT, germ tube. Protuberances were not seen at 0 or 4 h.
Data for 0·004 and 0·008% not shown.

This implies that there is specific inhibition of morphogenesis occurring, rather than a total inhibition of growth. A possible explanation for the predominance of budded forms may be that the presence of TTO represents an 'environmental stress' and that under these conditions growth by budding is favoured [14]. Most publications describing inhibition of GTF do not mention the morphologies of those cells not bearing germ tubes, therefore comparisons are limited. However, Odds *et al.* [27] note that in the presence of azoles, development of hyphae was severely restricted and that growth occurred in the form of clumped budding yeast cells.

Inhibition of GTF was shown to be reversible, based on results of the pre-exposure experiments. Compared to their corresponding controls, cells pre-exposed to TTO appeared to have a concentration dependent 'lag phase' in germination, as seen by the differences in GTF at 1 h (Figs 3 and 4). However, after 2 h, GTF was approaching that of control cells, except for those cells pre-exposed to 0·25% TTO. Viable counts from cells pre-exposed to 0·25% TTO indicated that there was a decrease in viability over the course of the experiment. The lag phase in germination and the profound inhibition of germination after pre-exposure to 0·25% TTO suggest that there are restorative or repair mechanisms at work in the yeast cells before germination can take place. Ellepola & Samaranayake [15] found a similar effect for some antifungal agents when they treated cells for 1 h and then induced germ tubes for 1 h in serum. They theorized that treatment with antifungal agents induced a post antifungal effect whereby the growth of the test organisms was suppressed after limited exposure to, and subsequent removal of, an antifungal agent.

The MIC/MFC results, and the results of GTF for 10 isolates in the presence of 0·031% TTO, showed a degree of variability between isolates. Variation between isolates has been seen by other authors in germ tube inhibition studies [15,28]. The correlation observed between MFC and degree of GTF suggests that the results obtained by these two quite different assays are not completely unrelated, and that the results of one assay may give an indication of the likely result in the other assay. Other studies have also found that *in vitro* MIC/MFC data correlated with inhibition of GTF in the presence of antifungal agents [29]. However, Ellepola & Samaranayake [15] showed no relationship.

Effects on membranes are considered to be a primary mode of action of essential oils on microbial cells [30–32]. It has been suggested that where membrane integrity is adversely affected, membrane-associated functions may also be compromised [33]. For example, alteration of cytoplasmic membrane permeability by essential oils has been demonstrated by the leakage of intracellular potassium ions and 260 nm absorbing material [30–32,34]. Membrane associated enzymes that may be important in GTF include enzymes involved in cell wall synthesis (chitin, mannan and 1,3-β-D-glucan synthases) and adenosine triphosphatase (ATPase) [14,35]. It follows that in yeast cells, other membranes and their functions, such as mitochondrial membranes and respiration, may also be affected. Inhibition of respiration in yeast cells by essential oils and terpenic oil components has been demonstrated [30,36,37]. Boonchird & Flegel [38] found that the terpenes eugenol and vanillin both inhibited GTF by *C. albicans*. They speculated that if eugenol inhibited respiration and energy production, as has been shown previously, then given the relationship between respiration and GTF, the lack of energy production prevented the morphological transition from blastoconidia to hypha [38]. However, inhibition of respiration may cause a non-specific inhibition of growth, rather than the specific inhibition of GTF [14].

The ability of TTO, and TTO products, to inhibit and kill *C. albicans in vitro* has been demonstrated previously [4,6]. The present study demonstrates that TTO affects the ability of blastoconidia to germinate. In terms of the pathogenesis of *Candida* infections, this is another step in the infection process where TTO has an effect. Given the putative increased virulence of hyphae compared to blastoconidia, the inhibition of hyphal development is an advantageous characteristic for a therapeutic agent. TTO is limited to use as a topical antimicrobial agent, and is therefore only relevant for the treatment of superficial candidal diseases.

A report published recently described the treatment of acquired immune deficiency syndrome (AIDS) patients with fluconazole-refractory oral candidiasis with a melaleuca oral solution. This study found that after 28 days of therapy with a melaleuca oral solution, 67% of patients showed a clinical response (either cured or improved) [11]. Although this appears to be the only recently published report describing the effectiveness of a TTO product against candidal disease, the results are encouraging. In addition, evidence suggests that the use of intravaginal TTO products provides at least symptomatic relief in vaginal candidiasis [39, C. F. Carson *et al.*, unpublished results]. The ability of TTO to prevent GTF may explain, in part, the reported symptomatic relief. Currently available intravaginal tea tree oil products contain between 3 and 10% tea tree oil [6], and work is required to determine the *in vivo* efficacy of these products as well as the potential for tea tree oil to irritate mucous membranes. The incidence of adverse reactions to tea tree oil on mucous membranes is unknown, how-

ever, no adverse reaction were seen in a study by Peña [39] in which 130 patients were treated with intravaginal tea tree oil preparations.

In conclusion, TTO reversibly inhibits GTF in *C. albicans*. Inhibition could be explained by a generalized inhibition of growth at the higher concentrations of oil. However, at lower concentrations, GTF was specifically inhibited while growth continued by budding. These findings may be due to effects on cellular membranes and associated functions, including the inhibition of respiration.

Acknowledgements

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