

Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil

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ABSTRACT

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Aims: To investigate the *in vitro* antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil.

Methods and Results: Activity was investigated by broth microdilution and macrodilution, and time kill methods. Components showing the most activity, with minimum inhibitory concentrations and minimum fungicidal concentrations of $\leq 0.25\%$, were terpinen-4-ol, α -terpineol, linalool, α -pinene and β -pinene, followed by 1,8-cineole. The remaining components showed slightly less activity and had values ranging from 0.5 to 2%, with the exception of β -myrcene which showed no detectable activity. Susceptibility data generated for several of the least water-soluble components were two or more dilutions lower by macrodilution, compared with microdilution.

Conclusions: All tea tree oil components, except β -myrcene, had antifungal activity. The lack of activity reported for some components by microdilution may be due to these components becoming absorbed into the polystyrene of the microtitre tray. This indicates that plastics are unsuitable as assay vessels for tests with these or similar components.

Significance and Impact of the Study: This study has identified that most components of tea tree oil have activity against a range of fungi. However, the measurement of antifungal activity may be significantly influenced by the test method.

Keywords: *Candida albicans*, 1,8-cineole, monoterpenes, tea tree oil, terpinen-4-ol.

INTRODUCTION

The essential oil of *Melaleuca alternifolia*, also known as tea tree oil, is commonly used in Australia as a topical therapeutic agent. The medicinal uses of tea tree oil relate primarily to the anti-inflammatory (Brand *et al.* 2002; Koh *et al.* 2002) and antimicrobial (Carson *et al.* 2002; Hammer *et al.* 2002) properties of the oil. Use as a topical antimicrobial agent is supported by a growing body of clinical data indicating that tea tree oil is effective in the treatment of infections or conditions such as herpes labialis (Carson *et al.* 2001), acne (Bassett *et al.* 1990), tinea (Tong *et al.* 1992;

Satchell *et al.* 2002a), onychomycosis (Buck *et al.* 1994), dandruff (Satchell *et al.* 2002b) and oral candidiasis (Vazquez and Zawawi 2002), and in the clearance of methicillin-resistant *Staphylococcus aureus* carriage (Caelli *et al.* 2000). In addition, several recent publications have characterized the *in vitro* activity and mechanisms of action of tea tree oil against bacteria (Cox *et al.* 2000; Mann *et al.* 2000; Carson *et al.* 2002) and, to a lesser extent, fungi (Hammer *et al.* 2000, 2002). However, little is known about the *in vitro* activity of tea tree oil components against fungi.

The components of tea tree oil, of which there are *ca* 100, are mostly monoterpenes, sesquiterpenes and their related alcohols (Brophy *et al.* 1989). The chemical composition of tea tree oil is well characterized and the International Standard ISO 4730 for oil of *Melaleuca*, terpinen-4-ol type (tea tree oil) contains a chromatographic profile that

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stipulates minimum and maximum percentage composition values for 14 components (International Organisation for Standardisation 1996). The three major components, terpinen-4-ol, γ -terpinene and α -terpinene, comprise *ca* 70% of whole oil while ρ -cymene, terpinolene, α -terpineol and α -pinene account for *ca* 15% of the oil (Brophy *et al.* 1989). Given the lack of data relating to the antifungal activity of the components of tea tree oil, the purpose of this study was to examine the *in vitro* susceptibility of some medically important fungi to tea tree oil components, using several different investigative tools.

MATERIALS AND METHODS

Test organisms

A total of 14 fungal isolates were obtained from the Discipline of Microbiology at The University of Western Australia and the Division of Microbiology and Infectious Diseases at The Western Australian Centre for Pathology and Medical Research. They included reference and clinical isolates and were *Candida albicans* ATCC 10231, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 90018, *Saccharomyces cerevisiae* ATCC 10716, *Trichosporon* sp., *Rhodotorula rubra*, *Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes* var. *interdigitale*, *T. mentagrophytes* var. *mentagrophytes*, *Aspergillus niger*, *A. flavus*, *A. fumigatus* and *Penicillium* sp. Yeast isolates were grown and maintained on Sabouraud dextrose agar (SDA) stored at 4°C, and filamentous fungi were grown and maintained on potato dextrose agar slopes stored at room temperature.

Tea tree oil and components

Tea tree oil (batch 971) was kindly supplied by Australian Plantations Pty Ltd (Wyrallah, NSW, Australia) and its composition complied with the International Standard ISO 4730 (International Organisation for Standardisation 1996). The following tea tree oil components, listed together with their percentage composition in batch 971, were investigated for antifungal activity: (+)-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); α -pinene (2.5%) (Aldrich); 1,8-cineole (2.1%) (Sigma); ρ -cymene (1.5%) (Aldrich); (+)-aromadendrene (1.0%) (Fluka); (+)-limonene (0.9%) (Sigma); β -myrcene (Sigma); (+)- β -pinene (Fluka); (\pm)-linalool (Sigma) and R-(–)-(α)-phellandrene (Fluka). The concentrations of β -myrcene, β -pinene, linalool and α -phellandrene in batch 971 of tea tree oil were not determined. All components

were of $\geq 97\%$ purity, except for terpinolene, β -myrcene and α -terpinene which were *ca* 90% pure.

Broth microdilution assay

Yeast inocula were prepared by growing isolates on SDA for 24–48 h at 35°C and then suspending growth in *ca* 2 ml of sterile distilled water (SDW). The density of this suspension was adjusted to 1 McFarland, and then serially diluted in SDW to correspond to a final inoculum concentration of $1.5\text{--}3.0 \times 10^3$ CFU ml⁻¹. Inocula for the remaining fungi were prepared as described previously (Hammer *et al.* 2002). Final inocula concentrations for all organisms were confirmed by viable counts.

Broth microdilution assays were performed according to National Committee for Clinical Laboratory Standards (NCCLS) methods (NCCLS 1997, 1998) with minor modifications. Briefly, doubling dilutions of components, with final concentrations ranging from 8 to 0.002% (v/v) were prepared in 96-well microtitre trays (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in RPMI Medium 1640 (Gibco BRL, Grand Island, NY, USA). Tween 80 (Sigma) was included at a final concentration of 0.001% (v/v) to enhance the solubility of each component or tea tree oil. The activity of terpinen-4-ol, terpinolene, 1,8-cineole, γ -terpinene, α -terpinene, ρ -cymene, α -terpineol and β -myrcene against *C. albicans* ATCC 10231 was also determined with a final concentration of 0.1% Tween 80 to ascertain whether an increased concentration of surfactant significantly influenced results. For yeasts, minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) were determined by subculturing 10 μ l from each well of the microtitre tray, spot inoculating onto SDA and incubating aerobically at 35°C. MICs were determined as the lowest concentration of agent resulting in the maintenance or reduction of the inoculum and MFCs were determined as the lowest concentration of agent resulting in no growth. For the remaining fungi, MICs were determined visually as described by the NCCLS (1998) and MFCs were determined by subculture as described previously (Hammer *et al.* 2002). All isolates were tested on at least two separate occasions and were re-tested if resultant MIC or MFC values differed. Modal values were then selected.

Broth macrodilution assay

The activity of tea tree oil and components against *C. albicans* ATCC 10231 was also determined by the broth macrodilution method. Doubling dilutions of tea tree oil or component, with final concentrations ranging from 2 to 0.016% (v/v) were prepared in RPMI Medium 1640 in 0.5 ml volumes in glass McCartney bottles, with a final

concentration of 0.1% (v/v) Tween 80. Initial work with a final concentration of 0.001% (v/v) Tween 80 showed that results for terpinolene and γ -terpinene were not reproducible whereas results with 0.1% Tween 80 were. Inocula were prepared as described for the microdilution assay and volumes of 0.5 ml were added to each dilution of oil or component. Dilutions were incubated at 35°C, either statically or with shaking. After 24 h incubation, MICs and MFCs were determined as described above. For α -terpineol, terpinen-4-ol, α -terpinene and ρ -cymene, MICs and MFCs were also determined after 48 h by incubating dilutions for a further 24 h.

Time kill assay

Inocula for the time kill assays were prepared by inoculating one to two colonies of *C. albicans* ATCC 10231 into ca 8 ml of Sabouraud dextrose broth (SDB) and incubating for 18 h at 35°C with shaking. Cells were then collected by centrifugation for 3 min at 1300 g, washed twice in SDW, and finally resuspended in phosphate-buffered saline (PBS) pH 7.4 to 6×10^6 – 1.3×10^7 CFU ml⁻¹. This was halved upon inoculation, resulting in a starting inoculum concentration of ca 5×10^6 CFU ml⁻¹. Treatments containing component or whole oil at one or more final concentrations ranging from 1 to 0.125% (v/v) were prepared in 1 ml volumes of PBS with a final concentration of 0.001% Tween 80, which was similar to controls. At 1-min intervals, 1 ml of inocula was added to each treatment and mixed for 20 s. Treatments were incubated with shaking at 35°C and samples were taken at 0.5 and 30 min, and at 1, 2, 3, 4 and 6 h. Viable counts were performed by serially diluting each sample 10-fold in SDW and spreading 100 μ l volumes from the appropriate dilutions onto SDA in duplicate. Alternatively, duplicate pour plates were prepared by aliquoting 1 ml of the appropriate dilution into the centre of an empty 90 mm plastic Petri dish and adding ca 19 ml of molten, cooled SDA. Petri dishes were swirled during and after the addition of agar to ensure even mixing. After incubation at 35°C, plates with 30–300 colonies were counted and viable counts determined. The limit of detection, calculated from 30 colonies in the 10⁻¹ dilution, was 3×10^3 CFU ml⁻¹ for spread plates or 3×10^2 CFU ml⁻¹ for pour plates. Assays were repeated at least twice and mean and standard error values were calculated from viable count data.

Statistical analyses

Viable count data from time kill assays were compared using a Student's *t*-test (two-tailed, two-sample assuming unequal variance). *P*-values of <0.05 were considered significant.

RESULTS

Broth microdilution assay

Data obtained by broth microdilution are shown in Table 1. In addition, MICs of α -pinene were 0.008% for *A. niger* and 0.016% for *A. flavus*, *A. fumigatus* and *Penicillium* sp. MFCs of α -pinene were 0.03% for *Penicillium* sp. and 0.016% for *A. niger*, *A. flavus* and *A. fumigatus*. For the dermatophytes, MICs of α -pinene were <0.004% and further testing of these organisms, and yeasts other than *C. albicans* ATCC 10231, was not pursued. Components with the lowest MICs and MFCs were terpinen-4-ol and α -terpineol, followed by 1,8-cineole. In contrast, α -terpinene, γ -terpinene and ρ -cymene showed little activity. Comparison of the different fungal groups showed that the dermatophytes were most susceptible to components, with the lowest MICs and MFCs for each component often occurring within this group. The group with the highest MICs was the yeasts and the highest MFCs, disregarding values of >8, were observed among the nondermatophyte filamentous fungi. MICs and MFCs obtained with the increased Tween concentration (0.1%) were either equivalent or one concentration lower for terpinen-4-ol, 1,8-cineole and tea tree oil, compared with values obtained with 0.001% Tween. For ρ -cymene, γ -terpinene and β -myrcene all values remained unchanged at >8% whereas values for α -terpinene were 8% with increased Tween. For terpinolene, values were considerably lower with 0.1% Tween 80, with an MIC of 1.0% and MFC of 2.0%, compared with values of >8% obtained with 0.001% Tween.

Broth macrodilution assay

By the macrodilution method, all components except β -myrcene showed activity at $\leq 2.0\%$ (Table 2). Comparison of values obtained at 24 and 48 h showed that MICs and MFCs did not change for terpinen-4-ol and α -terpineol (data not shown). However, MICs and MFCs for α -terpinene and ρ -cymene were one or more concentrations higher at 48 h compared with 24 h, for assays conducted standing and with shaking. Similarly, comparison of results obtained with standing or shaking did not differ for terpinen-4-ol, α -terpineol, terpinolene and tea tree oil at 24 h. However, for γ -terpinene and α -terpinene, MICs were 1.0% when obtained standing, compared with 0.5% obtained with shaking. Susceptibility data obtained by micro- and macrodilution methods were equivalent or differed by only one dilution for terpinen-4-ol, α -terpineol, α -pinene, aromadendrene, α -phellandrene and tea tree oil. In contrast, values were two or more concentrations lower by macrodilution for terpinolene, 1,8-cineole, γ -terpinene, α -terpinene, ρ -cymene, limonene and linalool.

Table 2 *In vitro* activity of tea tree oil and components (% v/v) against *Candida albicans* ATCC 10231 obtained by the broth microdilution and macrodilution methods

Tea tree oil/ component	Microdilution*		Macerdilution†	
	MIC	MFC	MIC	MFC
Tea tree oil	0.5	0.5	0.25	0.25
Terpinen-4-ol	0.25	0.25	0.12	0.25
γ -Terpinene	>8	>8	0.5	1
α -Terpinene	>8	>8	0.5	1
Terpinolene	>8	>8	0.5	0.5
α -Terpineol	0.12	0.25	0.12	0.25
α -Pinene	0.12	0.12	0.12	0.25
1,8-Cineole	4	8	0.5	0.5
ρ -Cymene	>8	>8	0.5	0.5
Aromadendrene	0.25	0.5	0.25	1
Limonene	>8	>8	0.5	1
β -Myrcene	>8	>8	>2	>2
β -Pinene	0.002	0.002	<0.016	<0.016
Linalool	1	1	0.06	0.12
α -Phellandrene	4	8	1	2

MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration.

*Final concentration of 0.001% Tween 80, data obtained at 48 h.

†Final concentration of 0.1% Tween 80, data obtained at 24 h.

Time kill assays

Results of time kill assays are shown in Fig. 1. Data for 1% γ -terpinene, α -terpinene and ρ -cymene were very similar and were indistinguishable by statistical analyses. The results for γ -terpinene only are shown as a representative (Fig. 1e). Treatments causing decreases in viability of >3 log CFU ml⁻¹ within 30 min were 0.25% terpinen-4-ol, 1% tea tree oil, and 0.5 and 1.0% 1,8-cineole. At 30 min, no viable organisms were recovered from either 1,8-cineole treatment. Treatments causing similar decreases in viability but over a longer time period were 0.25% α -terpineol, 1% α -terpinene, 1% γ -terpinene, 1% ρ -cymene, and 0.25 and 0.5% tea tree oil. Treatments having only moderate or negligible effects on *C. albicans* viability were 0.12% α -terpineol, terpinen-4-ol and tea tree oil, 0.25% 1,8-cineole and 1% terpinolene.

DISCUSSION

Quantification of the antimicrobial activity of particular essential oil components appears to be heavily influenced by the test method, as evidenced by the large differences between the MICs and MFCs obtained by the broth micro- and macrodilution methods. While technical factors such as

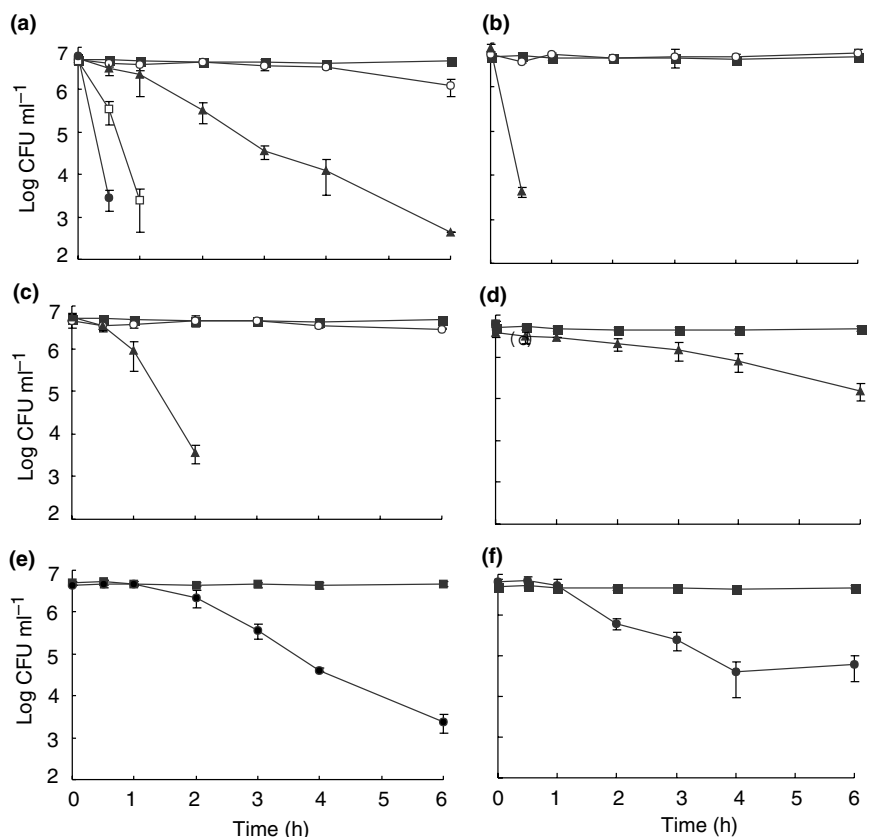


Fig. 1 Time kill curves of (a) tea tree oil, (b) terpinen-4-ol, (c) α -terpineol, (d) 1,8-cineole, (e) γ -terpinene and (f) terpinolene against *Candida albicans* ATCC 10231. Cells were treated with 0% (■), 0.12% (○), 0.25% (▲), 0.5% (□) or 1.0% (●) component or tea tree oil (v/v). Mean \pm S.E.M.

different assay volumes, extent of sealing and the concentration of Tween 80 may have contributed to the contradictory results, differences may also relate specifically to the physical, molecular and chemical characteristics of tea tree oil components. Most terpenes are of only limited solubility in aqueous media. For example, the solubilities of γ -terpinene, α -terpinene, ρ -cymene, terpinolene and limonene have been reported as being between 1.0 and 8.2 ppm (Griffin *et al.* 1999). A major consideration for antimicrobial activity assays is, therefore, how to achieve and maintain adequate solubilization of the compound and physical contact with the test organism. Surfactants have often been incorporated into these assays to address this issue (Janssen *et al.* 1987). In the current study, increasing the Tween 80 concentration from 0.001 to 0.1% in the microdilution assay did not result in significantly lower MICs or MFCs, with the exception of terpinolene. This suggests that the lack of activity observed for these components in the microdilution assay was not solely due to inadequate solubilization. The prospect that the tea tree oil components may be dissolving into or becoming otherwise irreversibly associated with the polystyrene of the microtitre tray is supported by data in the literature. Tea tree oil is known to interact with certain plastic types and has been shown to both deform and migrate through polymers such as low-density polyethylene (Rowe 1999), although these effects are poorly documented. The sorption of these less water-soluble components into polystyrene has also been used as a technique for removing them from solutions of tea tree oil (Brand *et al.* 2001). Removal of these compounds by their association with the polystyrene results in less of the component being in solution and available to interact with the test organism, and may explain why MICs and MFCs were considerably lower when performed in glass bottles. Interestingly, MICs and MFCs for terpinen-4-ol, α -terpineol and tea tree oil did not differ significantly between methods, suggesting that the use of microtitre trays, although not ideal, may still be acceptable for these components and oil. Component solubility must be considered when designing assays for evaluating the antimicrobial activity of essential oil components such as terpenes. Methods such as disc or well diffusion are particularly unsuitable given that the size of the zone of inhibition is dependent on the diffusion of mostly water-insoluble compounds through an aqueous agar medium (Janssen *et al.* 1987; Carson and Riley 1995).

Methodological considerations aside, most of the components tested showed antifungal activity, and by grouping those components similar in chemical composition and structure, generalizations about their antifungal activity can be made.

The monoterpene alcohols terpinen-4-ol, α -terpineol, 1,8-cineole and linalool had relatively good antifungal activity with MICs and MFCs for some components that

were slightly lower than those of tea tree oil. In addition, terpinen-4-ol, α -terpineol and 1,8-cineole showed relatively rapid killing effects against *C. albicans* in time kill assays. Previously published data for both fungi and bacteria are in agreement with these results (Moleyar and Narasimham 1986; Carson and Riley 1995; Griffin *et al.* 1999; Adegoke *et al.* 2000; Cox *et al.* 2001; Inouye *et al.* 2001). Although linalool differs slightly from the other monoterpene alcohols because it is acyclic, this component still showed significant antifungal activity. This suggests that the presence of the alcohol moiety is a greater determinant of antifungal activity than whether the component has a cyclic or acyclic structure. The antimicrobial activity of terpenes, including the monoterpene alcohols, has been attributed to their interactions with cellular membranes (Sikkema *et al.* 1995). At relatively low concentrations, these interactions may result in changes such as inhibition of respiration (Uribe *et al.* 1985) and alteration in permeability (Uribe *et al.* 1985; Cox *et al.* 2000) and at higher concentrations effects such as total loss of homeostasis, gross membrane damage and death may occur (Carson *et al.* 2002). The monoterpene alcohols are thought to be particularly antimicrobially active because of their relatively high water solubility and the presence of the alcohol moiety (Griffin *et al.* 1999; Dorman and Deans 2000).

The monocyclic terpenes γ -terpinene, α -terpinene, terpinolene, ρ -cymene, limonene and α -phellandrene showed MICs and MFCs by macrodilution that were one or two concentrations higher than those for tea tree oil. Similarly, time kill assays showed these components to have antifungal activity, although the rate of killing for γ -terpinene, α -terpinene, terpinolene and ρ -cymene could not be regarded as rapid. Relatively slow kill rates have been reported previously for these compounds (Cox *et al.* 2001). Earlier reports of antimicrobial activity for these components are confounded by methodological issues and a lack of meaningful data for the filamentous fungi. As a result, activity ranging from negligible (Carson and Riley 1995; Dorman and Deans 2000) through to moderate or good (Moleyar and Narasimham 1986; Himejima *et al.* 1992; Griffin *et al.* 1999; Adegoke *et al.* 2000; Cox *et al.* 2001) has been reported for these compounds. While not as active as the monoterpene alcohols, these compounds are likely to be active by similar mechanisms but activity may be limited by their low solubilities in both aqueous media and microbial membranes. Aromadendrene, the only sesquiterpene tested, showed activity similar to the monocyclic terpenes, although a previous report found little antimicrobial activity, albeit by well diffusion (Dorman and Deans 2000).

Myrcene, an acyclic monoterpene, showed no antifungal activity, which is consistent with the little amount of published data available for this component (Dorman and

Deans 2000; Jeon *et al.* 2001). The lack of activity observed for this acyclic monoterpene suggests that the cyclic structure of the cyclic monoterpenes may be contributing significantly to their activity. However, generalizations from this study are limited since only one nonalcohol acyclic terpene was tested.

The bridged bicyclic terpenes α - and β -pinene showed considerable antifungal activity, with β -pinene showing the most. In general, these data are in agreement with previous studies (Himejima *et al.* 1992; Adegoke *et al.* 2000), although some studies have demonstrated little activity for these components (Raman *et al.* 1995; Consentino *et al.* 1999; Mourey and Canillac 2002). The present study showed that β -pinene was more active against *C. albicans* than α -pinene, whereas previously, activities have been shown as equivalent for *C. utilis* (Himejima *et al.* 1992) or α -pinene was shown as the more active against *C. albicans* (Griffin *et al.* 1999). There is no clear consensus yet as to which pinene isomer is more antimicrobially active and the differing activities of the enantiomers of both compounds ought not to be discounted (Lis-Balchin *et al.* 1999). The solubility of terpenes is hypothesized as correlating with their antimicrobial activity (Sikkema *et al.* 1995). However, the pinenes are examples of compounds with very low water solubilities (Griffin *et al.* 1999) but relatively high antimicrobial activities. The reasons for this are so far unknown, but may relate to properties of these components other than solubility.

With the use of appropriate methods, this study has identified that most of the components of tea tree oil have activity against *C. albicans* and other fungi. This contradicts several previous reports (Raman *et al.* 1995; Consentino *et al.* 1999; Cox *et al.* 2001) and challenges beliefs about the various attributes and properties of tea tree oil components, such as that tea tree oil contains a single 'active' component terpinen-4-ol while many of the other components lack activity (Mann *et al.* 2000).

The importance of investigating the activity of tea tree oil components lies in gaining an understanding of the activity of each component, and of how each component contributes to the activity of the whole oil. Although some of the components tested in this study are present at only very low levels in whole oil, each may contribute to total activity and attempts to eliminate components considered inactive may, therefore, be counter-productive. In addition, several aspects of the properties of tea tree oil components such as synergistic action between two or more components or other beneficial pharmacological or medicinal properties have not yet been explored fully.

In conclusion, this study showed that α - and β -pinene and the monoterpene alcohols had the lowest MICs and MFCs against fungi, followed by the monocyclic terpenes. Data from this study suggests that the use of polystyrene

microtitre trays in microdilution assays may result in an underestimation of the antimicrobial activity of some essential oil components and should, therefore, be avoided. Appropriate methods for determining the susceptibility of microorganisms to essential oil components require further investigation, as does the activity of these components against bacteria.

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