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Antifungal activity
of tea tree oil

Activity against yeasts, dermatophytes and other filamentous fungi

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Foreword

The aim of this project was to investigate comprehensively the in vitro activity and mechanism of action of tea tree oil and components against fungi. Fungi are significant human pathogens, causing common superficial infections like tinea or athlete's foot and vaginal thrush.

This report covers studies on the in vitro activity of both tea tree oil and its components against yeasts, dermatophytes and other filamentous fungi. Activity was assessed by standard in vitro susceptibility and time-kill assays. This report also describes several different studies on the mechanism of action of tea tree oil against yeasts, in particular *C. albicans*.

This project was funded by Australian Bodycare Pty Ltd and matching funds provided by the Federal Government. This report, a new addition to RIRDC's diverse range of over 900 research publications, forms part of our Tea Tree Oil R&D Program, which aims to support the continued development of a profitable tea tree oil industry.

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Simon Hearn
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Abbreviations

CCCP	carbonylcyanide *m*-chlorophenyl hydrazone
cfu	colony forming units
DES	diethyl stilboestrol
DPH	1,6-diphenyl-1,3,5-hexatriene
g	force of gravity
GC	germinated conidia
GTF	germ tube formation
HS	horse serum
ISO	International Standards Organisation
M	molar
MB	methylene blue
MFC	minimum fungicidal concentration
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standards
NGC	non-germinated conidia
OD	optical density
PBS	phosphate buffered saline
PDA	potato dextrose agar
PDB	potato dextrose broth
PM	plasma membrane
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SDW	sterile distilled water
SEM	standard error of the mean
sp.	species
spp.	species (plural)
TTO	tea tree oil
U	unit
YEPG	yeast extract peptone glucose (broth)

Contents

Executive Summary

The first step in investigating the activity of an antimicrobial compound is often to determine which concentrations are inhibitory and which concentrations are lethal to a range of microorganisms. In the current project, inhibitory and lethal concentrations were determined for a range of fungi, including dermatophytes and other filamentous fungi. Dermatophytes are of significance because they cause superficial skin and nail infections in humans, and the remaining fungi are of importance as contaminants of air and air-conditioning systems. Dermatophytes (n = 106) belonging to the genera *Epidermophyton*, *Microsporum* and *Trichophyton* had minimum inhibitory concentrations (MICs) of tea tree oil ranging from 0.004 - 0.06% and minimum fungicidal concentrations (MFCs) ranging from <0.03 - 1% (v/v). Another agent used to treat dermatophyte infections, griseofulvin, was also tested and MICs ranged from 0.25 - 2 μg/ml, similar to previously published values. Ranges of tea tree oil MICs and MFCs for the filamentous fungi *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium* spp. (n = 78) were 0.008 - 0.25% and 0.06 - 8% (v/v), respectively. The least susceptible species was *Aspergillus niger*, with two isolates having MFCs of 8%.

The next part of this project was to investigate the activity of the different components of tea tree oil against six yeast isolates and eight species of dermatophytes and filamentous fungi. Eight tea tree oil components were chosen because they are present in the greatest proportions and make up the majority of the oil. The components terpinen-4-ol and α-terpineol had the greatest activity against yeasts, followed by 1,8 cineole. The components with the least anti-yeast activity were α-terpinene, γ-terpinene, terpinolene and ρ-cymene, which showed little inhibitory or fungicidal activity at the highest concentration of 8%. Terpinen-4-ol, α-terpineol and α-pinene had the most activity against dermatophytes and filamentous fungi, 1,8-cineole and terpinolene had moderate activity and α-terpinene, γ-terpinene and ρ-cymene showed little activity, as determined by the broth microdilution assay. However, comparison of susceptibility data obtained for *C. albicans* by both the broth macrodilution and microdilution methods showed that MICs and MFCs for some components, in particular those showing little activity by the microdilution assay, were considerably lower when determined by the macrodilution method. This disparity may be caused by differences between the assay methods such as the volumes used in each and the use of polystyrene or glass for each assay.

The remaining experiments conducted during this project were undertaken to provide a better understanding of how tea tree oil and components act against fungi.

Time kill experiments were conducted to determine the rate at which tea tree oil kills fungi, and at which concentrations. Time kill studies with *C. albicans* showed that organisms were rapidly killed

when treated with 0.5 and 1.0% tea tree oil whereas treatment with 0.25% produced a slower kill, and very little killing was evident with 0.12%. The components terpinen-4-ol, 1,8-cineole and terpinolene produced relatively rapid rates of kill at concentrations of approximately 0.5%, however, the components γ-terpinene, α-terpinene and ρ-cymene produced only moderate kill rates at 1%. Time kill experiments with tea tree oil at 4 × MFC for dermatophytes and 1 × MFC for filamentous fungi demonstrated a comparatively slow rate of kill, with three of the four test organisms still detected after 8 h treatment with tea tree oil. However, no organisms could be recovered after 24 h treatment.

These time-kill experiments with filamentous fungi showed that *Aspergillus* spp. were reasonably 'resistant' to killing at some concentrations. This prompted us to investigate what may be contributing to the 'resistance' of this fungus species. The conidia (sometimes referred to as 'spores') of *Aspergillus niger* were germinated in the laboratory and the susceptibility of these germinated conidia to tea tree oil was compared to that of non-germinated conidia. Germinated conidia were significantly more susceptible to tea tree oil than non-germinated conidia. These results indicate that the intact outer layers of the conidia may be responsible for the reduced susceptibility of these structures to tea tree oil, however, when there is a breach in this outer hull, organisms are rendered susceptible to the oil.

Previous studies of the mechanism of action of tea tree oil or similar compounds have indicated that tea tree oil and components alter the permeability of microorganisms, which is suggestive of the membrane being compromised or damaged. Therefore the ability of tea tree oil and components to alter the permeability of *C. albicans* was studied, using two methods. When cells of *C. albicans* were treated with 0.12% tea tree oil, negligible permeability changes occurred, whereas moderate changes occurred with 0.25% and rapid changes occurred at 0.5 and 1%. When the same studies were conducted with individual tea tree oil components rather than whole oil, terpinen-4-ol, α-terpineol and 1,8-cineole caused the largest permeability changes at the lowest concentrations. As a generalisation, each component caused permeability changes at, but not below, MIC amounts. Changes in permeability caused by treatment with amounts in excess of the MIC were more rapid and greater than those evident at MIC levels.

Since the permeability studies indicated that the cell membrane was adversely affected by tea tree oil and components, further experiments on membrane properties and functions were conducted. The measurement of membrane fluidity is a measurement of the rate of movement of the phospholipid molecules within the plasma membrane. This parameter is highly regulated by microorganisms and changes in membrane fluidity may have serious repercussions on cell functioning. The membrane fluidity of cells treated with tea tree oil or components for 10 and 30 min was significantly increased after 30 min treatment with all components except γ-terpinene. Furthermore, 1,8 cineole caused a

significant increase in membrane fluidity after only 10 min. These increases in fluidity were interpreted as showing that the molecular forces between the lipid molecules of the plasma membrane were decreased, perhaps because the tea tree oil components have inserted into the plasma membrane. This would also cause a generalised expansion of the membrane and may also explain why the permeability of cells treated with either tea tree oil or components was altered.

Another membrane-associated function that is very important for the functioning and stability of yeast cells is the plasma membrane ATPase enzyme. This enzyme maintains cell homeostasis and osmotic stability by regulating the concentrations of ions inside the cell. Assays to investigate the effects of tea tree oil on this enzyme were conducted. The efficiency of this enzyme can be estimated by measuring decreases in the pH of the cell suspension. This is because when glucose is added to non-growing yeast cells, it is rapidly taken into the cells by their inbuilt transport systems. To power this transport system, protons are pumped out of the cells by the plasma membrane ATPase, which results in a decrease in the pH of the external medium that the cells are suspended in. Results from the current study showed that tea tree oil inhibited glucose-induced acidification of the external medium of yeast cell suspensions. This occurred at approximately MIC concentrations, was dose-dependent and was apparent within a relatively short amount of time (10 - 20 min).

In other studies investigating the role of the PM ATPase, cells were pre-treated with diethylstilboestrol (DES), a compound that specifically inhibits the PM ATPase. When these cells were then treated with tea tree oil they were shown to be acutely susceptible to tea tree oil compared to cells that were not pre-treated with diethylstilboestrol. That is, cells with no functioning PM ATPase were acutely susceptible to tea tree oil, suggesting that this enzyme is crucial for cell survival.

In experiments where cells were grown for 24 h in the presence of low quantities of oil, changes in growth rate and membrane properties were evident. Growth rate and total biomass were reduced when cells were grown in the presence of 0.03 and 0.06% tea tree oil and cells had increased membrane fluidity, compared to control cells grown without tea tree oil. This increased membrane fluidity may represent an adaptive response enabling cells to maintain normal cell functions. Other studies showed that cells grown with tea tree oil did not accumulate trehalose, which is a disaccharide that has been shown to accumulate inside cells as a stress response to a range of external challenges. It was anticipated that trehalose would be accumulated since this effect has been shown for a range of other compounds, and it was therefore postulated that the presence of tea tree oil may inhibit respiration to such a degree that the production of metabolites such as trehalose is not possible.
Tea tree oil and some components were shown to have antifungal activity against both yeasts and filamentous fungi and our data suggest that some mechanisms of action may be related, either directly or indirectly, to alterations in membrane properties and functions. These data support the use of tea

tree oil as a therapeutic agent since they show unequivocally that tea tree oil has fungicidal activity against a range of organisms. However, published trials investigating the clinical efficacy of tea tree oil products are sorely needed as the publication of trial data in reputable, prominent medical journals will raise the profile of tea tree oil and tea tree oil products in both community and medical sectors.

Chapter 1: Introduction

With the resurgence of interest in alternative health care and natural medicines in the 1990s, tea tree oil has become a popular alternative medicine. A survey of alternative medicine use amongst patients attending a Sydney hospital emergency department showed that 52% of subjects reported using alternative medicines and that of all reported medicines, topically applied or inhaled tea tree oil was the most common (13.3% of the total) (Kristoffersen *et al.*, 1997). Current use of tea tree oil is diverse, with the oil being applied for complaints ranging from inflamed insect bites, infected cuts, acne and tinea, to foot odour and dandruff. It is also included in a range of products such as lip balm, toothpaste, antiseptic handwashes and deodorants. In addition, tea tree oil has been added to a variety of pet products such as shampoos.

1.1 Medically important fungi

Fungi are capable of causing a range of both superficial and systemic infections. Since tea tree oil is suitable only for topical application (including mucous membranes such as the vaginal or oral mucosa), only superficial fungal infections amenable to topical treatment will be discussed here. In addition, fungi are capable of surviving and growing in a wide range of environmental situations. The colonisation and growth of fungi in air conditioning systems or a high load of fungal spores in indoor air have been implicated as factors affecting the health of building inhabitants. Since tea tree oil has been suggested as a potential agent to reduce fungal loads in air and/or air conditioning systems, these fungi will also be discussed below briefly.

Yeasts

Yeasts are the most commonly isolated fungi causing human disease (Warren & Hazen, 1995). Members of the genus *Candida*, and in particular *Candida albicans*, are the most important in terms of frequency of isolation and severity of disease (Hazen, 1995). The next most commonly isolated pathogenic *Candida* species are *C. guilliermondii*, *C. parapsilosis* and *C. tropicalis* (Warren & Hazen, 1995; Richardson & Warnock, 1997). *Candida* species can be found as commensals of human skin, mucosa and the gastrointestinal tract (Cannon *et al.*, 1995; Warren & Hazen, 1995). In addition to their role as pathogens, *C. albicans* is the species most frequently isolated from both the normal oral cavity and the female genital tract, whereas the species *C. parapsilosis* and *C. guilliermondii* are more commonly isolated from skin (Richardson & Warnock, 1997). While *Candida* yeasts can cause deep-seated infections in severely immunocompromised hosts, they are more often responsible for superficial infections, such as vaginal or oral candidiasis, and onychomycosis (Richardson & Warnock, 1997).

In addition to *Candida*, members of other yeast genera such as *Hansenula, Malassezia, Rhodotorula, Sporobolomyces* and *Trichosporon* have emerged over the last decade or so as important opportunistic or nosocomial pathogens (Hazen, 1995).

Dermatophytes

The dermatophytes are members of the genera *Trichophyton*, *Epidermophyton* and *Microsporum,* and these fungi cause infections of the keratinised tissue of humans and other animals (Weitzman *et al.*, 1995a). Dermatophytic infections are limited to the superficial keratin-containing skin, hair or nails because host factors such as non-specific inhibitory factors in serum and the inhibition of fungal keratinases prevent deeper infections (Weitzman *et al.*, 1995a). The infections caused by these fungi are generally named according to the site they infect (eg. tinea capitis for the scalp). Tinea of the feet (tinea pedis) and to a lesser extent the nails (tinea unguium) are generally amenable to topical treatment and both of these infections have been the subject of clinical trials using tea tree oil.

Filamentous fungi associated with air, or air conditioning systems

Many species of fungi are commonly found in indoor air. The most prevalent of these are *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium*, *Eurotium* and *Wallemia* (Maroni *et al.*, 1995). Of these fungi, almost all are capable of causing opportunistic infections in immunocompromised hosts, however, these fungi are of interest in the present study because of their association with, and capacity to colonise, airconditioning systems, and the potential health risks associated with this. The presence of fungi in indoor air has been implicated in atopic allergic dermatitis and respiratory allergy (Maroni *et al.*, 1995). In addition, exposure of children to fungi in their homes has been associated with asthma, atopy and respiratory symptoms, especially in winter (Garrett *et al.*, 1998).

Fungi are commonly found in air handling units in buildings that have air-conditioning or heating systems. Growth of fungi has been seen on many components of these systems such as filters, coils and ducts (Levetin *et al.*, 2001), and the kinds of fungi commonly found are members of the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Hyalodendron* and *Penicillium* (Levetin *et al.*, 2001). Since the presence of fungi in indoor environments is associated with detrimental health effects, methods and procedures of controlling fungi within these systems are desirable. However, the inevitable presence of moisture within these systems favours fungal growth and often hampers control mechanisms.

1.2 Previous reports of the in vitro antifungal activity of tea tree oil

1.2.1 Yeasts

Previous studies have shown that a range of yeasts from the genera *Candida, Malassezia* and *Trichosporon* are susceptible *in vitro* to concentrations of tea tree oil of less than 1.0%. Since *Candida* yeasts (in particular *C. albicans*) are commonly chosen as test organisms, a moderate amount of susceptibility data are available for these organisms. Individual MICs and MIC_{90}s that have been reported for *C. albicans*, by either broth or agar dilution assays include (%) 0.04 (Beylier, 1979), 0.2 (Griffin & Markham, 2000), 0.25 (Vazquez *et al.*, 2000), 0.3 (Christoph *et al.*, 2000) and 0.44 (Nenoff *et al.*, 1996). Several other *Candida* species, such as *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. kefyr* and *C. krusei*, have been tested against tea tree oil *in vitro* and MICs ranged from 0.25 to 0.5% and MFCs ranged from 0.5 to 1.0% (Vazquez *et al.*, 2000; Banes-Marshall *et al.*, 2001; D'Auria *et al.*, 2001). *Malassezia* yeasts have also been found previously to be susceptible to tea tree oil with MICs in the range of 0.06 – 0.44% (Nenoff *et al.*, 1996; Griffin & Markham, 2000). Tea tree oil has been shown to have activity against single isolates of *T. cutaneatum, Schizosaccharomyces pombe* and *Debaromyces hansenii* with MICs of 0.22% (Nenoff *et al.*, 1996), 0.5% and 0.5%, respectively (D'Auria *et al.*, 2001).

1.2.2 Dermatophytes

Two studies used the disc diffusion method to investigate the activity of tea tree oil against dermatophytes. In both studies, zones of inhibition were seen adjacent to discs containing either 10 or 20 μl of neat tea tree oil, using isolates of *Epidermophyton floccosum, M. audonii, M. canis, T. mentagrophytes, T. rubrum* and *T. tonsurans* (Ånséhn, 1990; Concha *et al.*, 1998). The exception was one strain of *E. floccosum* which showed no zone of inhibition (Concha *et al.*, 1998). Several studies have investigated the activity of tea tree oil against dermatophytes in more depth and showed MICs of 0.7% for *E. floccosum* (Christoph *et al.*, 2000), 0.11 – 0.5% for *M. canis* (Nenoff *et al.*, 1996; D'Auria *et al.*, 2001), 0.25% for *M. gypseum* (D'Auria *et al.*, 2001) 0.12 – 0.75% for *T. mentagrophytes* (Bassett *et al.*, 1990; Nenoff *et al.*, 1996; Griffin & Markham, 2000; D'Auria *et al.*, 2001) and 0.12 – 1.0% for *T. rubrum* (Bassett *et al.*, 1990; Nenoff *et al.*, 1996; Griffin & Markham, 2000; D'Auria *et al.*, 2001). MFCs of tea tree oil have been determined as follows; 0.25 – 0.5% for *M. canis* and *T. mentagrophytes*, 0.5% for *M. gypseum*, 0.25 – 0.5% and 0.25 – 1.0% for *T. rubrum* (D'Auria *et al.*, 2001).

1.2.3 Other filamentous fungi

Similar to the dermatophytes, the activity of tea tree oil against other filamentous fungi has been investigated by several methods. With a few exceptions, these fungi are susceptible. All isolates of

Aspergillus niger, Rhizopus oligosporus and *Penicillium* spp. showed zones of inhibition to either 20 µl or 35 µl oil on a paper disc (Concha *et al.*, 1998; Chao *et al.*, 2000). MICs for the filamentous fungi, mostly obtained by the agar dilution method, were in the range of 0.2 – 1.0% for isolates of *A. flavus, A. niger* and *Penicillium*, *Rhizopus* and *Scopulariopsis* spp. (Beylier, 1979; Bassett *et al.*, 1990; Southwell, 1993; Rushton *et al.*, 1997; Christoph *et al.*, 2000; Griffin & Markham, 2000). However, isolates of *A. fumigatus* and *A. nidulans* were not inhibited at 2% tea tree oil in another study (Vazquez *et al.*, 2000).

1.3 General considerations

The available data suggest that tea tree oil has activity against yeasts, dermatophytes and other filamentous fungi, however, these data are derived from many different publications and are not necessarily directly comparable. Thus a clear picture of the range and nature of antifungal activity is not evident, an issue that this study specifically addresses.

Chapter 2: Objectives

The antifungal activity of tea tree oil has not been extensively investigated. The availability of comprehensive in vitro susceptibility data may impact on the way that tea tree oil could be used in the treatment of superficial fungal infections and for other non-medical applications.

The aims of this research project, carried out as part of RIRDC's Tea Tree Oil Research and Development Program, were to:

- Determine the in vitro susceptibility of a wide range of fungi, including yeasts, dermatophytes and other filamentous fungi, to tea tree oil
- To examine the in vitro activity of the major components of tea tree oil against yeasts and filamentous fungi
- To investigate the mechanism of action of tea tree oil and components, against *Candida albicans* and several other yeasts

Chapter 3: Materials and methods

3.1 Microbial isolates

Clinical and reference isolates were obtained from (1) the Department of Microbiology at The University of Western Australia, (2) the Division of Microbiology and Infectious Diseases at the Western Australian Centre for Pathology and Medical Research (PathCentre) and (3) the Mycology Section of the Department of Microbiology at Royal Perth Hospital. The species used in the study are shown in Table 3.1.

Table 3.1 Species of fungi used in the present study

Grouping	Organism
Yeasts	*Candida albicans*
	C. parapsilosis
	Rhodotorula rubra
	Saccharomyces cerevisiae
	Trichosporon spp.
Dermatophytes	*Epidermophyton floccosum*
	Microsporum canis
	M. gypseum
	Trichophyton mentagrophytes var *interdigitale*
	T. mentagrophytes var *mentagrophytes*
	T. rubrum
	T. tonsurans
Other filamentous fungi	*Aspergillus flavus*
	A. fumigatus
	A. niger
	Cladosporium spp.
	Fusarium spp.
	Alternaria spp.
	Penicillium spp.

3.1.1 Viable counting methods

Viable counts were performed by diluting each suspension of organisms in a series of 10-fold dilutions in sterile distilled water (SDW), 0.85% saline or buffer. Colonies were then enumerated on agar by the three techniques described below. For the Miles-Misra and spread plate methods, agar plates were surface dried for approximately 30 min prior to inoculation.

Miles-Misra counts were performed by spot inoculating two to four replicate 10 μl drops onto the agar surface. After the spots had dried, plates were incubated and colonies were counted. Spread plates were performed by aliquoting 100 μl of the appropriate dilution into the middle of an agar plate and then spreading the sample over the agar surface with a sterile glass spreader. Duplicate spread plates were used on all occasions. Plates with 30 to 300 colonies were counted and if one or both spread plates had colony numbers within this range, viable counts were determined. The lower limit of detection, calculated from 30 colonies in a 10^{-1} dilution on a spread-plate, was 3×10^3 cfu/ml.

Viable organisms were enumerated by the pour-plate method by placing 1 ml of the appropriate dilution into the centre of an empty 90 mm plastic petri dish. Molten Sabouraud dextrose agar (SDA) (18 ml) that had been cooled to approximately 50°C was then added to the sample in the petri dish, which was swirled during and after the addition of agar to ensure even mixing. Pour plates were prepared in duplicate on all occasions. Plates were incubated for up to 72 h at 35°C and plates with between 30 and 300 colonies were counted. The lower limit of detection, based on 30 colonies per plate, was 300 cfu/ml.

3.2 Chemicals and growth media

The chemicals used throughout this project were obtained from BDH, Kilsyth, VIC, Australia; Sigma Chemical Company, St Louis, Missouri, USA; Aldrich Chemical Co. Inc. Milwaukee, Wisconsin, USA; Fluka Chemie AG, Buchs, Switzerland; ICN, Aurora, Ohio, USA; Tokyo Chemical Industries Co. Ltd., Tokyo, Japan; Janssen Biotechnology, Olen, Belgium; and Searle Diagnostics, High Wycombe, Bucks, England.

Culture media and manufacturers were as follows; Sabouraud dextrose agar/broth (Oxoid Ltd., Basingstoke, Hampshire, England), Potato dextrose agar/broth (Oxoid) and Yeast extract peptone glucose (broth) (made from ingredients from Oxoid).

3.2.1 Tea tree oil and components

Melaleuca alternifolia (tea tree) oil was kindly donated by Australian Plantations Pty Ltd., Wyrallah, NSW. Batch 97/1 was used for all studies and had the composition shown in Table 3.2, as determined

by gas-chromatography mass spectrometry performed by the Wollongbar Agricultural Institute, Wollongbar, NSW.

Individual tea tree oil components were obtained as follows; (+)-terpinen-4-ol (Fluka Chemie AG, Buchs, Switzerland), γ-terpinene (Aldrich Chemical Company Inc., Milwaukee, WI, USA), α-terpinene (Sigma Chemical Co., St Louis, MO, USA), terpinolene (Fluka), α-terpineol (Aldrich), 1,8-cineole (Sigma), α-pinene (Sigma) and ρ-cymene (Aldrich).

Table 3.2 Composition of *M. alternifolia* oil batch 97/1

Component	Percentage	Component	Percentage
1. terpinen-4ol	41.5	9. aromadendrene	1.0
2. γ-terpinene	21.2	10. δ-cadinene	1.0
3. α-terpinene	10.2	11. limonene	0.9
4. terpinolene	3.5	12. ledene	0.9
5. α-terpineol	2.9	13. globulol	0.6
6. α-pinene	2.5	14. sabinene	0.4
7. 1,8-cineole	2.1	15. viridiflorol	0.3
8. ρ-cymene	1.5		

3.3 In vitro susceptibility assays for determining MICs and MFCs

3.3.1 Inocula preparation

Yeast inocula were prepared by growing isolates for 24 - 48 h on SDA at 35°C. Growth was then suspended in approximately 2 ml of 0.85% saline or SDW. The density of this suspension was adjusted in SDW to 1 McFarland, which corresponds to approximately 1.0×10^7 cfu/ml. This was serially diluted in SDW as necessary to correspond to a final inocula concentration range of $1.5 - 3.0 \times 10^3$ cfu/ml for the broth microdilution assay. Final inocula concentrations were confirmed by Miles-Misra viable counts.

Dermatophyte inocula were prepared by subculturing isolates onto Potato dextrose agar (PDA) slopes and incubating for 7 d at 30°C (Norris *et al.*, 1999). Slopes were then flooded with 0.85% saline. 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 in

SDW to correspond to the required final inocula concentrations of approximately 2.5×10^3 - 2.5×10^4 cfu/ml (Hazen, 1998) as confirmed by Miles-Misra viable counts.

Inocula for the other filamentous fungi were prepared as for the dermatophytes (described above), with the following modifications. Isolates of *Cladosporium* and *Alternaria* were grown on PDA slopes for 7 d at 30°C and the remaining fungi except for *Fusarium* were incubated at 35°C for 7 d (National Committee for Clinical Laboratory Standards, 1998). *Fusarium* spp. were incubated for 48 to 72 h at 35°C and then at approximately 28°C for the remaining 4 or 5 d. Slopes were flooded with phosphate buffered saline (PBS) containing 0.05% Tween 80 instead of 0.85% saline (Del Poeta *et al.*, 1997) and the concentrations of these suspensions were adjusted and diluted as described above. Final inocula concentrations were 0.4×10^4 - 5.0×10^4 cfu/ml (National Committee for Clinical Laboratory Standards, 1998) as confirmed by spread-plate viable counts.

3.3.2 Broth microdilution assay

The broth microdilution assays were based on reference methods M27-P and M38-P recommended by the National Committee for Clinical Laboratory Standards for yeasts and conidium-forming filamentous fungi, respectively (National Committee for Clinical Laboratory Standards, 1997; National Committee for Clinical Laboratory Standards, 1998). Microdilution trays contained a series of doubling dilutions of the test agent in 100 µl volumes of the growth medium RPMI 1640 (Gibco BRL) with L-glutamine, without sodium bicarbonate, buffered to pH 7.0 with morpholinopropane-sulfonic acid (Sigma Chemical Co.). Tea tree oil or components were tested in the range of 8% to 0.002% (v/v) and Tween 80 (Sigma) was included at a final concentration of 0.001% (v/v) to enhance oil solubility. The dermatophytes were also tested against griseofulvin (Sigma) and a stock solution was prepared at 6.4 mg/ml in dimethylsulfoxide (DMSO) and was diluted as required to result in final test concentrations of 4 - 0.06 µg/ml. The highest concentration of DMSO was 3.125% (v/v). One column served as growth control, containing only 100 µl media (with or without 0.001% Tween 80), and 100 µl inocula.

After inoculation, tests were incubated as follows; 48 h at 35°C for yeasts, 96 h at 30°C for dermatophytes (Norris *et al.*, 1999), 48 h at 35°C for *Aspergillus*, *Penicillium* and *Fusarium*, 48 h at 30°C for *Alternaria* and 72 h at 30°C for *Cladosporium*. After these incubation periods, subcultures of 10 µl were taken from each well and spot inoculated onto SDA. Subcultures were incubated at the temperatures appropriate for each species and after growth, MICs and MFCs were determined. For yeasts, the MIC was defined as the lowest concentration of oil resulting in the maintenance or reduction of the inoculum. For dermatophytes and other filamentous fungi MICs were determined visually with the aid of a reading mirror as follows. Growth in each well was compared to that of the

control and was scored numerically as follows: 4, no reduction in growth; 3, approximately 75% of the growth control; 2, approximately 50% of the growth control; 1, approximately 25% of the growth control; 0, optically clear or no visible growth (National Committee for Clinical Laboratory Standards, 1998). The MIC was determined as the lowest concentration of tea tree oil or griseofulvin corresponding to a 75% reduction in growth, compared to the control (Espinel-Ingroff *et al.*, 1997).

MFCs of tea tree oil were determined by subculturing 10 µl from wells not visibly turbid and spot inoculating onto SDA plates. For yeasts, the MFC was determined as the lowest concentration of oil resulting in the death of 99.9% of the inoculum. MFCs were not determined for griseofulvin as this agent is fungistatic only. Subcultures for dermatophytes were incubated at 30°C for at least 7 d (Aguilar *et al.*, 1999) and at 35°C for 48 h for *A. niger*. MFCs for dermatophytes and filamentous fungi were determined as the lowest concentration resulting in no growth in the subculture.

The MIC_{90} was determined as the lowest concentration of oil inhibiting 90% of isolates, while the MFC_{90} was defined as the concentration of tea tree oil fungicidal for 90% of the isolates tested. 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.

Chequerboard assays to assess combinations of agents

The activity of tea tree oil in combination with boric acid, nystatin or miconazole was investigated using *C. albicans* ATCC 10231 and *C. glabrata* ATCC 15545 as the test organisms. Concentrations of each agent ranged from 2 - 0.002% (w/v) for boric acid, 16 - 0.016 µg/ml for nystatin, 256 - 0.25 µg/ml for miconazole and 2 - 0.03% (v/v) for tea tree oil. Microdilution trays were prepared by adding 100 µl of RPMI Medium to columns 2 to 12 of the tray, adding a stock solution of tea tree oil in RPMI Medium to columns 1 and 2, and then diluting across the 12 columns of the microtitre tray but excluding the last column. The second agent was then diluted down the 8 rows of the tray, excluding the last row. The last column and row served as controls of each agent alone.

Trays were inoculated, incubated, subcultured and MICs were determined as described above. Synergy or antagonism between agents was determined by calculating the fractional inhibitory concentration (FIC) for each combination. The FIC for each agent was calculated by dividing the MIC in combination by the MIC alone, and then adding the FIC values for the two agents together. Values of < 0.5 were regarded as indicative of synergy, values of between 0.5 and 1.0 indicated additive activity and values exceeding 1.0 indicated antagonism (Hodges & Hanlon, 1991). Analyses were performed at least twice for each combination of agents.

3.4 Mechanism of action studies

3.4.1 Preparation of cells for mechanisms of action studies

For time kill, methylene blue, pre-treatment assays and membrane fluidity studies, yeast cells were prepared by inoculating 10 - 30 ml of SDB or yeast-extract-peptone-glucose broth (YEPG) with 1 - 2 colonies of each yeast isolate and incubating for 18 h at 35°C with shaking. Cells were then collected by centrifugation for 3 min at 3000 rpm (1300 × *g*), washed twice in SDW, and finally resuspended in the relevant buffer to approximately 1.0×10^7 cfu/ml with the use of a nephelometer.

For the acidification assays, cells were prepared as described above except that *S. cerevisiae* NCTC 10716 was grown at 30°C instead of 35°C. Also, cells were collected, washed twice and resuspended in cold SDW to approximately 10^7 cfu/ml. Cells were kept on ice until use.

For assays investigating the leakage of 260 nm-absorbing materials, cells were prepared by inoculating approximately 300 ml of SDB with *C. albicans* ATCC 10231 or *C. glabrata* ATCC 15545 and incubating for 18 h at 35°C with shaking. Cells were then collected by centrifugation, washed three times with PBS, and resuspended in PBS to 0.2 g wet weight cells/ml, corresponding to approximately 1.2×10^9 cfu/ml for *C. albicans* and 2.4×10^9 cfu/ml for *C. glabrata*. All centrifugation was conducted at 4°C at 8670 × *g* using a Beckman J2-21M/E Centrifuge, with a JA10 rotor. The centrifugation step to collect cells was for 15 min and all other centrifugation steps were for 10 min.

3.4.2 Time kill assays

Inocula preparation

Cells of *C. albicans* 10231 were prepared as described in section 3.4.1. Inocula for the dermatophytes and filamentous fungi (*Aspergillus* spp.) were prepared as described for the broth microdilution assay 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. An isolate each of *T. rubrum*, *T. mentagrophytes* var. *interdigitale*, *A. niger* and *A. fumigatus* was used in these assays. Starting inocula concentrations were approximately 10^6 cfu/ml for dermatophytes and *A. fumigatus*, and approximately 10^5 cfu/ml for *A. niger*.

Performance of the time-kill assay

Tea tree oil treatments were prepared in 1 ml volumes at twice the desired final concentrations in PBS, with final Tween 80 concentrations of 0.001% for yeasts and dermatophytes or 0.02% 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. Limits of detection were calculated based on a minimum of 30 colonies from the 10^{-1} dilution, taking into account different plating volumes for each organism and were 7.5×10^3 cfu/ml for dermatophytes and 3.0×10^3 for *Aspergillus* spp. Assays were performed 2 to 6 times. For *Aspergillus* and *Trichophyton* spp., colony count data for each experiment was converted to values relative to the colony count at time zero to normalise data and correct for slight variations in starting inocula concentrations between experiments. Mean and standard error values for each isolate at each time point were calculated and plotted against time, using a log scale.

3.4.3 Growth curves in the presence of tea tree oil

An overnight culture of *C. albicans* ATCC 10231 was prepared by inoculating one colony into approximately 10 ml of SDB and incubating with shaking at 35°C for 18 h. To start the experiment, 0.1 ml of this 18 h culture was added to each treatment. Treatments were prepared in 30 ml volumes in 150 ml conical flasks, containing 0, 0.016, 0.031, 0.062 and 0.125% tea tree oil in SDB with 0.001% Tween 80. Samples were taken immediately for optical density (OD) measurements and viable counts. Inoculated flasks were incubated at 35°C for 24 h with shaking at 125 rpm. Additional samples were taken at hourly intervals for optical density measurements and at 8 h and 24 h for viable counts. Viable counts were performed using the Miles-Misra method.

OD values were determined by measuring the absorbance of each sample at 540 nm (Catley, 1988) using a Perkin-Elmer UV/VIS Lambda 2 spectrometer, using Kartell disposable microcuvettes with a 10 mm path length. Each sample was measured twice by the spectrometer and mean values were calculated. Preliminary investigations showed that the presence of more than 0.016% tea tree oil interfered with OD measurements so all samples were diluted to contain less than or equal to 0.016% tea tree oil before the OD was determined. Each test sample was blanked on a solution containing the corresponding amount of tea tree oil. Where necessary, samples were diluted in SDB with 0.001% Tween 80 to keep OD below a reading of 1.0. These data were analysed by calculating the $\log_{10}$ value for each OD and plotting these data on a logarithmic scale. The mean generation time, or time required for the population to double, was also calculated.

After 24 h, wet cell weight was determined as an estimation of biomass. Cells were collected by centrifuging exactly 20 ml of culture for 5 min at 3000 rpm (1300 *g*) and then pouring off the supernatant. Any supernatant remaining in the centrifuge tube was removed with the use of a disposable plastic transfer pipette. Centrifuge tubes were weighed and the mass of cells determined. The wet weight of cells per ml of culture was then calculated. Growth experiments were repeated 3 - 4 times.

Assays investigating cells pre-conditioned with tea tree oil

Assays were performed as described above with a few modifications. Cells were pre-conditioned by inoculating 1 – 2 colonies of *C. albicans* 10231 into approximately 10 ml of 0.062% tea tree oil in SDB with 0.001% Tween 80. Control cells were grown without tea tree oil. Both cultures were grown for 24 h at 35°C with shaking (125 rpm), after which time cells were collected and resuspended in SDB to 0.2 g wet weight cells/ml. Suspensions of both pre-conditioned and control cells were diluted 1 in 10 in SDB and 0.1 ml of this was added to 30 ml of SDB with 0.001% Tween 80 and 0.062% tea tree oil. Samples were taken immediately for viable counts and OD measurements. Flasks were incubated for 24 h at 35°C with shaking (125 rpm) and further samples were taken at 14, 16, 18, 20, 22 and 24 h. At 24 h, wet weight was determined as described above. Assays were repeated at least three times.

3.4.4 Methylene blue dye exclusion assay

Treatments containing tea tree oil/component were prepared in 1 ml volumes at twice the desired final concentrations in PBS, with final concentrations of 0.001% Tween 80. At 1 min intervals, 1 ml of inocula (prepared as described previously) was added to each treatment and mixed for 20 s. Samples of 80 μl were taken from each treatment and added to 20 μl of 0.05% methylene blue for staining. This was mixed well and left for 5 min at room temperature. A wet mount was then prepared and cells were examined using a × 40 objective. A minimum of 100 cells in consecutive visual fields was recorded as stained uniformly blue or not. The percentage of cells stained blue in each sample was calculated. This assay was performed at least twice per treatment. Mean and standard error values were determined.

3.4.5 Leakage of 260 nm-absorbing materials

The assay to detect the leakage of 260 nm-absorbing materials caused by treatment with tea tree oil or components was based on that of Besson *et al*. (1989), with *C. albicans* 10231 as the test organism.

Treatments containing tea tree oil or components were prepared at twice the desired final concentration in 2 ml volumes in PBS with 0.002% Tween 80. Initial experiments showed that results for the component terpinolene were not consistent with a final concentration of 0.001% Tween 80, thus subsequent treatments containing terpinolene, γ-terpinene and α-terpinene were prepared in 0.2% Tween 80, which was halved after inoculation. Treatments and controls were inoculated with 2 ml of the suspension of organisms prepared as described in section 3.4.1. Solutions were mixed for approximately 10 s, and 20 s after the addition of inocula, a 150 μl sample was taken from each treatment and added to 1.35 ml PBS with 0.001% Tween 80 for a 1 in 10 dilution. These dilutions were then filtered with a 0.45 μm filter and the filtrate was collected. Treatments and controls were incubated at 35°C with shaking and additional samples were taken at 1, 2, 4 and 6 h.

Blanking solutions were prepared which contained the same concentrations of Tween 80 and/or components as treatments, and these were diluted 1 in 10 in PBS with 0.001% Tween 80 and were filtered as described above. The OD of the blanks was measured by dispensing 200 µl volumes into each of four wells of a 96-well SPECTRAplate microplate. The OD of the solutions was then read at 260 nm using a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). After the OD_{260} of blanks was determined, test filtrates were dispensed in the same manner into the corresponding wells of the microplate and the OD_{260} values of the test filtrates were then determined. The microplate reader took six individual measurements and calculated the average for each microplate well. The OD_{260} of all test filtrates were determined on the same day as each experiment was conducted. The OD_{260} values of the four blank filtrates were subtracted from the corresponding OD_{260} values for the four test filtrates and an average value for the four wells was obtained. Each treatment concentration was repeated at least three times, with the exception of treatments that produced no obvious leakage after 6 h incubation, which were repeated only twice. Mean, standard deviation and standard error values were determined.

3.4.6 Susceptibility of germinated and non-germinated *A. niger* conidia

The assay comparing the activity of tea tree oil against non-germinated and germinated conidia was performed according to the method of De Lucca *et al.* (1997), with a few modifications. Two isolates of *A. niger* were used in this assay and inocula were prepared by growing isolates on PDA at 30°C for 7 d. Conidia were harvested by flooding each slope with PDB and gently probing the growth. Conidial suspensions were adjusted as described previously to approximately 10^6 conidia/ml, and were then diluted 1/100 in potato dextrose broth (PDB) to approximately 10^4 conidia/ml. Part of this suspension (non-germinated conidia) was used immediately and part was incubated for 8 h at 30°C to produce germinated conidia. Attempts to germinate *A. fumigatus* conidia were unsuccessful.

Tea tree oil treatments ranged from 0.25 - 0.03% (final concentrations) and were prepared in PDB with 0.001% Tween 80. Conidia (both germinated and non-germinated) were treated by adding 45 µl of the conidial suspension to 405 µl of each treatment or control and incubating for 30 min at 30°C. Colony counts were performed from the controls (0% tea tree oil) by spread plating either 50 µl (non-germinated) or 100 µl (germinated) aliquots onto each of four PDA plates. Colony counts from treatments were performed by adding 0.45 ml SDW to each treatment to dilute it, and spread plating either 100 µl (non-germinated) or 200 µl (germinated) aliquots onto each of four PDA plates. The dilution step was employed to counter the antimicrobial effects of the tea tree oil on the fungi. Viable count plates were then incubated at 35°C and colonies were counted. Assays were performed 2 - 4

times per isolate per tea tree oil concentration. Data are expressed as proportions of the time zero non-germinated conidia viable count result.

3.4.7 Acidification of the external medium during treatment with tea tree oil

The ability of yeast cells to acidify the external medium after the addition of glucose, but in the presence of tea tree oil was examined, based on the methods of Lunde & Kubo (Lunde & Kubo, 2000), with some modifications.

Cells of *C. albicans*, *C. glabrata* and *S. cerevisiae* were prepared as described in section 3.4.1 and cell density was adjusted to approximately 10^8 cfu/ml. Amounts of a 10% tea tree oil stock solution were added to aliquoted cell suspensions to correspond to final tea tree oil concentrations of 0, 0.1, 0.2, 0.3 and 0.4%. After the addition of tea tree oil, cell suspensions were incubated for 5 min at 30°C, and then 1 ml of a 20% (w/v) glucose solution was added to each control or treatment at timed intervals to result in a final glucose concentration of 2%. After the addition of glucose, treatments were mixed thoroughly for approximately 20 s with a vortex mixer and time zero pH readings were taken within 30 s of the addition of glucose. The pH of samples was determined using a pH electrode (TPS Pty. Ltd., Brisbane, QLD). Controls and treatments were incubated at room temperature and the pH of each was determined at 0, 5, 10, 20, 30, 40, 50 and 60 min. Experiments were performed at least twice per treatment per test organism.

The addition of tea tree oil alone caused a slight decrease in the pH of each solution, therefore values were normalised by dividing the pH measurements that were taken at, and after, 5 min by the reading taken at time zero for that particular tea tree oil concentration.

3.4.8 Effect of pre-treatment of *C. albicans* with various substances on subsequent susceptibility to tea tree oil

These assays were based on those described by Koshlukova *et al*. (1999). Cells of *C. albicans* ATCC 10231 were pre-treated with carbonylcyanide *m*-chlorophenyl hydrazone (CCCP), diethylstilboestrol (DES) or calcium ions and were then post-treated with either nothing or several different concentrations of tea tree oil. For some assays, cells were also post-treated with 2M NaCl which was included as a positive control. The buffer used for assays with CCCP and DES was PBS and succinate buffer was used for assays with cations.

Stock solutions of CCCP and DES were prepared and diluted in methanol (w/v). Stock solutions of CCCP were stored at -20°C and solutions of DES were stored at room temperature protected from light. The stock solution of calcium ions was prepared as a 1 M solution of $CaCl_2.2H_2O$ which was

sterilised by autoclaving. Further dilutions were made in SDW. The optimal pre-treatment concentrations of CCCP and DES and post-treatment concentrations of tea tree oil were determined in a series of preliminary investigations. Pre-treatment concentrations were deemed too high if they alone caused a loss of viability compared to non pre-treated cells. Final pre-treatment concentrations were 200 μM CCCP, 100 μM and 125 μM DES, and 100 mM Ca^{2+}.

Assays were performed by adding 0.8 ml of cells to each pre-treatment or control prepared in 0.2 ml volumes. For controls, cells were added to either PBS or succinate buffer only. The PBS control also contained vehicle, which was a final concentration of 4% (v/v) methanol. Pre-treatments were incubated for 2 h at 35°C with shaking. After this time, 0.2 ml of pre-treated cells was added to 0.8 ml of each different post-treatment. Post-treatments were typically 0, 0.2, 0.3, 0.4 and 0.5% tea tree oil, although there was some variation. All post-treatment solutions (except 2M NaCl) contained a final concentration of 0.001% Tween 80. Also, where relevant, concentrations of the pre-treatment agent were maintained in the post-treatment phase. For example, when the cells pre-treated with CCCP were added to the tea tree oil or control post-treatment, a final concentration of 200 μM CCCP was maintained throughout.

After 1 h of post-treatment, viable counts were performed immediately using SDA spread and pour plates. The assay was repeated at least twice per treatment and means, standard deviations and standard errors of the viable counts were calculated.

3.4.9 Trehalose accumulation during treatment with tea tree oil

Cells were prepared by inoculating 1 – 2 colonies of *C. albicans* ATCC 10231 or *S. cerevisiae* NCTC 10716 into 40 ml of YEPG in a 150 ml flask. Cells were incubated for 18 - 24 h at 30°C for *S. cerevisiae* or 35°C for *C. albicans*, with shaking at 125 rpm. Cells were then diluted 1 in 10 in fresh medium by adding 30 ml of this overnight culture to 270 ml of YEPG in a 1 litre flask. These cultures were grown for 5 h at the appropriate temperature for each organism, with shaking at 125 rpm to obtain early to mid-exponential phase organisms. Cells were then divided into 100 ml volumes in 500 ml flasks. Volumes of a 12.5% stock solution of tea tree oil in SDW were added to flasks to result in final concentrations of 0, 0.03 and 0.06% tea tree oil. Cells were then incubated for an additional 3 h and samples of 10 ml (*C. albicans*) or 20 ml (*S. cerevisiae*) were collected at 0, 1, 2 and 3 h.

For heat shock treatments, cells were prepared as described above except that all incubations for both organisms were conducted at 30°C. After the 5 h growth period, a 50 ml aliquot of cells was incubated at 45°C in a waterbath with occasional shaking and after 90 min cells were collected (Attfield, 1987). Stationary phase cells were obtained by inoculating 40 ml of YEPG in 150 ml flasks with 1 – 2

colonies of each organism and incubating at 30°C for *S. cerevisiae* or at 35°C for *C. albicans* for 48 h with shaking at 125 rpm. Cells were then collected, washed and stored as described below.

The OD_{600} of all samples was determined prior to centrifugation, using the appropriate blank. After cells were collected by centrifugation they were washed twice in ice cold SDW to remove free glucose (Lee & Goldberg, 1998). After the second wash the supernatant was poured off and the cell pellet was frozen at -80°C until further analysis (Hounsa *et al.*, 1998).

Trehalose was extracted from yeast cells by resuspending pellets in 10 - 20 volumes of water (approximately 1.5 ml) and incubating for 20 min at 95°C. The cell debris was removed by centrifugation and the supernatant collected (Lee & Goldberg, 1998). Trehalose amounts were determined by treating the supernatant with trehalase, an enzyme that hydrolyses trehalose to glucose. Forty microlitres of a 500 mU/ml solution of trehalase was added to 0.3 ml of cell supernatant. This was incubated for 6 - 8 h at 37°C, after which time glucose levels were determined. Pre-existing levels of glucose in each sample were also determined and subtracted from the total glucose.

Glucose levels were determined by the glucose oxidase/peroxidase method and reagents were used according to the instructions supplied by the manufacturer. Briefly, 0.4 ml of glucose oxidase/peroxidase reagent was added for each 0.2 ml of cell supernatant. This was mixed and incubated at 37°C for exactly 30 min. The reaction was then stopped by adding 0.4 ml of 12N H_2SO_4. The OD of each cell supernatant was then read against the reagent blank at 540 nm. Cellular trehalose content was expressed as μg trehalose per mg dry weight, which was determined from an OD_{600}/dry weight standard curve. Assays were repeated at least twice.

3.4.10 Changes in membrane fluidity after treatment with tea tree oil

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

Cells of *C. albicans* ATCC 10231 were cultured with tea tree oil as described in section 3.4.3, in YEPG broth. After cells had grown for 24 h with or without tea tree oil, they were collected from each treatment by centrifugation for 3 min at 3000 rpm (1300 × *g*), washed twice in PBS, and finally resuspended in PBS to an OD_{540} of between 0.40 and 0.42. Cells were then labelled and fluorescence intensity was determined. Experiments were repeated 2 - 4 times.

Immediate changes in membrane fluidity after treatment with tea tree oil or components

Cells of *C. albicans* ATCC 10231 were prepared as described in section 3.4.1 and were resuspended to approximately 1×10^8 cfu/ml in PBS with 0.001% Tween 80. Aliquots of 9.75 ml of this suspension were dispensed for each treatment and 15 ml was dispensed for the control.

Stock solutions of tea tree oil or components were prepared as 10% (v/v) solutions in PBS with 0.001% Tween 80. Stock solutions were mixed thoroughly then 0.25 ml of each was added to the dispensed cells. After the addition of tea tree oil or component treatments were mixed thoroughly. Treatments and controls were incubated at 35°C with shaking at 150 rpm. Samples of 5 ml were taken at 0 (control cells only), 10 and 30 min, and cells were immediately collected, washed twice in PBS with 0.5% Tween 80 and then washed twice in PBS without Tween. Cells were finally resuspended in PBS to an OD_{540} of between 0.40 and 0.42. Cells were then labelled and fluorescence intensity was determined. Experiments were repeated at least twice per treatment.

Cell labelling and fluorescence measurements

The fluorescent probe used in these assays was 1,6-diphenyl-1,3,5-hexatriene (DPH) and a stock solution was prepared by dissolving the powder in dimethylformamide to a concentration of 50 mM (w/v). From this, a working stock solution of 400 μM was prepared, also in dimethylformamide, and all stock solutions were stored at room temperature protected from light. In all instances 20 μl of this 400 μM stock solution was added to 4 ml volumes of cells to result in a final probe concentration of 2 μM (Ansari *et al.*, 1993a). After the addition of probe, cells were incubated for 30 min at 35°C in the dark to allow for incorporation of the probe (Swan & Watson, 1997). Fluorescence intensity was then determined using a Varian Cary Eclipse Fluorescence Spectrophotometer. The spectrophotometer was programmed to measure the fluorescence intensity of each sample every six seconds over a one min period and the average of these values was calculated. All fluorescence intensity measurements were made at excitation and emission wavelengths of 350 and 430 nm, respectively (Slavik, 1994). On all occasions the spectrophotometer was blanked on unlabelled control cells, which were from the time zero point for the assay investigating immediate changes in membrane fluidity. For both assays, the fluorescence intensity of cells prepared as above but unlabelled was also determined on occasions to investigate the possibility that residual tea tree oil or components may have been contributing to fluorescence intensity values. Relative intensity values were determined by dividing each fluorescent intensity measurement by the fluorescent intensity value of control cells.

3.4.11 Statistical analyses

Arithmetic means, standard deviations and standard errors were determined where appropriate. Unless stated otherwise, data sets were compared using a Student's t-Test, two-tailed, two-sample assuming unequal variance. *P* values of < 0.05 were considered significant.

Chapter 4: Results

4.1 In vitro susceptibility data

4.1.1 Tea tree oil

NOTE: MICs and MFCs for dermatophytes and tea tree oil are the same as those shown in RIRDC Publication No. 01/11.

The in vitro susceptibility of a range of dermatophytic and other filamentous fungi are shown in Tables 4.1 and 4.2. MICs of tea tree oil for all fungi (*n* = 184) ranged from 0.004% to 0.25%, and MFCs ranged from <0.03% to 8.0%. Generally, MIC_{90} and MFC_{90} values were lower for dermatophytes, as compared to the filamentous fungi.

MICs for tea tree oil against all dermatophytes (*n* = 106) ranged from 0.004% for *T. tonsurans* to 0.06% for *T. mentagrophytes* var *mentagrophytes* (Table 4.1). All dermatophyte species had MIC_{90}s of 0.03%, except for *T. tonsurans* which had a lower MIC_{90} of 0.016%. MFCs ranged from 0.03% for *T. rubrum* to 1% for *T. mentagrophytes* var *mentagrophytes* and *T. mentagrophytes* var *interdigitale*. MIC and MFC values for each isolate were at least one dilution different and, for some isolates differed by up to six dilutions.

MICs of tea tree oil against the non-dermatophytic filamentous fungi (*n* = 78) ranged from 0.008 to 0.25%. MIC_{90}s were 0.12% for all species or genera except *Penicillium* spp. which had MIC_{90}s of 0.06% (Table 4.2). MFCs ranged from 0.06% for *Alternaria* spp. to 8.0% for *A. niger*. MIC_{90}s were 2% for *Alternaria* spp., *A. fumigatus*, *Cladosporium* spp., *Fusarium* spp. and *Penicillium* spp., 4% for *A. flavus* and 8% for *A. niger*. Of the 14 isolates of *A. niger* tested, only two had MFCs of 8%. Comparison of MICs and MFCs for each isolate commonly showed differences of more than two dilutions, and for some isolates MICs and MFCs differed by up to eight dilutions.

Table 4.1. In vitro susceptibility of dermatophytes to tea tree oil and griseofulvin determined by the broth microdilution method

Organism (*n*)	Tea tree oil (% v/v)						Griseofulvin (μg/ml)		
	MIC			MFC			MIC		
	Range	50%	90%	Range	50%	90%	Range	50%	90%
E. floccosum (15)	0.008 - 0.03	0.016	0.03	0.12 - 0.25	0.25	0.25	0.25 - 1	0.5	1
M. canis (16)	0.004 - 0.03	0.016	0.03	0.06 - 0.25	0.25	0.25	0.25 - 1	0.5	1
M. gypseum (6)	0.016 - 0.03			0.25 - 0.5			0.5 - 2		
T. interdigitale[a] (21)	0.008 - 0.03	0.016	0.03	0.25 - 1	0.5	1	0.25 - 1	0.5	0.5
T. mentagrophytes[b] (14)	0.008 - 0.06	0.016	0.03	0.25 - 1	0.5	0.5	0.5 - 2	1	1
T. rubrum (19)	0.008 - 0.03	0.016	0.03	<0.03 - 0.25	0.12	0.25	0.25 - 2	0.5	1
T. tonsurans (15)	0.004 - 0.016	0.008	0.016	0.12 - 0.5	0.25	0.5	0.5 - 1	1	1

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

Table 4.2 In vitro susceptibility of non-dermatophytic fungi (*n* = 78) to tea tree oil as determined by the broth microdilution method

Organism (*n*)	Tea tree oil (% v/v)					
	MIC			MFC		
	Range	50%	90%	Range	50%	90%
Alternaria spp. (10)	0.016 - 0.12	0.06	0.12	0.06 - 2	1	2
Aspergillus flavus (12)	0.06 - 0.12	0.12	0.12	2 - 4	2	4
A. fumigatus (12)	0.06 - 0.12	0.06	0.12	1 - 2	2	2
A. niger (14)	0.06 - 0.12	0.12	0.12	2 - 8	4	8
Cladosporium spp. (10)	0.008 - 0.12	0.06	0.12	0.12 - 4	0.5	2
Fusarium spp. (10)	0.008 - 0.25	0.12	0.12	0.25 - 2	0.5	2
Penicillium spp. (10)	0.03 - 0.06	0.03	0.06	0.5 - 2	1	2

Table 4.3 In vitro susceptibility of eight fungal isolates to tea tree oil and components, as determined by the broth microdilution method

Organism	Tea tree oil or component (% v/v)															
	Tea tree oil		Terpinen-4-ol		α-Terpineol		1,8 Cineole		α-Terpinene		γ-Terpinene		ρ-Cymene		Terpinolene	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
E. floccosum	0.03	0.25	0.008	0.016	0.016	ND[b]	0.06	0.5	0.12	0.25	1	2	4	4	0.12	ND[b]
M. canis	0.016	0.25	0.008	0.12	0.03	0.25	4	4	1	4	4	>8	8	>8	0.5	2
T. interdigitale[a,c]	0.03	0.5	0.016	0.25	0.008	0.12	0.5	4	1	2	4	8	8	>8	1	1
T. rubrum[c]	0.016	0.12	0.016	0.06	0.016	0.06	0.25	1	0.5	1	1	2	4	8	0.5	1
A. niger[c]	0.12	4	0.06	0.5	0.03	0.5	8	>8	8	>8	>8	>8	>8	>8	2	2
A. flavus	0.06	4	0.016	0.5	0.016	0.5	0.5	>8	8	>8	>8	>8	>8	>8	4	4
A. fumigatus[c]	0.12	2	0.016	0.5	0.016	0.5	4	>8	8	>8	>8	>8	>8	>8	2	2
Penicillium sp.	0.03	2	0.008	0.5	0.016	0.5	4	>8	4	8	8	>8	4	>8	4	4

[a] *T. mentagrophytes var. interdigitale*. [b] MFCs were not reproducible thus no value is given. [c] Isolates used in the time-kill assays.

4.1.2 Components of tea tree oil

Filamentous fungi

Terpinen-4-ol and α-terpineol showed MIC and MFC values that were similar to those of tea tree oil, for each of the test isolates (Table 4.3). Preliminary MICs and MFCs for α-pinene against dermatophytes were very low, with values of <0.004%, thus the testing of α-pinene against dermatophytes was not pursued. For the filamentous fungi, MICs of α-pinene were 0.008% for *A. niger* and 0.016% for *A. flavus*, *A. fumigatus* and *Penicillium* sp. MFCs were 0.03% for *Penicillium* sp. and 0.016% for the remainder. 1, 8-Cineole showed both inhibitory and fungicidal activity against the four dermatophytes and moderate inhibitory, but not fungicidal activity, against the filamentous fungi. The components α-terpinene, γ-terpinene, terpinolene and ρ-cymene showed only slight to moderate inhibitory and fungicidal activity. Interestingly, whilst MICs of terpinolene were several concentrations higher than those of tea tree oil, MFCs of terpinolene were similar to those of tea tree oil. Generally, the dermatophytes were more susceptible to components than the filamentous fungi, having lower MICs and MFCs.

Yeasts

Results obtained by the broth microdilution method are shown in Table 4.4. Terpinen-4-ol and α-terpineol were similar in activity, both having low MIC values of 0.06 - 0.25% and MFC values of 0.12 - 0.25%. These values were slightly lower than those obtained for tea tree oil. The component with the next lowest MICs was 1,8 cineole, with MIC/MFC results ranging from 1% for *S. cerevisiae* and *R. rubra* to 8% for *C. parapsilosis*. In general, MICs and MFCs for 1,8-cineole were lower for non-*Candida* species than for *Candida* spp. The components α-terpinene, γ-terpinene and terpinolene did not inhibit any of the *Candida* isolates at 8%, but inhibited the remaining three test organisms at concentrations of 8% or less. For those components showing inhibitory or fungicidal activity at or below 8%, MIC and MFC values were either equivalent or differed by one dilution only. Concentrations of ρ-cymene up to and including 8% did not inhibit or kill any of the yeast isolates (data not shown in Table).

Data obtained for tea tree oil components against *C. albicans* 10231 by the broth macrodilution method are shown in Table 4.5, along with data obtained by the broth microdilution method for comparison. Reproducible results were not attainable for terpinolene and γ-terpinene when Tween 80 was used at a final concentration of 0.001%, so the final concentration of Tween 80 was increased to 0.1%.

Table 4.4 In vitro susceptibility of yeasts to tea tree oil and components, as determined by the broth microdilution method.

Organism	Tea tree oil or component (% v/v)													
	Tea tree oil		Terpinen-4-ol		α-Terpineol		1,8 Cineole		α-Terpinene		γ-Terpinene		Terpinolene	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans ATCC 10231	0.5	0.5	0.25	0.25	0.12	0.25	4	8	>8	>8	>8	>8	>8	>8
C. albicans ATCC 90028	0.25	0.5	0.12	0.25	0.12	0.25	4	4	>8	>8	>8	>8	>8	>8
C. parapsilosis ATCC 90018	0.25	0.5	0.12	0.25	0.25	0.25	8	8	>8	>8	>8	>8	>8	>8
S. cerevisiae ATCC 10716	0.25	0.5	0.12	0.25	0.12	0.12	1	1	8	>8	8	>8	nd	nd
R. rubra	0.06	0.5	0.25	0.25	0.12	0.12	1	1	8	8	8	>8	nd	nd
Trichosporon sp.	0.12	0.12	0.12	0.25	0.06	0.12	2	4	4	4	8	8	4	4

nd, not done

Table 4.5 In vitro susceptibility of *C. albicans* 10231 to tea tree oil and components as determined by broth macro- and microdilution methods

Tea tree oil or component		Microdilution[1]		Macrodilution[2]			
				Standing		Shaking	
	Time (h)	MIC	MFC	MIC	MFC	MIC	MFC
Tea tree oil	24			0.25	0.25	0.25	0.25
	48	0.5	0.5				
Terpinen-4-ol	24			0.12	0.25	0.12	0.25
	48	0.25	0.25	0.12	0.25	0.12	0.25
α-Terpineol	24			0.12	0.25	0.12	0.25
	48	0.12	0.25	0.12	0.25	0.12	0.25
Terpinolene	24			0.5	0.5	0.5	0.5
	48	>8	>8				
1,8-Cineole	24			0.5	0.5	0.5	0.5
	48	4	8				
γ-Terpinene	24			1	1	0.5	1
	48	>8	>8				
α-Terpinene	24			1	1	0.5	1
	48	>8	>8	2	2	1	>2
ρ-Cymene	24			0.5	0.5	0.5	0.5
	48	>8	>8	1	1	>2	>2

[1]Final concentration of 0.001% Tween 80; [2]Final concentration of 0.1% Tween 80

MICs and MFCs for tea tree oil were both 0.25% by the macrodilution method, compared to 0.5% obtained by the microdilution method. Terpinen-4-ol and α-terpineol showed a similar pattern with MIC and MFC values either equivalent or one concentration lower by macrodilution as compared to microdilution. MICs and MFCs obtained by macrodilution for terpinolene, 1,8-cineole, γ-terpinene, α-terpinene and ρ-cymene were considerably lower than those obtained by the microdilution method. For example, terpinolene, γ-terpinene, α-terpinene and ρ-cymene showed no inhibitory or fungicidal activity at or below 8% by the microdilution method but all gave MICs and MFCs of 0.5 or 1% at 24 h by the macrodilution method.

Data obtained with standing or shaking varied little for most components. The exception was ρ-cymene where MICs and MFCs obtained with standing were 1% whereas values obtained with shaking were >2%, both at 48 h. A comparison of values obtained at 24 and 48 h for terpinen-4-ol and α-terpineol showed no difference. However, both MIC and MFC values increased by one or more concentrations for α-terpinolene and ρ-cymene from 24 to 48 h.

4.1.3 Tea tree oil combined with other antifungal agents

For *C. albicans* ATCC 10231, MICs of each agent alone were 0.5%, 0.25%, 2.0 μg/ml and 128 μg/ml for tea tree oil, boric acid, nystatin and miconazole, respectively. The lowest ∑ FIC values for each combination were 0.25 for tea tree oil and boric acid or nystatin, and 0.04 for tea tree oil and miconazole. Miconazole did not show fungicidal activity and showed only inhibitory activity over a range of concentrations. The ∑ FIC values for all three combinations indicated synergy although this was to a much greater extent for the combination of tea tree oil and miconazole than for the other combinations. For *C. glabrata* ATCC 15545, MICs of each agent alone were 0.25%, 1.0%, 8.0 μg/ml and 32 μg/ml for tea tree oil, boric acid, nystatin and miconazole, respectively. The lowest ∑ FIC values were 0.16 for tea tree oil and boric acid, 0.25 for tea tree oil and nystatin, and 0.19 for tea tree oil and miconazole. Again these values indicate synergy for all combinations.

4.2 Mechanism of action studies

4.2.1 Time kill studies

Candida albicans

Tea tree oil

Time kill data are shown in Fig 4.1. The treatment of cells with 0.12% tea tree oil did not cause a significant decrease in viability, as compared to control cells, until 6 h ($P = 0.035$). The viability of cells treated with 0.25% tea tree oil differed significantly from control cells at 2, 3 and 4 h ($P < 0.001$). Viable cells were recovered from the 0.25% treatment at 6 h on one occasion only. Cells treated with 0.5% tea tree oil showed significant decreases in viability at 30 min and 1 h ($P < 0.001$) and no organisms were recovered at 2 h. Similarly, treatment with 1% tea tree oil caused a significant decrease in cell viability after 30 min ($P < 0.001$) and after 1 h treatment viable cells could not be recovered. Decreases in cell viability of ≥ 3 $\log_{10}$ cfu/ml (a 99.9% decrease) were seen after treating cells for 6 h with 0.25%, 1 h with 0.5% and after 30 min with 1% tea tree oil.

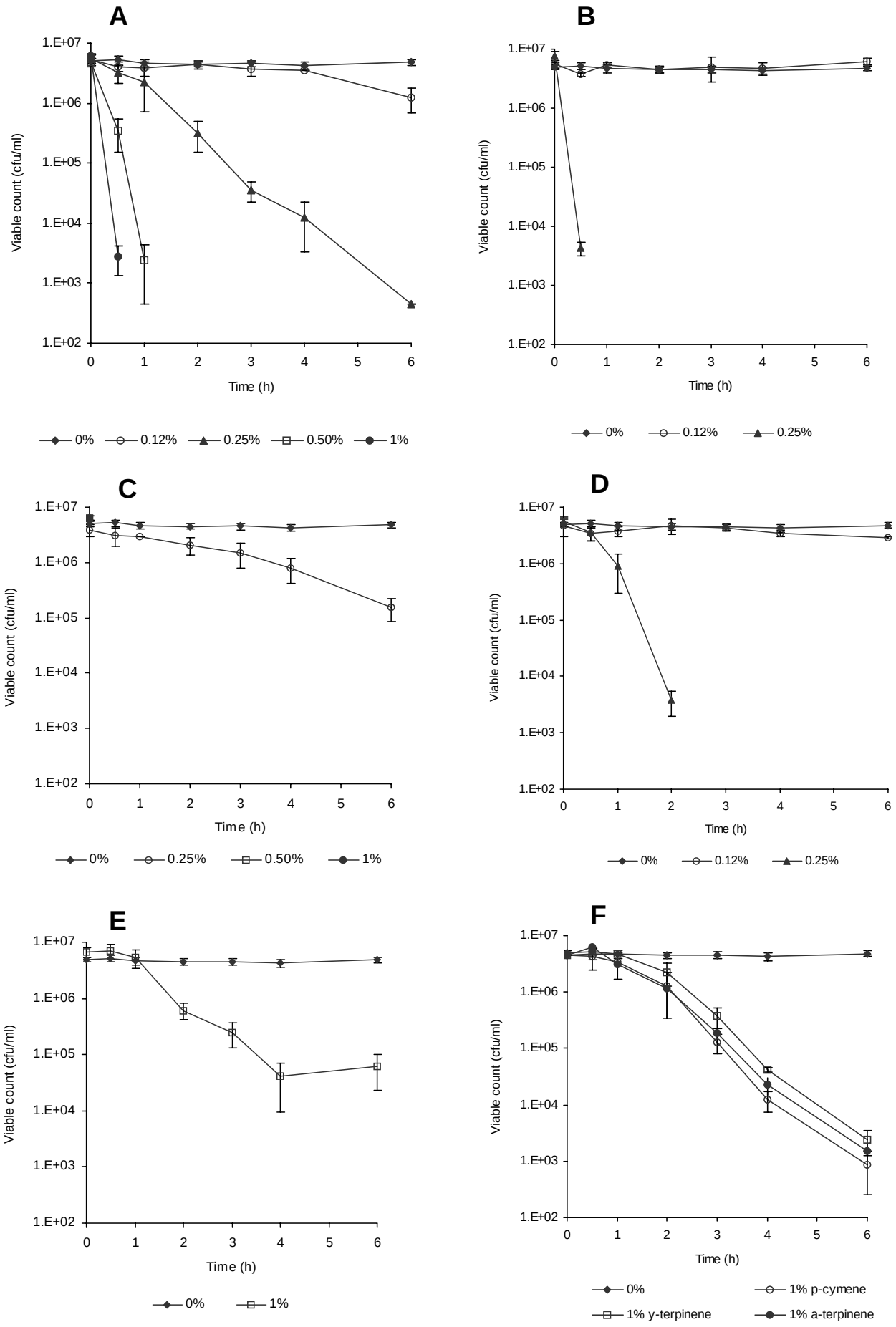

Fig 4.1 Time kill curves of *C. albicans* ATCC 10231 treated with (A) tea tree oil, (B) terpinen-4-ol (C) 1,8-cineole (D) α-terpineol (E) terpinolene (F) γ-terpinene, ρ-cymene and α-terpinene

Components

Viable counts of cells treated with 0.12% terpinen-4-ol did not differ from control cells at any time. Treatment of cells with 0.25% terpinen-4-ol produced a significant decrease in viability at 30 min ($P < 0.001$) and viable cells could not be detected at 1 h. The viability of cells treated with 0.12% α-terpineol differed significantly from control cells at 6 h ($P = 0.011$) whereas cells treated with 0.25% α-terpineol differed significantly from control cells at 1 h ($P = 0.002$) and 2 h ($P < 0.001$). A decrease in cell viability of $> 3 \log_{10}$ cfu/ml was seen after 2 h treatment with 0.25% α-terpineol, after which time viable cells could not be detected.

The viability of cells treated with 0.25% 1,8-cineole differed significantly from control cells at 4 and 6 h ($P < 0.01$), although the decrease in viability was only approximately 1 $\log_{10}$ cfu/ml. No viable cells could be detected after 30 min treatment with both 0.5% and 1% 1,8-cineole. Cells treated with 1% terpinolene showed significant decreases in cell viability after 2, 3, 4, and 6 h ($P < 0.001$). However, the decrease in cell viability was not greater than 3 $\log_{10}$ cfu/ml.

Results obtained for cells treated with 1% γ-terpinene, α-terpinene or ρ-cymene were similar. Significant decreases in viability as compared to controls were seen at 3, 4 and 6 h ($P < 0.001$) for all of these components. However, a decrease of $> 3 \log_{10}$ cfu/ml as compared to controls was seen for all treatments at 6 h only.

Dermatophytes and other filamentous fungi

Time kill experiments were conducted at 0.5% tea tree oil for *T. rubrum* and 2% tea tree oil for *T. mentagrophytes* var. *interdigitale*, corresponding to 4 × MFC for each organism (Fig 4.2). Similar trends were seen for both organisms in terms of their loss of viability over the course of the experiment. Both dermatophytes showed a $> 1 \log_{10}$ difference in viable count between treatment and control by 60 min, however, a $> 3 \log_{10}$ reduction in viability was not achieved by 6 h. After 24 h, no viable organisms could be detected in the tea tree oil treatments.

Time kill experiments were conducted with tea tree oil at 1 × MFC, which was 4% for *A. niger* and 2% for *A. fumigatus* (Fig 4.2). Viable counts from tea tree treatments did not differ from control counts by more than 1 $\log_{10}$ until between 6 and 8 hours of incubation with tea tree oil. At 24 h, viable counts of control organisms were 7.91×10^{-1} for *A. niger* and 5.02×10^{0} for *A. fumigatus*, however, no viable organisms could be detected in the tea tree oil treatments. Compared to time kill data obtained for *Trichophyton* spp., the 'slower' rate of kill for *Aspergillus* spp. is most likely to be due to the difference in tea tree oil concentration, since 4 × MFC was the concentration used with dermatophytes, compared to 1 × MFC which was used with *Aspergillus* spp.

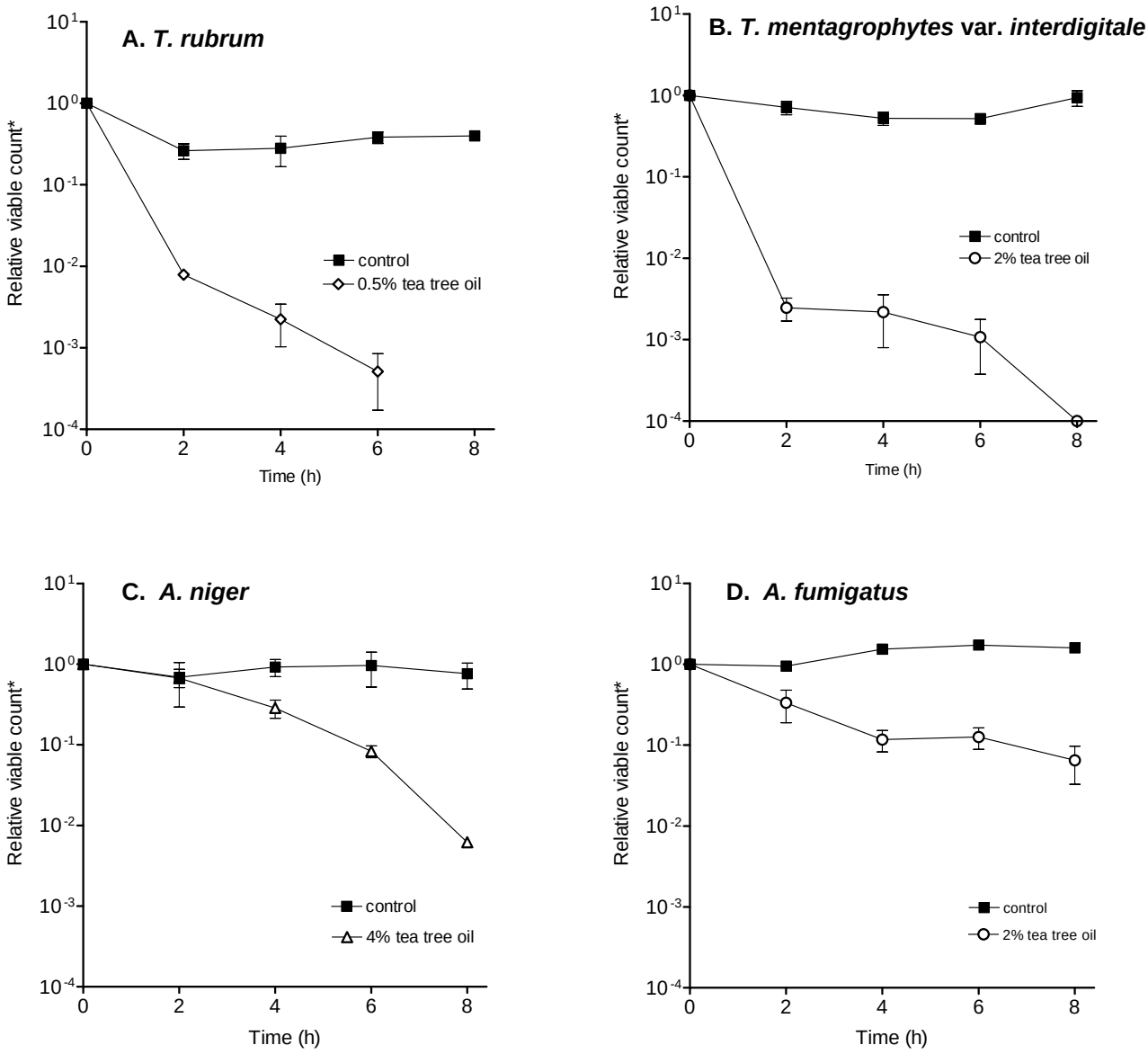

Fig 4.2 Time kill curves for one isolate each of *T. rubrum* (A), *T. mentagrophytes* var. *interdigitale* (B), *A. niger* (C) and *A. fumigatus* (D). *Relative viable count values were determined by dividing all viable count values by the cfu/ml count for the control at time zero. Mean ± SEM plotted against time.

4.2.2 Susceptibility of germinated *Aspergillus niger* conidia

Germinated *A. niger* conidia were much more susceptible to tea tree oil than non-germinated conidia (NGC). The lowest concentration of tea tree oil tested was 0.03%, and at this concentration the viability of germinated conidia (GC) differed significantly from control GC for isolate 2 only (P = 0.009) (Fig. 4.3). However, the viability of GC treated with 0.06% tea tree oil differed significantly from control GC for both isolate 1 (P = 0.019) and isolate 2 (P = 0.005). No GC were recovered from tea tree oil treatments of 0.12% or 0.25%. The concentration of 0.12% corresponded to the MIC of tea tree oil for both organisms as determined by the broth microdilution assay. The viability of NGC treated with tea tree oil at 0.25% or less was not affected, compared to control NGC ($P > 0.05$). Significantly less control GC were recovered than control NGC ($P = 3.3 \times 10^{-5}$).

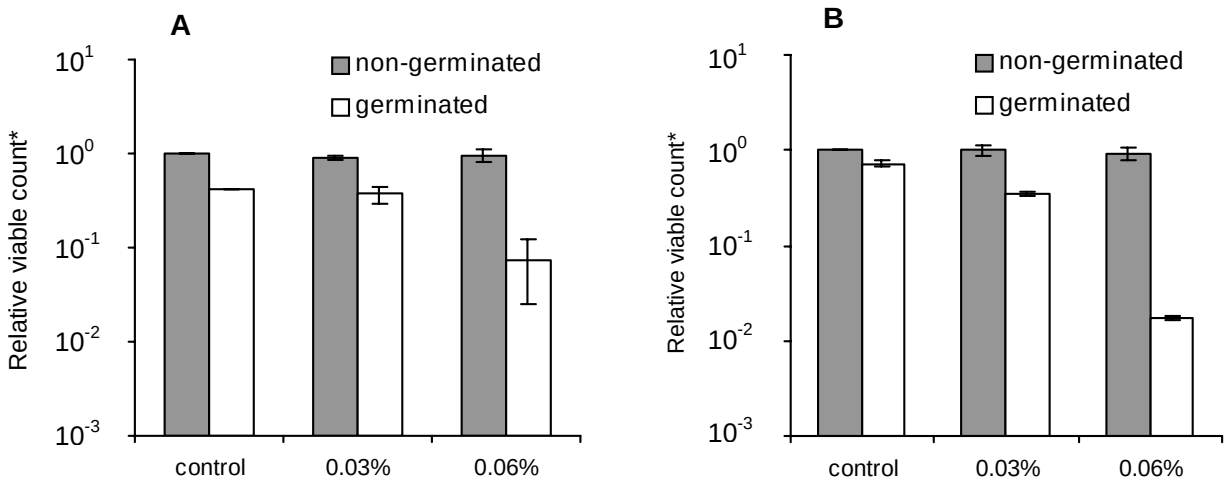

Fig 4.3 Susceptibilities of non-germinated and germinated conidia of two *Aspergillus niger* isolates (A, B) to several concentrations of tea tree oil. *Relative viable count values were derived by dividing all viable count values by the cfu/ml count for the non-germinated conidia control at time zero. Mean ± SEM plotted against time.

4.2.3 Growth curves with tea tree oil

The growth of *C. albicans* in the presence of tea tree oil is shown in Table 4.6. Cells grown in the presence of 0.016% tea tree oil did not differ significantly from control cells whereas cells grown with 0.03% (1/16 × MIC) and 0.06% (1/8 × MIC) showed reduced growth, as determined by wet weight yield and $\log_{10}(OD_{540})$ values. However, net increases in cfu/ml for cells grown in the presence of any tea tree oil concentration did not differ significantly from control cells at 8 or 24 h. Initial experiments with 0.12% (¼ × MIC) tea tree oil showed no increase in OD over the 24 h test period thus lower concentrations were used for all subsequent experiments.

The growth curves for control cells and cells grown with 0.016% tea tree oil were very similar with the exponential growth phase starting at approximately 2 h and finishing at approximately 12 h. Comparison of $\log_{10}$ (OD_{540}) values showed that control cells and cells grown in the presence of 0.016% tea tree oil were significantly different at 24 h only ($P = 0.024$). The wet weight cell yield at 24 h for cells grown with 0.016% tea tree oil was less than the control but this difference was not significant.

For cells grown in the presence of 0.03% tea tree oil, $\log_{10}$ (OD_{540}) values differed significantly from controls from 3 h onwards ($P < 0.05$) and for 0.06% tea tree oil $\log_{10}$ (OD_{540}) values differed significantly from controls from 5 h onwards ($P \leq 0.037$). Growth curves for cells grown in the presence of 0.06% tea tree oil were more variable compared to the other concentrations of oil, and mean generation time values varied from 1.76 h to 3.75 h. After 24 h, wet weight cell yield for cells grown with both 0.03 or 0.06% tea tree oil differed significantly from controls and from each other.

Cells pre-conditioned with 0.06% tea tree oil

No appreciable increase in OD_{540} was seen for both pre-conditioned and non-pre-conditioned cells from 0 to 10 h, thus growth from 14 to 24 h only was investigated further. Figure 4.4 demonstrates that differences between the two growth curves were not great. Log_{10} (OD_{540}) values were significantly different at 22 and 24 h only ($P = 0.023$ and 0.004, respectively). The net $\log_{10}$ increase in cfu/ml from 0 to 24 h for pre-conditioned cells was 3.32×10^2 cfu/ml whereas non-pre-conditioned cells increased by 1.76×10^2 and this difference was significant ($P = 0.012$). However, wet weight cell yields at 24 h were not significantly different ($P = 0.25$).

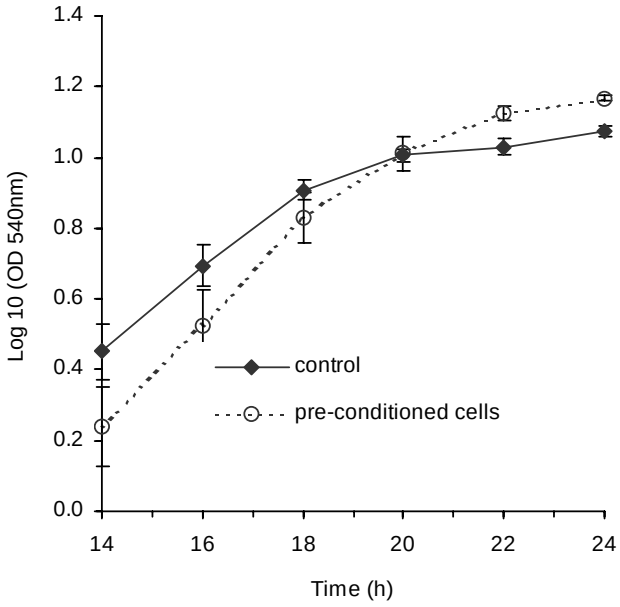

Fig 4.4 Growth of pre-conditioned *C. albicans* ATCC 10231 in the presence of 0.06% tea tree oil. Cells pre-conditioned with 0.06% tea tree oil were compared to cells not pre-conditioned with tea tree oil. Mean (± SEM).

Table 4.6 Growth parameters of *C. albicans* ATCC 10231 in the presence and absence of tea tree oil

Tea tree oil (%)	Mean cell yield in mg wet wt/ml (± SEM)	Mean generation time* Hours (range)	Relative increase in $\log_{10}$ cfu/ml (SEM)	
			8 h	24 h
0	30.63 ± 0.72	1.38 (1.29 - 1.48)	4.63×10^{1} (1.22×10^{1})	3.64×10^{2} (9.11×10^{1})
0.016	30.02 ± 1.08	1.39 (1.33 - 1.45)	2.91×10^{1} (4.9×10^{0})	9.41×10^{2} (6.3×10^{2})
0.031	27.43 ± 0.61	1.35 (1.26 - 1.42)	2.20×10^{1} (7.8×10^{0})	5.30×10^{2} (2.3×10^{2})
0.062	22.45 ± 0.28	2.49 (1.76 - 3.75)	2.89×10^{0} (1.37×10^{0})	3.45×10^{2} (5.26×10^{1})

*average of 3-4 experiments

4.2.4 Changes in permeability assessed by methylene blue uptake

Some of the results of the methylene blue dye exclusion assays are shown in Fig 4.5 with the remainder described below. There was no significant increase in the percentage staining of control cells from 0 h to 6 h, with 4.65% and 5.79% of cells staining blue at each time point, respectively.

After 6 h of treatment with 0.12% tea tree oil the percentage of cells staining blue was 8.90%, which was not significantly different from controls. Significant increases in numbers of cells staining blue were seen after 2 h treatment with 0.25 and 0.5%, and after 1 h treatment with 1% tea tree oil ($P < 0.05$).

Terpinen-4-ol and α-terpineol showed similar trends whereby treatment with 0.12% of either component did not cause a significant increase in the percentage of cells staining blue over the 6 h time period, but significant increases were seen with 0.25%, with >80% of cells stained blue at 30 min ($P < 0.01$).

1,8-Cineole at 0.25% did not cause an increase in the percentage of cells staining blue, whereas cells treated with 0.5% and 1% differed significantly from controls after 2 h and 1 h respectively. After 6 h treatment with terpinolene at 1%, the percentage of cells staining blue over the 6 h time period was 25.43% (SEM 18.88) although this increase was not significant.

γ-Terpinene, ρ-cymene and α-terpinene, all examined at 1%, did not cause significant increases in the percentages of cells staining blue over the 6 h time period. By 6h, the percentages of cells staining blue had increased to between 9.85 and 12.95, compared to 5.79% of control cells staining blue.

4.2.5 Changes in permeability determined by the leakage of 260 nm-absorbing materials

The appearance of intracellular components in the external medium is an indication of an increase in membrane permeability. Nucleotides and their components, such as purines, pyrimidines and amino acids absorb strongly at 260 nm and as such can be quantified spectrophotometrically.

The leakage of 260 nm-absorbing material from cells, induced by treatment with tea tree oil or components, as measured by OD_{260}, is shown in Fig 4.6 or described in the text below. *C. albicans* ATCC 10231 was tested against tea tree oil and components whereas *C. glabrata* ATCC 15545 was tested against tea tree oil only. Two different Tween concentrations were used in the assays with *C. albicans*, and to assess whether the increased Tween concentration enhanced or diminished leakage, both the 0% controls and the 1% tea tree oil treatments were conducted at both Tween concentrations.

Both showed no significant differences. Analysis of leakage from *C. albicans* control cells over time, at both Tween concentrations, showed slight increases in the OD_{260}. With 0.001% Tween 80, the mean OD_{260} values were 0.012 at 0 h and 0.045 at 6 h and with 0.1% Tween 80 the mean OD_{260} values increased from 0.011 to 0.035. As compared to time zero values these differences were significant from 2 h (0.001% Tween) or 4 h (0.1% Tween) onwards. Similarly, mean values for control cells of *C. glabrata* increased from 0.019 at 0 h to 0.09 at 6 h, and values differed significantly from time zero from 2 h onwards.

The treatment of *C. albicans* with 0.12% tea tree oil did not cause appreciable leakage of 260 nm-absorbing materials over the 6 h time period. However, treatment with 0.25, 0.5 and 1% was significantly different from controls at 6 h, 2 h and 30 min, respectively. Treatment with 1% tea tree oil caused significantly more leakage than 0.5% at 4 and 6 h only. When *C. glabrata* was treated with 0.25% tea tree oil a slight increase in OD_{260} was seen but this did not differ significantly from controls. Treatment with 0.5 and 1.0% tea tree oil resulted in significant increases in OD_{260} after 4 h and 2 h, respectively.

Treatment of *C. albicans* with 0.12% terpinen-4-ol did not cause significant increases in OD_{260} as compared to controls, whereas changes caused by treatment with 0.25% were significant at 1, 4 and 6 h. Treatment with either 0.12 or 0.25% α- terpineol did not cause any significant increases in OD_{260} as compared to controls.

Treatment with 0.5 and 1% 1,8-cineole caused significant increases in the OD_{260} after 1 h, as compared to controls. However, these two treatment concentrations caused very similar patterns of leakage and differed significantly from each other at 4 h only. No appreciable increase in OD_{260} was seen after treatment with 0.25%, however, measurements were significantly different from controls at 1 and 2 h.

Terpinolene at 0.25, 0.5 and 1.0% caused significant increases in OD_{260} values after 1, 1 and 2 h, respectively. The treatments of 0.5 and 1% did not differ significantly from each other whereas 0.25 and 0.5% were significantly different at 2, 4 and 6 h. The treatment of cells with 1% α-terpinene or γ-terpinene did not cause large increases in the OD_{260} (data not shown), however, OD_{260} values for γ-terpinene-treated cells differed significantly from controls after 2 h.

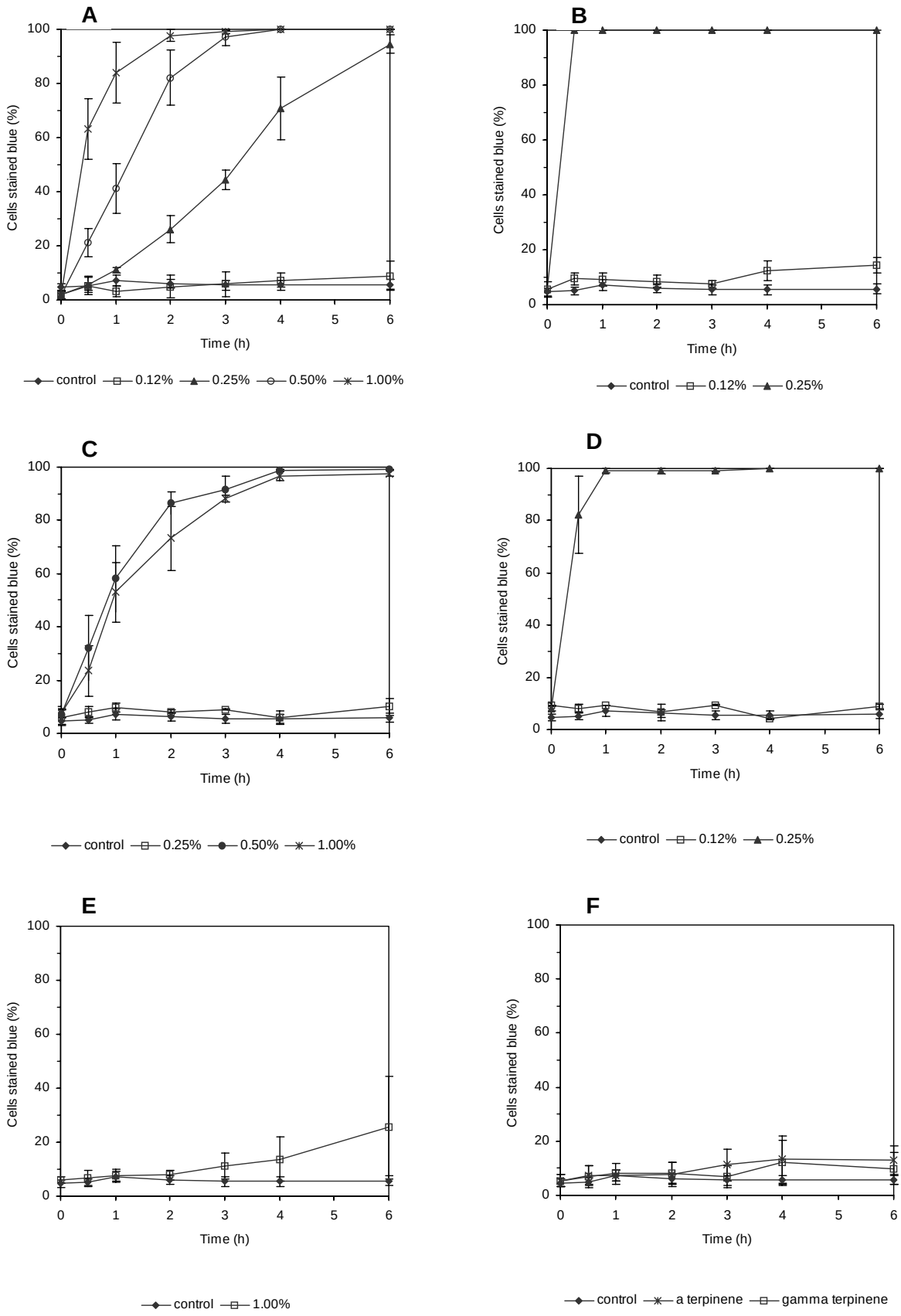

Fig 4.5 Alterations in cell permeability assessed by the loss of impermeability to methylene blue dye, caused by (A) tea tree oil, (B) terpinen-4-ol (C) 1,8-cineole (D) α-terpineol (E) terpinolene (F) γ-terpinene

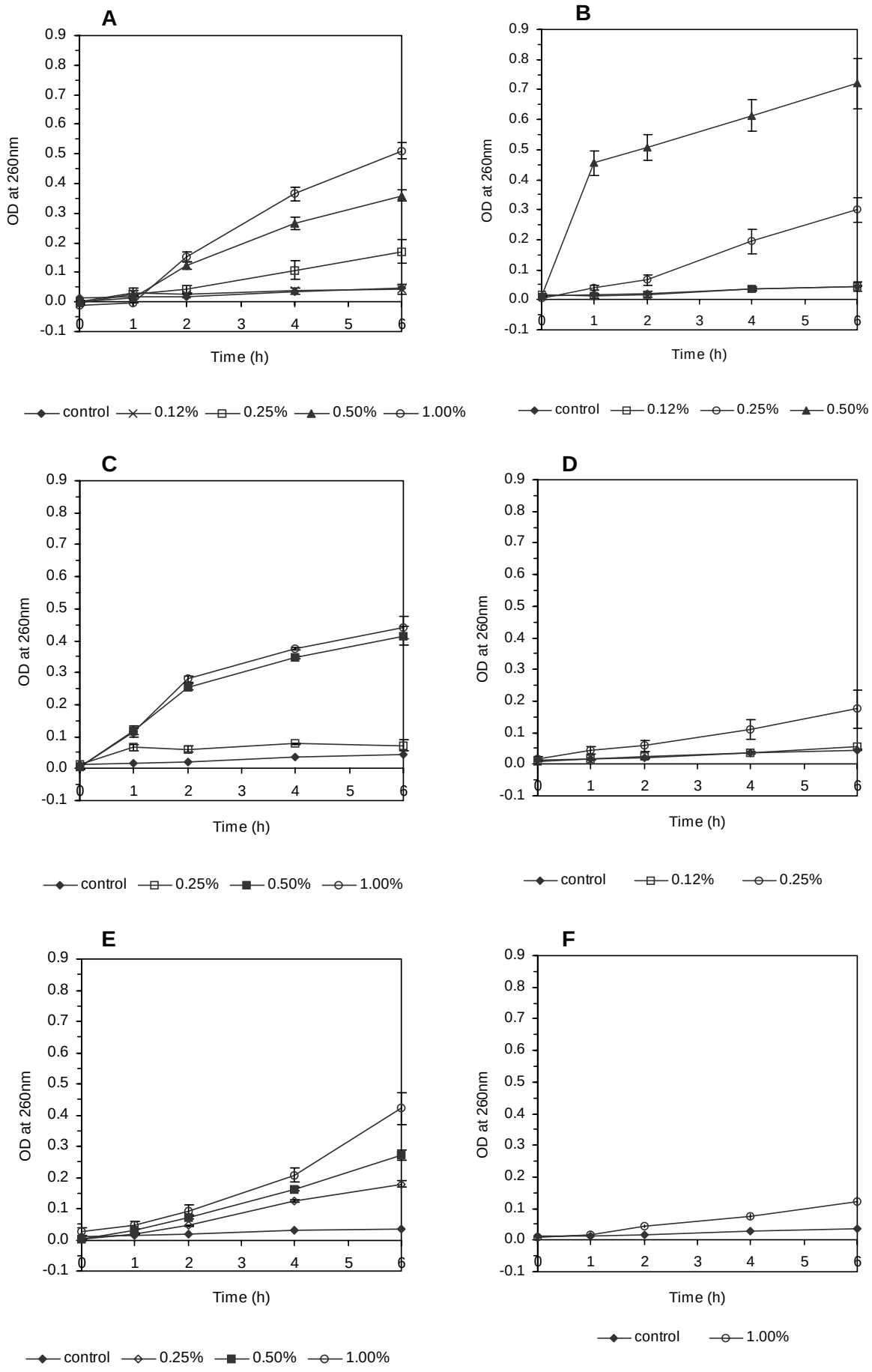

Fig 4.6 Leakage of 260 nm-absorbing materials caused by (A) tea tree oil, (B) terpinen-4-ol (C) 1,8-cineole (D) α-terpineol (E) terpinolene (F) γ-terpinene

4.2.6 Susceptibility to tea tree oil of *C. albicans* cells pre-treated with CCCP, DES and Ca^{2+}

CCCP

The viability of control cells post-treated with 0.2% tea tree oil did not differ significantly from control cells post-treated with no tea tree oil (Fig 4.7). However, significant differences in viability were seen when control cells were post-treated with 0.3% and 0.4% tea tree oil ($P < 0.01$ for both). Pre-treatment with 200 μM CCCP alone did not cause a significant decrease in cell viability after comparing cells pre-treated with 200 μM CCCP and controls cells that were both post-treated with no tea tree oil.

When both control cells and CCCP pre-treated cells were post-treated with tea tree oil, significant differences were evident. Comparison of the viability of cells post-treated with 0.2% tea tree oil showed that significantly fewer viable CCCP pre-treated cells were recovered ($P = 0.0094$). Where CCCP pre-treated cells were post-treated with 0.3% or 0.4% tea tree oil, no viable organisms could be recovered, in contrast to the equivalent control cells where viable cells were still recoverable.

When control cells were post-treated with a combination of 0.3% tea tree oil and 200 μM CCCP, the numbers of cells recovered did not differ significantly from control cells that had been post-treated with 0.3% tea tree oil alone, indicating that the effects of CCCP were not immediate and that cells required pre-treatment for up to 2 h before the effects of CCCP were apparent.

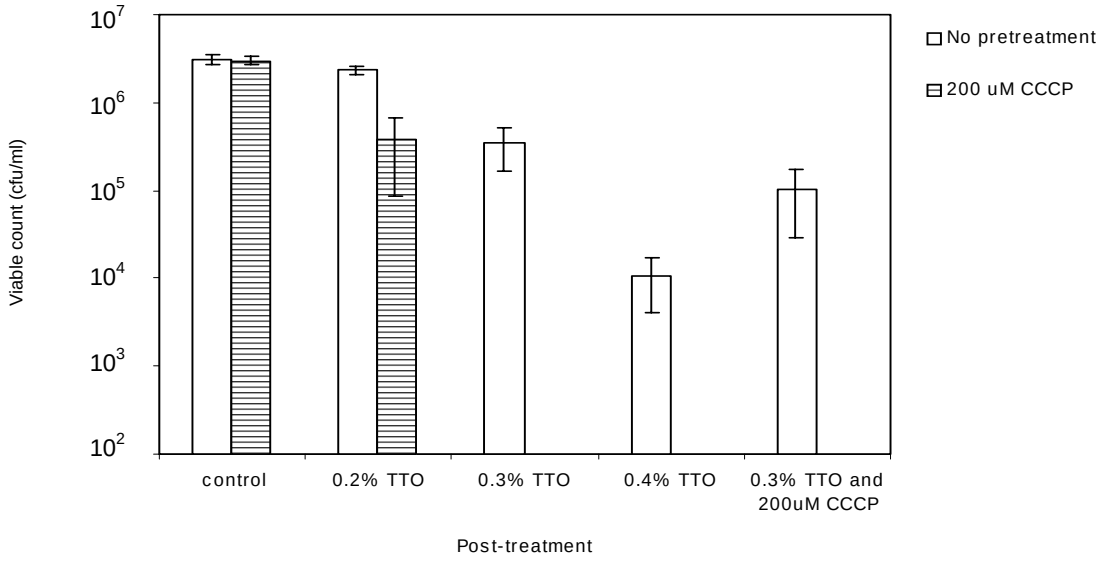

Fig 4.7 Viability of *C. albicans* ATCC 10231 cells pre-treated with CCCPthen post-treated with tea tree oil. Cells were pre-treated for 2 h with CCCP or vehicle then post-treated for 1 h with 0, 0.2 or 0.3% tea tree oil, or other treatments as necessary. Mean ± SEM.

DES

The results of the pre-treatment of *C. albicans* ATCC 10231 with 100 μM and 125 μM DES and post-treatment with tea tree oil are shown in Fig 4.8. The control cells post-treated with tea tree oil are the same as those described in section 6.1.3.1 above. Pre-treatment of cells with both 100 μM and 125 μM DES alone caused a decrease in cell viability as compared to control cells ($P < 0.01$).

Comparison of all cells post-treated with 0.2% tea tree oil showed that significantly fewer viable DES pre-treated cells were recovered ($P < 0.05$ for both DES concentrations). Where cells were post-treated with 0.3% and 0.4% tea tree oil, no DES pre-treated cells were recovered, regardless of DES concentration.

Analysis of cells post-treated with 2M NaCl showed that the viability of control cells was not affected, as compared to control cells that were not post-treated. The viability of cells pre-treated with both concentrations of DES and post-treated with 2M NaCl differed significantly from both non-post-treated DES pre-treated cells, and control cells post-treated with 2M NaCl.

The viability of control cells post-treated with a combination of 125 μM DES and 0.3% tea tree oil was reduced as compared to the post-treatment of 0.3% alone, but the difference was not significant.

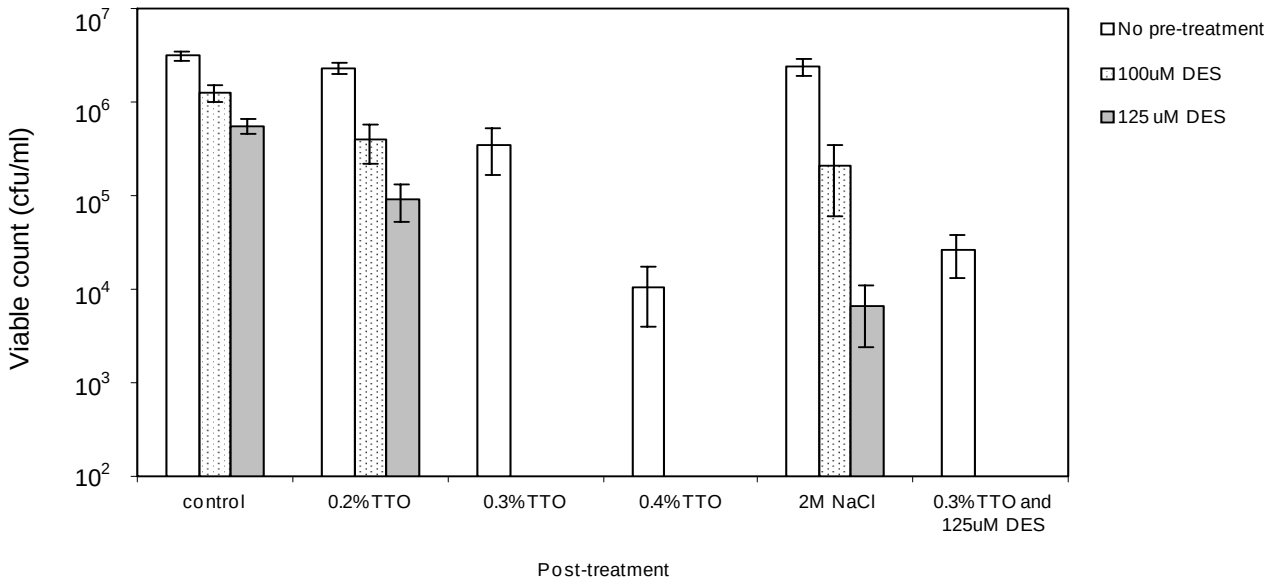

Fig 4.8 Viability of *C. albicans* ATCC 10231 cells pre-treated with diethylstilboestrol (DES), then post-treated with tea tree oil. Cells were pre-treated for 2 h with DES or vehicle then post-treated for 1 h with 0, 0.2 or 0.3% tea tree oil, or other treatments as necessary. Mean ± SEM.

Calcium

For control cells, post-treatment with 0.2% tea tree oil did not cause a significant decrease in viability whereas post-treatment with 0.3% and 0.4% tea tree oil did, as compared to control cells post-treated with no tea tree oil ($P = 0.001$ for both) (Fig 4.9). The same trend was seen for calcium pre-treated cells, whereby post-treatment with 0.2% tea tree oil did not cause a significant decrease in viability but post-treatment with 0.3 and 0.4% did ($P < 0.01$). The pre-treatment of cells with calcium did not appear to alter susceptibility to tea tree oil, given that the comparison of viable counts from each post-treatment did not show significant differences.

Viable organisms were not consistently recovered from the 0.4% and 0.5% post-treatments. Organisms were recovered from the control cells post-treated with 0.4% tea tree oil (9.2×10^3 cfu/ml) on one occasion of 5 repeats and once each from calcium pre-treated cells that were post-treated with 0.4% (3.1×10^2 cfu/ml) and 0.5% (3.45×10^2 cfu/ml). These post-treatment concentrations had been repeated 5 and 4 times, respectively.

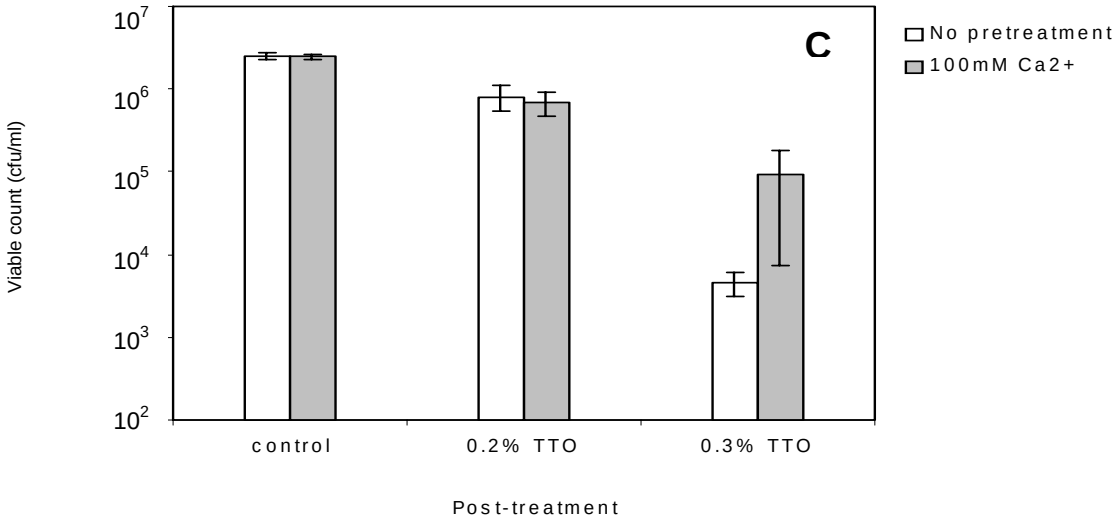

Fig 4.9 Viability of *C. albicans* ATCC 10231 cells pre-treated with calcium, then post-treated with tea tree oil. Cells were pe-treated for 2 h with calcium then post-treated for 1 h with 0, 0.2 or 0.3% tea tree oil, or other treatments as necessary. Mean ± SEM.

4.2.7 Inhibition of medium acidification in the presence of tea tree oil

The mean pH of the external medium of control cells after 60 min had changed from 7.0 to 4.9 for *S. cerevisiae*, from 6.9 to 5.3 for *C. albicans* and from 6.6 to 4.5 for *C. glabrata* (Fig 4.10). Expressed as relative decreases in pH, these correspond to mean decreases of 0.71, 0.77 and 0.68 for each organism, respectively. The relative decreases in pH for control cells differed significantly from time zero measurements after 5 min for *S. cerevisiae* and *C. albicans* and after 10 min for *C. glabrata*.

For *S. cerevisiae*, relative changes in pH during treatment with 0.1, 0.2, 0.3 and 0.4% tea tree oil differed significantly from controls from 40, 20, 30 and 30 min onwards, respectively ($P < 0.05$). The tea tree oil treatment of 0.1% differed significantly from the other tea tree oil treatments of 0.2, 0.3 and 0.4% from 20, 40 and 30 min onwards, respectively ($P < 0.05$). The tea tree oil treatments of 0.2, 0.3 and 0.4% did not differ significantly from each other.

For *C. albicans*, relative decreases in pH during treatment with 0.1% tea tree oil did not differ significantly from controls. Relative decreases in pH during treatment with 0.2, 0.3 and 0.4% differed significantly from controls at 30, 20 and 20 min, respectively. The 0.1% treatment differed significantly from the 0.2, 0.3 and 0.4% treatments from 10 - 20 min onwards. Also, the 0.4% treatment differed significantly from treatments containing both 0.2% (10 min onwards) and 0.3% (20 - 50 min, but not 60 min) tea tree oil.

For *C. glabrata*, relative decreases in pH during treatment with 0.1% tea tree oil did not differ significantly from controls. Relative changes in pH after treatment with 0.2, 0.3 and 0.4% differed significantly from controls after 10, 5 and 5 min, respectively. Comparison of different tea tree oil treatments showed that 0.1 and 0.2% were not significantly different from each other whereas 0.3% differed from 0.1% from 20 min onwards and 0.4% differed from all other treatments from 10 min onwards.

Where cells were treated with 0.4% tea tree oil there was an initial decrease in pH but after 10 – 20 min the pH then began to slowly rise. For example, for *S. cerevisiae* the lowest relative pH was 0.844 at 20 min, however, this had risen to 0.89 by 60 min. This trend was evident with all three test organisms, although, comparison of data from the time point with the lowest values with data at 60 min did not show significant differences.

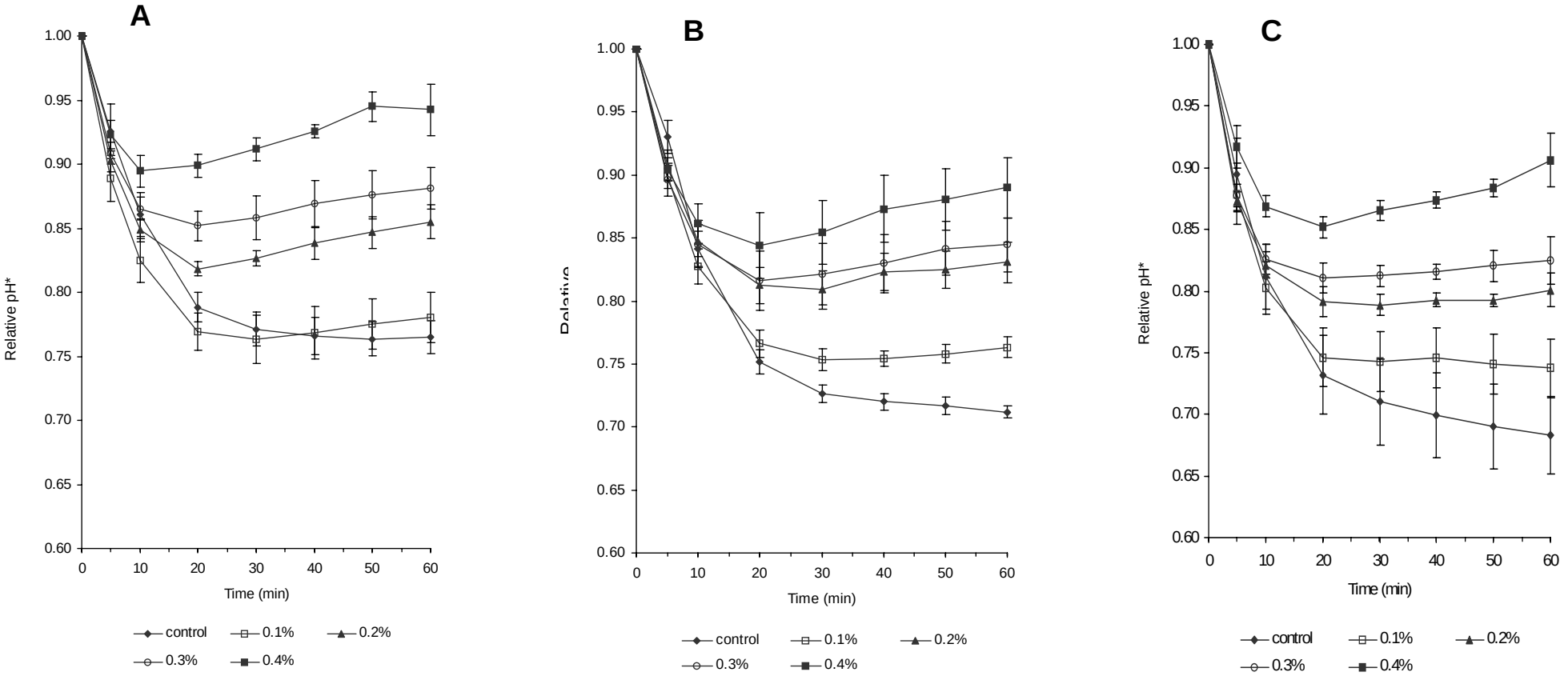

Fig 4.10 Relative decrease in the pH of the external medium of suspensions of *C. albicans* (A), *S. cerevisiae* (B) and *C. glabrata* (C) in the presence of tea tree oil, following the addition of glucose. *Relative pH was derived by dividing pH values for 5 min and onwards by the pH value for that treatment at 0 min.

4.2.8 Accumulation of trehalose during treatment with tea tree oil

The trehalose content of cells of *S. cerevisiae* and *C. albicans*, expressed as milligrams trehalose per gram dry weight, is shown in Fig 4.11. Assays were performed in duplicate only, so the ranges expressed below give both values and statistical analyses were limited. Baseline levels of trehalose were low, and were between 0.24 and 0.64 mg/g dry wt, for both organisms.

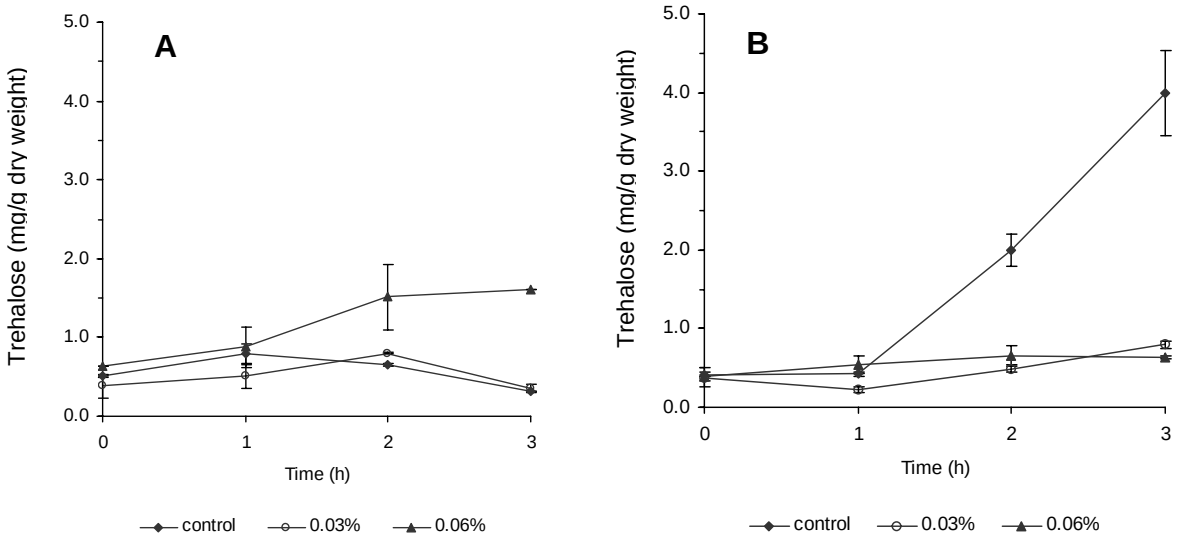

Fig 4.11 Trehalose content of yeasts treated with tea tree oil. *S. cerevisiae* NCTC 10716 (A) and *C. albicans* ATCC 10231 (B) were grown in YEPG containing 0, 0.03 or 0.06 (% v/v) tea tree oil and levels of intracellular trehalose were determined at hourly intervals. Mean ± SEM

Control cells of *S. cerevisiae* did not accumulate trehalose, with levels being slightly lower at 3 h than at 0 h. Levels of trehalose in the presence of 0.03% tea tree oil did not change over time, but when *S. cerevisiae* was grown with 0.06% tea tree oil small amounts of trehalose were accumulated (1.60 – 1.61 mg/g dry wt) which represented an approximately 2.5-fold increase compared to 0 h. Comparison of the trehalose content at 3 h of control cells and those grown with 0.06% tea tree oil showed a significant difference ($P < 0.01$). The levels of trehalose accumulated by cells treated with 0.06% tea tree oil did not approach the levels accumulated after heat shock (12.91 – 30.68 mg/g wet wt), which were approximately 25 to 60-fold higher than baseline levels. *S. cerevisiae* did not accumulate trehalose under the conditions used in the current study to produce stationary phase cells (0.98 – 1.09 mg/g wet wt).

Levels of trehalose in cells of *C. albicans* treated with tea tree oil approximately doubled from 0 to 3 h. However, trehalose levels in control cells increased by 4 to 6-fold between 1 and 2 h and had approximately doubled again by 3 h (3.45 – 4.53 mg/g dry wt). The differences between the control and treatments were not statistically significant. Increases in the trehalose content in cells of *C.*

albicans after heat shock were approximately 20 – 23-fold (8.68 – 8.79 mg/g dry wt) and increases in stationary phase cells levels were approximately 29-fold (10.62 – 12.96 mg/g dry wt), as compared to baseline levels.

4.2.9 Membrane fluidity changes

***C. albicans* grown for 24 h in the presence of tea tree oil**

Values for control cells, measured by the fluorescence intensity (arbitrary units) of DPH in *C. albicans* ranged from 30.66 – 52.04. Because of this variation in baseline measurements, all subsequent measurements were converted to relative values by dividing each measurement by the intensity value of control cells at time zero. The relative increase in fluorescence intensity seen for cells grown with 0.016% tea tree oil was not significant compared to control cells, whereas increases seen for cells grown with 0.03 and 0.06% were (Fig. 4.12). Also, the relative intensities of cells grown with 0.03% and 0.06% differed significantly from cells grown with 0.016% but not from each other. Intensity measurements of tea tree oil-grown but unlabelled cells were determined twice and were –0.07 and 0.23 (0.016%), 0.36 and 0.57 (0.03%) and 0.33 and 0.85 (0.06%). At most these readings represented approximately 1.6% of the total fluorescence intensity measurement.

***C. albicans* treated for 10 and 30 min with tea tree oil or components**

The membrane fluidity of *C. albicans* cells treated with tea tree oil or components, as determined by changes in the fluorescence intensity of DPH, is shown in Fig 4.13. Fluorescence intensity measurements were converted to relative values as described above since actual fluorescence measurements of control cells ranged from 34.61 – 54.31 for individual experiments. Analysis of relative changes in fluorescence intensity showed that treatment with 0.25% tea tree oil, terpinen-4-ol, 1,8 cineole, α-terpinene and terpinolene caused significant increases at 30 min, 0.25% α-terpineol caused a significant decrease and terpinolene was not significant. In addition, treatment with 0.25% 1,8-cineole was the only treatment to cause a significant increase at 10 min. Fluorescence intensity increased significantly from 10 to 30 min for tea tree oil, terpinen-4-ol, 1,8-cineole, α-terpinene and terpinolene treatments. No significant changes occurred in control cells between 0, 10 and 30 min, as determined by comparing both raw data values and relative changes in fluorescence intensity. Intensity measurements of unlabelled cells for each treatment (blanked on unlabelled untreated control cells) were between –2.12 and 0.22 (actual measurements) and represented –6.8 to 0.81% of total measurements, with the average being –1.98%. The –6.8% deviation was for α-terpineol at 10 min on one occasion only. The fluorescence measurements of treated but unlabelled cells were considered to not contribute significantly to overall measurements.

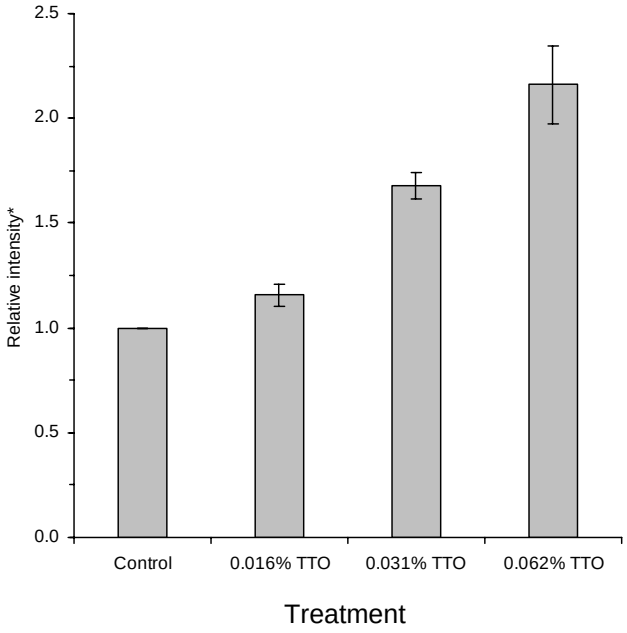

Fig 4.12 Membrane fluidity of cells grown for 24 h with tea tree oil. Cells of *C. albicans* ATCC 10231 were grown with 0, 0.016, 0.031 or 0.062% (v/v) tea tree oil for 24 h, collected and washed, labelled with diphenylhexatriene (DPH) and fluorescence intensity was then determined.

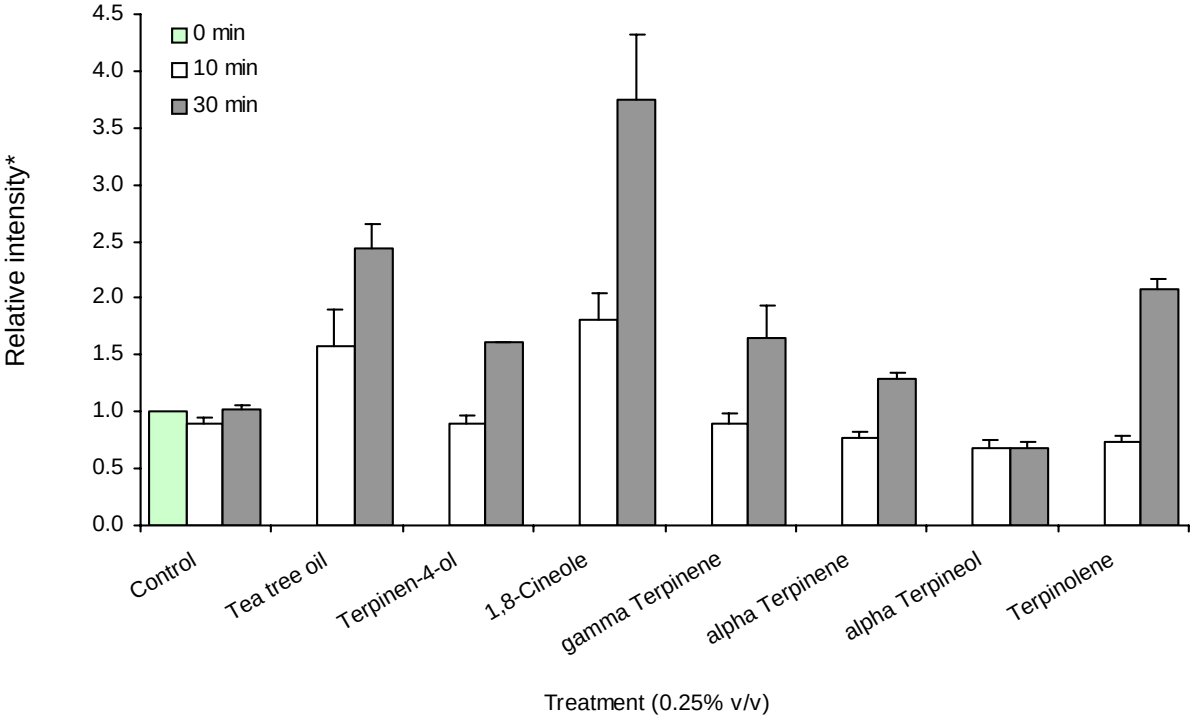

Fig 4.13 Effects of tea tree oil and components on the fluidity of the plasma membrane of *C. albicans* cells. Cells of *C. albicans* ATCC 10231 were treated with 0.25% (v/v) tea tree oil or components for 10 and 30 min, after which time cells were washed, incubated with DPH for 30 min, and fluorescence intensity was then determined. *Relative intensity was determined by dividing all intensity values by that of control cells at time zero. Mean ± SEM.

Chapter 5: Discussion

Results from the range of experiments conducted in this study have shown that tea tree oil and components have activity against yeasts, dermatophytes and other filamentous fungi. These results will be interpreted and discussed below.

5.1 In vitro susceptibility data

5.1.1 Tea tree oil

Dermatophytes

Dermatophytes were both inhibited and killed by tea tree oil. The data obtained in the current study can be compared to previously published results, except where methodological differences are too great. An example of this is two previously published reports that used the disc diffusion method (Ånséhn, 1990; Concha *et al.*, 1998), which gives a qualitative indication of the anti-dermatophytic activity of tea tree oil but does not give specific, quantitative data, such as MICs.

MICs determined by the agar dilution method in other studies were 0.08% (Inouye *et al.*, 2001b), 0.3-0.4% (Griffin & Markham, 2000), 0.11-0.44% (Nenoff *et al.*, 1996), and 0.75% (Bassett *et al.*, 1990) for *T. mentagrophytes* and 0.04% (Inouye *et al.*, 2001b), 0.5% (Bassett *et al.*, 1990), and 1% (Griffin & Markham, 2000) for *T. rubrum*. Also, MICs of 0.11% have been shown for *M. canis* (Nenoff *et al.*, 1996). The overall range of MICs from these agar dilution studies was 0.04 to 1%, and these are mostly higher than the MICs obtained in the current study. Two additional studies determined tea tree oil susceptibility by a broth dilution method. MIC_{90}s of 0.25% and MFC_{90}s of 0.5% were obtained for *M. canis*, *T. mentagrophytes* and *T. rubrum* (D'Auria *et al.*, 2001). Similarly, MICs of 0.7% for *E. floccosum* and 0.6% for *T. rubrum* were obtained (Christoph *et al.*, 2000).

The MICs obtained for dermatophytes in the current study ranged from 0.004 – 0.06%, and all were notably lower than any previously reported values. However, MICs obtained for dermatophytes by the agar dilution method are often several-fold higher than those obtained by the broth dilution method (Niewerth *et al.*, 1998). In addition, it seems plausible that these differences in MICs are due to assay differences such as growth medium, and the temperature and time of incubation. Also, the criteria by which the MIC is determined may influence the final result. Despite these methodological differences, the MFCs obtained in the study by D'Auria *et al.* (2001) were either the same or only one dilution different to those obtained in the present study. This suggests perhaps that MFCs, as an indicator of antifungal activity, are less subject to variation than MICs.

Other filamentous fungi

The filamentous fungi were also inhibited and killed by tea tree oil, although some isolates were less susceptible than others with MFCs as high as 8%. Very little tea tree oil susceptibility data have been published for the filamentous fungi, however, two agar diffusion studies reported that isolates of *A. niger*, *Penicillium* spp., and *Rhizopus oligosporus* were inhibited by either 35 μl or 20 μl of tea tree oil (Concha *et al.*, 1998; Chao *et al.*, 2000).

MICs obtained by the agar dilution method in previous studies were 0.25% (Bassett *et al.*, 1990) and 0.4 - 0.7% (Griffin & Markham, 2000) for *A. flavus*, and 0.016% (Beylier, 1979), 0.2% (Southwell, 1993) and 0.3 - 0.4% (Griffin & Markham, 2000) for *A. niger*. These are all higher than those obtained in the present study. Using the broth microdilution method, two reports showed MICs of >0.08% (Inouye *et al.*, 2001a) and >2% (Vazquez *et al.*, 2000) for *A. fumigatus*. In the present study, MICs for *A. fumigatus* ranged from 0.06 – 0.12%, which is closer to the values obtained by Inouye *et al*. (2001) than those of Vazquez *et al*. (2000). This was despite the fact that the latter study used a broth microdilution method with RPMI 1640 and conditions very similar to those used in the present study. One difference between these two methods was that Vazquez *et al.* (2000) used a final inoculum concentration of 10^6 conidia/ml, which was much higher that that used in the present study (0.4×10^4 – 5.0×10^4), and this may have a significant influence on results. Very few MFCs of tea tree oil for filamentous fungi have been published. In addition, published values have simply indicated that the test organism was not killed at the highest concentrations chosen for the assay. For example, MFCs of tea tree oil of >0.08% for *A. fumigatus* (Inouye *et al.*, 2001a) and >2% for *A. fumigatus* and *A. nidulans* (Vazquez *et al.*, 2000) have been reported.

Data from the current study also showed that *Fusarium, Cladosporium* and *Alternaria* were inhibited and killed by 0.008 to 2% tea tree oil, however, no comparable MIC or MFC data have been published previously. The only study investigating the effects of tea tree oil on these fungi used growth inhibition assays and showed that the growth of two isolates each of *Fusarium* and *Alternaria* was inhibited by tea tree oil (Bishop & Thornton, 1997).

Tea tree oil in combination with boric acid, nystatin or miconazole

For *C. albicans* ATCC 10231, MICs of each agent alone were 0.5%, 0.25%, 2.0 μg/ml and 128 μg/ml for tea tree oil, boric acid, nystatin and miconazole, respectively. The lowest ∑ FIC values for each combination were 0.25 for tea tree oil and boric acid or nystatin, and 0.04 for tea tree oil and miconazole. Miconazole did not show fungicidal activity and showed inhibitory activity only, over a range of concentrations. The ∑ FIC values for all three combinations indicated synergy although this was to a much greater extent for the combination of tea tree oil and miconazole than for the other

combinations. For *C. glabrata* ATCC 15545, MICs of each agent alone were 0.25%, 1.0%, 8.0 μg/ml and 32 μg/ml for tea tree oil, boric acid, nystatin and miconazole, respectively. The lowest ∑ FIC values were 0.16 for tea tree oil and boric acid, 0.25 for tea tree oil and nystatin, and 0.19 for tea tree oil and miconazole. Again these values indicate synergy for all combinations.

5.1.2 Components

Yeasts

Using the broth microdilution method, the two components terpinen-4-ol and α-terpineol had the lowest MICs and MFCs, and were similar in activity. A second group of components including terpinolene, ρ-cymene, γ-terpinene and α-terpinene showed little activity. The activity of 1,8-cineole was approximately midway between these two groups. In contrast, data obtained by the macrodilution method showed little variation in the activity of components, with all components except α-terpinene and ρ-cymene having MIC and MFC values between 0.12 and 2%. Few publications have described the activity of tea tree oil components against yeasts.

The activity of the main components of several essential oils (not including tea tree oil) against *M. furfur* and *T. beigelii* has been investigated by a disc diffusion method (Adam *et al.*, 1998). Either no zones or only small zones of inhibition of 1 - 4 mm were found for γ-terpinene, α-pinene, ρ-cymene and 1,8-cineole. However, the relatively small amount of each agent (5 μl of component per disc) may explain the small or absent zones. The activity of the components of *Aframomum danielii* extract against six food spoilage yeasts has been investigated and MICs of 4.9 – 312 μg/ml and MFCs of 9 – 1250 μg/ml have been found for α-terpinene (Adegoke *et al.*, 2000). Also, MICs of α-pinene and 1,8-cineole ranged from 78 – 156 μg/ml and MFCs ranged from 312 – 625 μg/ml. In another study, MICs of 50 and 100 μg/ml terpinolene were found for *S. cerevisiae* and *C. utilis*, respectively (Himejima *et al.*, 1992). For comparison, 1250 μg/ml is the highest value listed above and is equivalent to 0.125% (w/v), which means the values cited above were slightly lower than those seen in the present study.

Two publications specifically investigating the activity of the components of tea tree oil (Carson & Riley, 1995c; Griffin *et al.*, 1999b) reported similar overall trends to those found by the microdilution method in the present study, each using one strain of *C. albicans*. Also, the study by Griffin *et al.* (1999) showed that 1,8 cineole and terpinolene had 'intermediate' activity, in contrast to Carson and Riley (1995) who showed that while 1,8 cineole had intermediate activity, the activity of terpinolene was more similar to the group of components with 'low' activity. As a whole, the results obtained by microdilution in the present study are most similar to those of Carson and Riley (1995), largely due to similarities between the methods used in the two studies. A more recent study determined MICs and

MFCs of components using glass tubes with shaking and found MICs of 0.125%, and MFCs of 0.5, 0.5 and 1.0% for terpinen-4-ol, γ-terpinene and 1,8-cineole, respectively (Cox *et al.*, 2001a). These results most closely resemble those obtained in the present work by the macrodilution method, again due to methodological similarities.

The tea tree oil components showed a range of activity, although the possibility of synergistic interactions between these components was not explored. One study investigated potential synergy between terpinen-4-ol, γ-terpinene, ρ-cymene and 1,8-cineole and showed mostly additive activity when components were investigated in combination, suggesting that synergistic activity against *Candida* was minimal (Cox *et al.*, 2001a).

With regard to the activity of components against the dermatophytes and filamentous fungi, great differences in the in vitro activity of the components were apparent, with components such as terpinen-4-ol and α-terpineol showing considerably more in vitro activity than ρ-cymene and γ-terpinene, although different results may be obtained using a macrodilution method. Previously published MIC data are 0.08% terpinen-4-ol for *A. fumigatus* (Inouye *et al.*, 2001a), 0.08% α-terpineol for *E. floccosum* and *T. rubrum*, 0.16% α-terpineol for *A. niger* and *Penicillium notatum* (Christoph *et al.*, 2000) and 0.05% eucalyptol (1,8-cineole) and 0.05% α-terpinene for *A. niger* (Moleyar & Narasimham, 1986). MICs and MFCs of α-terpinene have been reported as 0.062% and 0.125% for an isolate of *A. flavus* and 0.031% and 0.031% for an isolate *A. parasiticus* (Adegoke *et al.*, 2000). Of these previously published values, those in most agreement with the current study are the results for terpinen-4-ol and α-terpineol, whereas the low MICs previously reported for 1,8-cineole and α-terpinene were not confirmed.

Different results for the activity of tea tree oil components were seen depending on which susceptibility assay was used. Results for tea tree oil, terpinen-4-ol and α-terpineol were either equivalent or differed by only one dilution between methods, whereas results for the remaining components were considerably lower by the macrodilution method, compared to the microdilution method. Differences between the macro- and microdilution methods, such as assay volumes, degrees of sealing in each system and the vessels in which the tests are performed, may account for the dissimilar results. Previous studies with antifungal agents such as amphotericin B and fluconazole have generally found that MICs do not differ greatly when determined by both methods (Espinel-Ingroff *et al.*, 1995; Tornatore *et al.*, 1997; Pelletier *et al.*, 2000), which suggests that the differences seen in the current work not due to differences in volume but may be specific to the characteristics of tea tree oil components. Tea tree oil components show a range of solubilities (Griffin *et al.*, 1999b) and the components having the lowest solubility were those that showed high MICs in the

microdilution assay, but not in the macrodilution assay. This discrepancy may be related to the composition of the vessel in which the assay is performed, and the relationship between this and the solubility characteristics of tea tree oil components. Tea tree oil is known to interact with certain types of plastics and has been shown to migrate through, and deform, plastics such as low density polyethylene (LDPE) (Rowe, 1999). Similarly, in the microdilution assay the tea tree oil components may be dissolving into, or becoming irreversibly associated with, the polystyrene of the microdilution tray. Obviously less of the component is then in solution and available to interact with the microbial cells and this may explain, in part, the differences between the results obtained.

5.2 Mechanism of action studies

Tea tree oil and components were shown to have a range of immediate effects on *C. albicans* cells, such as altering permeability and membrane fluidity, and when cells were grown with tea tree oil they were shown to have adaptive responses, despite having decreased growth rates. These results will be discussed below.

5.2.1 Growth curves with tea tree oil, including studies with pre-conditioned cells

The rate of growth and final cell population of *C. albicans* at 24 h were diminished in the presence of 0.03 and 0.06%, but not 0.016% tea tree oil. These concentrations are all less than those shown to inhibit the growth of *C. albicans* in standard MIC assays.

Other essential oils and components such as cinnamon oil, thyme oil and carvacrol, have been shown to produce similar growth inhibiting effects, such as extending the lag phase and reducing the final cell yield, in both yeasts and bacteria (Ultee *et al.*, 1998; Ferhout *et al.*, 1999). These effects were apparent at concentrations below the MIC, similar to the present study. Essential oils (including tea tree oil) and components have been shown to inhibit respiration in fungi, including *C. albicans* (Bard *et al.*, 1988; Inouye *et al.*, 1998; Cox *et al.*, 2000) which may explain how tea tree oil is slowing microbial growth. In particular, one study reported that tea tree oil almost completely inhibited respiration at 0.75% and partially inhibited respiration in *C. albicans* at 0.125%, which was also the MIC of tea tree oil determined in their study (Cox *et al.*, 2000).

In contrast, the presence of some essential oils at very low concentrations can encourage microbial growth, as seen in the present study with 0.016% tea tree oil and in another study (Ferhout *et al.*, 1999). This enhanced growth may be because the organisms are using the essential oil as a growth substrate (Ferhout *et al.*, 1999; King & Dickinson, 2000) or because the presence of the oil induces the expression of stress responses, which confers protection to further stresses or insults and enabled enhanced growth (Ferhout *et al.*, 1999).

If the presence of tea tree oil induces stress responses, this would presumably be apparent in the studies with cells pre-conditioned with tea tree oil. However, cells that were pre-conditioned by growth with tea tree oil showed no significant differences from control cells, when both were subsequently grown in tea tree oil, suggesting that these cells had no greater overall fitness. In contrast, when *B. cereus* cells were exposed to sub-inhibitory amounts of carvacrol they had a greater survival rate upon subsequent exposure to carvacrol, compared to cells not pre-conditioned with carvacrol (Ultee *et al.*, 2000). This adaptation was attributed to a decrease in membrane fluidity and an alteration of membrane composition, which helped to maintain membrane function. In another study, the growth of pre-conditioned and non pre-conditioned *S. cerevisiae* cells was compared in media containing 20 mg/l cinnamic acid and the growth of non pre-conditioned cells was found to be significantly slower than the pre-conditioned cells (Chambel *et al.*, 1999). The greater fitness of pre-conditioned cells was attributed to a stimulation of membrane H^+-ATPase as a compensatory response to low levels of cinnamic acid. Previous studies with lipophilic antifungal compounds have shown that the H^+ ATPase of cells grown in the presence of these compounds often has a higher specific activity as compared to control cells (Alexandre *et al.*, 1993; Alexandre *et al.*, 1996; Mizoguchi & Hara, 1998; Chambel *et al.*, 1999). This is considered to be an adaptive response to counteract the non-specific leakage induced by these compounds (Chambel *et al.*, 1999; Cabral *et al.*, 2001) and also suggests that the functions of the PM ATPase are crucial in helping cells to survive the deleterious effects of the compound.

In the current study pre-conditioning did not enhance growth which suggests that either tea tree oil does not induce stress or compensatory responses, or that different kinds of assays are needed to show these effects. It is also possible that the effects of pre-conditioning may be more apparent if studies were conducted with only a single component of tea tree oil.

5.2.2 Assays investigating changes in permeability

Many compounds alter cell permeability. These include, but are not limited to, lipophilic compounds such as amphotericin B (Beggs, 1994), ketoconazole (Ånséhn & Nilsson, 1984), ibuprofen (Pina-Vaz *et al.*, 2000b), lidocaine (Pina-Vaz *et al.*, 2000a), ethanol (Mizoguchi & Hara, 1998), and terpenic oils or compounds such as thymol (Shapiro & Guggenheim, 1995), polygodial (Taniguchi *et al.*, 1988), geraniol (Bard *et al.*, 1988), oregano oil (Lambert *et al.*, 2001), and tea tree oil (Cox *et al.*, 2000). Changes in cell permeability may be interpreted as indicating that a compound has activity against the cell membrane (Denyer & Hugo, 1991) and, since terpenes alter both the permeability and properties of membranes (Sikkema *et al.*, 1995), it was considered essential to conduct these kinds of studies in this study.

Changes in cell permeability may be assessed by staining with the membrane-impermeable dye methylene blue (MB). This assay has commonly been used as an indication of yeast viability (Georgopapadakou *et al.*, 1987; Boyum & Guidotti, 1997; Laroche *et al.*, 2001) although the use of this particular dye has largely been superseded by fluorescent dyes, like propidium iodide (Pina-Vaz *et al.*, 2000b), ethidium bromide (Lambert *et al.*, 2001) and SYTOX green (Thevissen *et al.*, 1999). Another method used to assess alterations in cell permeability is the leakage of intracellular materials such as potassium ions, pentoses, nucleotides and proteins (Denyer & Hugo, 1991). In particular, the leakage of intracellular purines and pyrimidines can easily be assayed by measuring the absorbance of cell-free supernatants at 260 nm, the wavelength at which these compounds absorb strongly (Denyer & Hugo, 1991).

Tea tree oil, and several components, altered the permeability of *C. albicans* cells, as demonstrated by both the loss of selective permeability to methylene blue dye and the leakage of 260 nm-absorbing materials. Comparison of methylene blue staining from the current work, with propidium iodide staining of *C. albicans* after treatment with tea tree oil as determined previously (Cox *et al.*, 2000), showed similar results. After treatment of *C. albicans* cells with 0.25% tea tree oil for 30 min, Cox *et al.* (2000) found that approximately 25% of cells were stained with propidium iodide whereas in the present work only 5.72% of cells were stained with methylene blue after 30 min, but by 2 h this had increased to 30.63%.

As a generalisation, changes in permeability were dose-dependent and occurred at concentrations greater than or equal to the MIC, where MICs were determined by the macrodilution assay. The exception was terpinolene which caused permeability changes at concentrations below the MIC, however, this was only apparent by the assay for detecting the leakage of 260 nm-absorbing materials. The most rapid staining with MB was seen where cells were treated with 0.25% terpinen-4-ol and α-terpineol with 100% and 82.43% of cells stained after 30 min treatment, respectively. However, these concentrations are twice the MIC, equal to MFC concentrations and, as such, gross cellular damage may have occurred.

The components of tea tree oil showed different activities and this may be related to differences in their chemical composition, shape and solubilities. One distinct difference between components is that terpinen-4-ol, α-terpineol and 1,8-cineole are all oxygenated monoterpenes and the presence of the oxygen group may increase the solubility of these terpenes in non-polar solvents such as water. In fact, a correlation was seen in the present work between the solubility of components and permeability changes at low concentrations. For example, α-terpineol, terpinen-4-ol and 1,8-cineole caused permeability changes at 0.12, 0.12 and 0.25%, respectively, and of all the components tested these were also the most water soluble, with reported solubilities of 1827, 1491 and 907 ppm, respectively

(Griffin *et al.*, 1999b). Correlation of the particular activities of tea tree oil components with their solubilities and chemical properties will be discussed further in subsequent sections.

Comparison of results obtained by the dye exclusion and leakage assays showed that the overall trends in permeability changes caused by either tea tree oil or components were similar. The major difference between the two methods was that permeability changes were apparent by the methylene blue method either earlier, or in the absence of, the leakage of 260 nm-absorbing materials. A similar trend was seen previously where tea tree oil-treated *C. albicans* cells were permeable to propidium iodide but they did not leak potassium ions (Cox *et al.*, 2000). These results suggest that staining with membrane-impermeable dyes may be a more sensitive method for detecting leakage than the appearance of intracellular material external to cells. However, these results may simply reflect differences in the times required for dye molecules to diffuse into permeabilised cells compared to the time required for significant amounts of intracellular materials to diffuse out of cells. It has been suggested that the thickness and impermeability of the yeast cell wall may be responsible for preventing the diffusion of potassium ions into the external medium (Cox *et al.*, 2000). It is also recognised that whereas the first sign of changes in cell permeability may be the appearance of potassium ions in cell-free preparations, the appearance of 260 nm-absorbing materials in cell supernatants does not usually occur as quickly (Lambert & Hammond, 1973; Denyer & Hugo, 1991).

The changes in cell permeability induced by tea tree oil and components could be explained by proposing that the components of tea tree oil act as ionophores. The action of an ionophore is to shuttle ions such as K^+, Na^+ and H^+ across the plasma membrane (PM) (Kroll & Patchett, 1991). This dissipates the transmembrane electrochemical gradient or membrane potential (Δp), which consists of both an electrical potential ($\Delta\Psi$) and a pH gradient (ΔpH), and depolarises the membranes. This theory is supported by previous studies using model membranes which have shown that several cyclic hydrocarbons, including α-pinene, benzene and tetralin dissipate both ΔpH and $\Delta\Psi$ (Sikkema *et al.*, 1994). Further support comes from the demonstration of potassium ion leakage from tea tree oil-treated cells (Cox *et al.*, 2000). Also, several of the same lipophilic compounds that cause alterations in permeability and/or leakage, also dissipate Δp, suggesting that these effects often occur together. This effect has been seen for carvacrol and *B. cereus* (Ultee *et al.*, 1999), tetralin (a lipophilic organic compound) and a range of bacteria (Sikkema *et al.*, 1992) and ethanol dissipated Δp in *S. cerevisiae* (Cartwright *et al.*, 1986). The components of tea tree oil may dissipate Δp because of their lipophilic nature, which means that they preferentially partition into the lipophilic portion of the PM bilayer. This results in an expansion of the PM (Sikkema *et al.*, 1994) which may cause changes in PM properties and functions such as membrane polarity and fluidity, and the functioning of ion channels,

transport systems and other membrane-associated enzymes. One or more of these changes may cause an increase in the passive flux of ions across the membrane, resulting in the dissipation of Δp.

The proposed action of tea tree oil components as ionophores can only account for effects seen with low concentrations of oil or components since gross effects such as the leakage of large molecules like nucleotides are seen at higher concentrations of oil. Furthermore, since slight alterations in permeability or the dissipation of Δp alone are probably not sufficient to kill cells (Beggs, 1994), it is likely that there are other mechanisms causing more severe effects and these require investigation. Several studies have shown that where Δp has been dissipated there is a corresponding decrease in the internal pH of cells, and this has been shown after treatment with amphotericin B (Bracey *et al.*, 1998), ethanol or decanoic acid (Cartwright *et al.*, 1986; Alexandre *et al.*, 1998), carvacrol (Ultee *et al.*, 1999) or oregano oil (Lambert *et al.*, 2001). The decrease in internal pH caused by the rapid influx of protons may be detrimental to many cell functions, particularly the activity of enzymes, many of which operate optimally within specific pH ranges (Becker *et al.*, 1996).

These experiments have demonstrated that tea tree oil, and some components, alter the permeability of *C. albicans* and compromise the integrity of the cell membrane, possibly by acting as ionophores. The specific mechanisms by which permeability alterations and/or leakage occur have not been investigated fully and the results of further studies on several aspects of membrane functioning are discussed below.

5.2.3 Effects of pre-treatments on tea tree oil susceptibility

These experiments investigated changes in tea tree oil susceptibility in cells that had been pre-treated to inhibit particular cell functions. The pre-incubation of *C. albicans* cells with CCCP depolarises cellular membranes, which in yeasts includes plasma, mitochondrial and vacuolar membranes. Pre-incubation of cells with DES inhibits the plasma membrane ATPase, the main function of which is to maintain cell homeostasis by regulating internal pH and ion concentrations. In contrast, the pre-incubation of *C. albicans* with calcium ions may decrease susceptibility to tea tree oil since calcium ions are theorised to protect membranes by increasing their stability.

CCCP

CCCP is a proton ionophore that acts by shuttling ions across all cell membranes, resulting in their depolarisation. Where mitochondrial membranes are depolarised, respiration is uncoupled from oxidative phosphorylation, which prevents ATP production. In addition to energy production by mitochondria, the Δp of the PM is very important to the cell as it is used for facilitating the active transport of nutrients across the PM and for the export of toxic drugs (Koshlukova *et al.*, 1999).

Results showed that cells pre-treated with CCCP were acutely susceptible to tea tree oil, compared to non pre-treated cells. Since membrane depolarisation has varied effects on cell functioning, there may be several explanations for this result. Firstly, depolarisation of the PM causes dissipation of the transmembrane proton gradient, rendering cells unable to compensate for imbalances in ion homeostasis. Tea tree oil may represent a further challenge to cell homeostasis that proves lethal for cells, although the mechanisms by which this occurs are unknown. Next, depolarisation of the PM affects both the transport of solutes into the cell and the export of toxic substances out of the cell (Kroll & Patchett, 1991; van der Rest *et al.*, 1995). Of these, the inhibition of the export of toxic substances is perhaps the most relevant. *Candida* yeasts have two major families of membrane proteins that facilitate the transport of toxic compounds across membranes. The first of these, the ATP-binding cassette (ABC) transporters, is fuelled by ATP, whereas transport by the major facilitator superfamily (MFS) is driven by the PM potential (Del Sorbo *et al.*, 2000). These two systems are responsible for the export of a wide range of toxic substances (although it is not known whether this includes terpenes) and their overexpression has been implicated in drug resistance in yeasts (Kohli *et al.*, 2002). Depolarisation of the PM removes the driving force for the MFS transporters and depolarisation of mitochondrial membranes results in the depletion of the energy sources required to drive the ABC transporters, meaning that both transport systems would be severely compromised.

Lastly, and perhaps most importantly, depolarisation of the mitochondrial membranes would result in a drastic reduction in cellular energy production which would in turn compromise normal cell operations. In particular, the functioning of the PM ATPase and ABC transporters, which are both energy-dependent, would be compromised. If the PM ATPase was unable to function adequately, cells would have a decreased capacity to compensate for perturbations in cell homeostasis.

In summary, the pre-treatment of *C. albicans* cells with the ionophore CCCP rendered cells acutely susceptible to tea tree oil, which in turn demonstrated that Δp-dependent functions are critical for protecting cells against tea tree oil-induced damage. The nature of these functions has not yet been identified. Further studies with agents that specifically inhibit the two components of Δp, either $\Delta\Psi$ or ΔpH, may prove useful.

DES

Diethylstilboestrol (DES) is an inhibitor of the PM ATPase in yeasts and other organisms (Dawson *et al.*, 1986). The role of the PM ATPase, as mentioned briefly above, is to maintain cell homeostasis by regulating intracellular pH and the PM electrochemical gradient (Δp). In addition to the PM ATPase, yeast cells contain mitochondrial and vacuolar ATPases (van der Rest *et al.*, 1995) and each of these ATPases has inhibitors specific to that enzyme. DES specifically inhibits the PM ATPase (Henschke

& Rose, 1991) and has only slight inhibitory effects on mitochondrial and vacuolar ATPases (Henschke & Rose, 1991; van der Rest *et al.*, 1995).

Results showed that cells pre-treated with DES were significantly more susceptible to tea tree oil than cells pre-treated with vehicle only. This suggests that the PM ATPase has a critical role in protecting cells against damage or death induced by tea tree oil. This is not unexpected given that the PM ATPase is largely responsible for maintaining cell homeostasis. Also, an activated PM ATPase is one of the responses used by yeasts to counter the proton-translocating effects of different antifungal agents, as mentioned in section 5.2.1. The increased mortality of PM ATPase-depleted cells treated with tea tree oil may be because of the ionophoric action of the components of tea tree oil, which causes the movement of ions across the membrane. In the absence of the PM ATPase this ionic imbalance cannot be compensated for and the resulting loss of homeostasis may be lethal to cells.

Of note was a slight loss of viability in control cells pre-treated with DES, compared to those pre-treated with vehicle only. However, it has been noted previously that PM ATPase inhibitors can cause a non-specific increase in membrane permeability (Borst-Pauwels *et al.*, 1983) which may result in the death of some cells.

Calcium

The presence of additional cations has been shown to decrease the antimicrobial effects of agents such as miconazole, amphotericin B, polygodial (Yano *et al.*, 1991) and ethanol (Birch & Walker, 2000). This may be occurring by several mechanisms. Cations have been shown to inhibit interactions between antimicrobial agents and cell membranes (Ben-Josef *et al.*, 1999), or cations may form complexes with the antimicrobial agent which results in changes in its activity (Marshall & Piddock, 1994). Lastly, cations may stabilise cell membranes by interacting with membrane phospholipids which decreases the PM proton and anion permeability (Birch & Walker, 2000). This last mechanism has been proposed for instances where the susceptibility of microorganisms to compounds such as ethanol and polygodial has been significantly altered by the presence of cations.

The presence of cations did not significantly alter cell survival, which remained essentially unchanged in the presence of additional Ca^{2+} ions. This suggests that either the protective mechanisms of cations mentioned above are unlikely to be occurring, or if these mechanisms are occurring, their effects are so minimal as to be essentially overwhelmed by the effects of tea tree oil.

5.2.4 Inhibition of medium acidification in the presence of tea tree oil

When glucose is added to non-growing yeast cells, it is rapidly taken up by cells using their inbuilt transport systems (Manavathu *et al.*, 1999b; Lunde & Kubo, 2000). To power the transport system,

protons (hydrogen ions) are pumped out of the cells and this action results in a decrease in the pH of the external medium that the cells are suspended in. The enzyme responsible for the pumping out of protons is the PM H^+ATPase and it is located in the cell membrane. Since the PM H^+ATPase is largely responsible for regulating both intracellular pH and the PM electrochemical gradient (Δp), the proper functioning of this enzyme is considered essential for cell viability (Manavathu *et al.*, 1999b). Other lipophilic compounds such as decanoic acid, ethanol, nonylphenol, β-pinene and polygodial have been shown to inhibit glucose-induced medium acidification in yeasts (Uribe *et al.*, 1985; Alexandre *et al.*, 1993; Alexandre *et al.*, 1996; Karley *et al.*, 1997; Chambel *et al.*, 1999; Manavathu *et al.*, 1999b) and it was thought that tea tree oil may have similar effects on medium acidification.

Results showed an immediate glucose-induced decrease in pH for all cell suspensions, but by 10 min differences between tea tree oil-treated cells and controls became evident. After about 20 min, the pH of cell suspensions in the presence of tea tree oil had stopped decreasing whilst the external pH of control cells continued to decrease. There was also an obvious dose-dependent inhibition of medium acidification whereby smaller changes in external pH correlated with the presence of increasing amounts of tea tree oil. The concentrations that inhibited medium acidification were approximately equivalent to MIC amounts and results for all three test organisms were remarkably similar.

The inhibition of medium acidification seen in these studies suggests that the functioning of the PM ATPase is compromised. This may be occurring by direct effects of tea tree oil on the enzyme, or by indirect effects such as alterations in the lipid molecules surrounding the enzyme resulting in changes to the 'matrix' within which the enzyme normally functions (Kubo *et al.*, 2001). Alternatively, the inhibition of medium acidification may be caused by effects on cellular functions other than the PM or PM ATPase. The PM ATPase requires large amounts of ATP for normal functioning, but if the tea tree oil is inhibiting respiration (Uribe *et al.*, 1985; Cox *et al.*, 1998), the energy deficit caused by this may compromise ATPase function. The immediate decrease in pH (between 0 and 10 min) that was seen despite the presence of tea tree oil may be because cells were utilising intracellular ATP pools, but after the depletion of these pools, cells were then unable to continue the pumping out of protons, which coincided with the cessation of the decrease in external pH. Other studies have noted the depletion of intracellular ATP pools in cells treated with essential oil components (Helander *et al.*, 1998; Ultee *et al.*, 1999) and this was postulated as being due to either increased use or decreased production, or both. The dose-dependent inhibition of acidification that was seen may correspond to degrees of respiration inhibition caused by tea tree oil. Furthermore, if one or more tea tree oil components act as ionophores on both plasma and mitochondrial membranes, as suggested in earlier in this discussion, dissipation of Δp of the yeast mitochondria may, in part, explain how respiration is inhibited.

5.2.5 Trehalose accumulation

Trehalose is a non-reducing disaccharide that has been shown to have a range of physiological functions in both yeasts and bacteria. It has been shown to act as a protectant during freezing, a membrane stabiliser and a storage carbohydrate (Attfield, 1987; Majara *et al.*, 1996; Hounsa *et al.*, 1998). Trehalose is accumulated intracellularly in response to a range of environmental stresses such as osmotic, heat and cold shock, and treatment with agents such as ethanol, hydrogen peroxide and copper sulfate (Attfield, 1987; Majara *et al.*, 1996; Ribeiro *et al.*, 1999). Accumulation occurs in a manner very similar to the expression of heat shock proteins (hsps), both occurring rapidly after an increase in temperature and both declining when cells are returned to normal physiological temperatures (Attfield, 1987). The accumulation of trehalose is suggested to be a general cellular stress response (Attfield, 1987; Lee & Goldberg, 1998) and it was therefore of interest to see whether trehalose was accumulated by yeast cells during treatment with tea tree oil. However, results showed that high levels of trehalose were not accumulated by *S. cerevisiae* or *C. albicans* during treatment with tea tree oil. Control cells of *C. albicans* accumulated relatively small amounts of trehalose which may have been because the cells were just starting to enter stationary phase. In contrast, cells of *S. cerevisiae* accumulated some trehalose when treated with 0.06% tea tree oil, although levels did not approach those induced by heat shock. The differing responses of these yeasts may be partly due to the different metabolic characteristics of these yeasts, since *S. cerevisiae* is considered to be fermentative whereas *Candida* spp. are considered to be respiratory (García *et al.*, 1997). Also, *Saccharomyces* and *Candida* have been shown previously to have differing patterns of trehalose accumulation with *C. tropicalis* accumulating glycerol, but not trehalose, in response to salt stress (García *et al.*, 1997).

Data from the present study may indicate that these yeasts simply do not accumulate trehalose in response to treatment with tea tree oil. However, this seems unlikely since yeasts have been shown previously to produce trehalose in response to 'chemical' agents such as ethanol and copper sulfate and it appears to be a widespread general stress response (Attfield, 1987). An alternative explanation is that the yeast cells are unable to accumulate trehalose because tea tree oil is inhibiting growth and metabolism, disallowing energy-requiring processes such as trehalose production. In support of this theory, it has already been shown in this work that tea tree oil slows the growth of *C. albicans* in a dose-dependent manner, and studies by others have shown that tea tree oil inhibits respiration (Cox *et al.*, 2000). Although trehalose is not accumulated by these yeasts in response to tea tree oil, this does not rule out the possibility that other stress responses may occur.

5.2.6 Membrane fluidity

Membrane fluidity (MF) is a measure of the lateral motion of phospholipid molecules within a lipid bilayer and can be measured with the use of fluorescent probes (Lentz, 1988; Slavik, 1994). The probe used in this work, 1,6-diphenyl-1,3,5-hexatriene (DPH) inserts into the lipid bilayer between, and

parallel with, the fatty acyl chains of the lipid bilayer, although the DPH molecule will occasionally position itself between the monolayers of the membrane, where the ends of the fatty acyl chains meet (Lentz, 1988; Slavik, 1994; Laroche *et al.*, 2001). Membrane fluidity is most often measured by determining fluorescence polarisation or anisotropy, which is inversely related to MF, such that a decrease in anisotropy correspond to an increase in MF.

Changes after 24 h

The MF of cells grown for 24 h with low concentrations of tea tree oil was increased, and increases were dose-dependent with greater changes occurring with greater quantities of oil. Although the MF of yeast cells grown with sub-inhibitory concentrations of essential oils has not been investigated previously, increases have been seen where yeasts have been grown with sub-inhibitory concentrations of agents such as amphotericin B (Younsi *et al.*, 2000), decanoic acid (Alexandre *et al.*, 1996), copper sulfate (Fernandes *et al.*, 2000) and ethanol (Alexandre *et al.*, 1994). Also, fluconazole resistant strains of *C. albicans* have been shown to have increased MF as compared to susceptible control strains (Kohli *et al.*, 2002). The MF changes seen in all of these studies were accompanied by changes in membrane lipid composition. This is a well-known response of microorganisms to environmental stressors and some of the more common lipid changes in yeasts are an increase in unsaturated fatty acids (Georgopapadakou *et al.*, 1987; Alexandre *et al.*, 1993; Younsi *et al.*, 2000; Beney & Gervais, 2001), changes in the composition of the phospholipid headgroups, and the increased incorporation of sterols into the PM (Alexandre *et al.*, 1993; Younsi *et al.*, 2000). These modifications of membrane lipid composition have been proposed as occurring to re-establish and stabilise the membrane, to counteract the effects of the agent on the cell and to compensate for the lipid disordering caused by the agent (Weber & de Bont, 1996; Beney & Gervais, 2001). The accumulation of lipophilic agents in the lipid bilayer may cause an increase in the volume of either the lipid or headgroup area and changes in acyl chain or headgroup composition are said to compensate for these increases (Weber & de Bont, 1996). Furthermore, the partitioning of lipophilic compounds such as hydrocarbons into membranes is dependent on membrane composition and it has been shown that alterations in the membrane lipid composition decreases the partition coefficient for cyclic hydrocarbons into those membranes, which represents an adaptive response (Sikkema *et al.*, 1995). The increase in the MF of tea tree oil-grown cells is probably reflecting changes in the composition of the PM lipids, although analysis of the composition of the PM lipids of cells grown with tea tree oil would be required to determine if this has occurred. This may in turn alter the physical characteristics of the membrane and conformation and functioning of membrane embedded proteins (Slavik, 1994; Weber & de Bont, 1996).

A previous study where cells of *B. cereus* were adapted to grow in low concentrations of the terpene carvacrol found that these cells had lower MF than non-adapted cells (Ultee *et al.*, 2000), in contrast to results seen in the present study. This lowered MF was due to changes in fatty acid and head group

composition, where there was an increase in the proportion of shorter chain length fatty acids. Possible explanations for these dissimilar results are that the membrane compositional changes that occur in bacteria and yeasts may differ, and that different responses may occur after exposure to a single component compared to a whole essential oil. Since carvacrol is not dissimilar to several tea tree oil components, both in terms of solubility and structure, it would be interesting to grow *C. albicans* cells with sub-inhibitory concentrations of single components such as terpinen-4-ol and 1,8-cineole and investigate changes in MF and lipid composition, to determine how similar changes are to those elicited by carvacrol.

Immediate changes

Relatively short-term incubations of *C. albicans* cells with tea tree oil or several components caused significant increases in MF as determined by DPH fluorescence. For most components and tea tree oil, more than 10 but less than 30 min was required for effects to take place. The exception was 1,8-cineole, which caused significant changes after only 10 min and also caused the largest change in fluidity at 30 min. Changes in MF, such as those seen in the present work, are likely to be concentration, time and organism dependent although these variables were not investigated to any great extent in this work.

Previous studies have also found that the short-term treatment of yeasts or bacteria with terpenes such as geraniol, β-pinene or carvacrol caused increases in MF (Uribe *et al.*, 1985; Bard *et al.*, 1988; Ultee *et al.*, 2000). Furthermore, studies with cyclic hydrocarbons in artificial membrane systems have revealed a range of compounds that also decrease fluorescence polarisation measurements, which is equivalent to an increase in MF (Sikkema *et al.*, 1994; Engelke *et al.*, 1996). Interestingly, the study with β-pinene also showed that the greatest change in MF was actually in the mitochondria, with negligible MF changes occurring in the PM (Uribe *et al.*, 1985).

Evidence suggests that terpenes alter the physical properties of membranes as a result of their insertion between the fatty acyl chains of the lipid bilayer (Sikkema *et al.*, 1994). This disturbs the van der Waals interactions between acyl chains (Ultee *et al.*, 2000), disrupting lipid packing and decreasing lipid order (Weber & de Bont, 1996). This accumulation of hydrocarbon molecules in the bilayer results in an increase in lipid volume which causes membranes to swell and increase in thickness (Sikkema *et al.*, 1994). In the present study, the tea tree oil components, at equivalent concentrations, altered MF to varying degrees. This may reflect differences in the position of each terpene within the lipid bilayer. Cyclic hydrocarbons such as terpenes are known to accumulate in different parts of the lipid bilayer (Sikkema *et al.*, 1995; Bouchard *et al.*, 1996) and the position or region in which they accumulate determines which changes in membrane properties occur. The position of hydrocarbons within the bilayer is thought to depend on the hydrophobicity of the compound (Weber & de Bont,

1996) with the more hydrophobic compounds accumulating deeper within the fatty acyl chains whereas the less hydrophobic compounds, such as those with hydroxyl groups, interact more with the head group area of the phospholipids (Weber & de Bont, 1996). Some compounds have also been demonstrated to accumulate within the hydrophobic core of the lipid bilayer, also known as the bilayer midplane.

In addition to the position of terpenes within the bilayer, another important factor contributing to the capacity of each component to cause fluidity changes is their solubility, particularly in membranes. A direct relationship has been found between the hydrophobicity of a compound and both its ability to partition into lipid bilayers and its toxicity (Sikkema *et al.*, 1994; Weber & de Bont, 1996). The octanol/water partition coefficient (K_{ow}) is used to express the partitioning of a lipophilic compound in an octanol/water two phase system which mirrors the partitioning of these same compounds into biological membranes. As a generalisation, compounds with log K_{ow} values of 1 - 5 are considered toxic to microorganisms (Heipieper *et al.*, 1994). Compounds where the log K_{ow} value exceeds 5 are considered essentially non-toxic for microorganisms as the extremely low water solubility of these compounds limits their bioavailability (Weber & de Bont, 1996). Of the components tested in the present study, 1,8-cineole had the lowest log K_{ow} value of 2.84 and values for the remaining compounds fell between 3.26 for terpinen-4-ol and 4.36 for γ-terpinene, as shown in Table 1.2 (Griffin *et al.*, 1999a). There was no obvious correlation between log K_{ow} and changes in MF, with the exception of 1,8-cineole which had the lowest log K_{ow} and produced the largest change in MF.

These experiments have shown that the fluidity of the cell membranes of *C. albicans* is altered by treatment with tea tree oil and components. Several important methodological factors need to be considered. Firstly, fluidity in the current work was estimated by fluorescence intensity measurements whereas it is considered more appropriate to measure fluorescence polarisation or anisotropy. This is because lipid bilayers are considered to be highly anisotropic, which relates to the rates and axes of motion of the phospholipid molecules with the bilayer, and to the vast differences in the physical properties encountered in different areas within the bilayer (Yeagle, 1993; Weber & de Bont, 1996). Secondly, previous studies have shown that different membrane fluidity results can be obtained with different probes (Ansari & Prasad, 1993b; Swan & Watson, 1997). In particular, this may occur where the test compound accumulates in a particular region of the bilayer and the probe has accumulated elsewhere, meaning that changes induced by the accumulation of the test compound will not be reflected by probe movement. Sikkema *et al*. (Sikkema *et al.*, 1994) observed no changes in the MF of liposomes treated with cyclic hydrocarbons when TMA-DPH was used as a probe, compared to changes seen DPH. They interpreted this as indicative that the cyclic hydrocarbons partition into the more hydrophobic central part of the membrane which is where they suggest the probe DPH is also localised.

Much remains to be determined with regard to the ways that the components of tea tree oil, and other aromatic hydrocarbons, interact with cell membranes. The preferred sites of accumulation for each compound has yet to be determined, whether this is within both the inner and outer monolayers, or within the bilayer midplane of the PM. The effects of swelling and expansion of the membrane on membrane-associated functions or membrane-embedded enzymes also requires investigation. The possibility remains that fluidity changes also occur in mitochondrial and vacuolar membranes. The chemical composition, physical structure and solubility of each compound may play a defining role in this.

5.2.7 Summary of mechanism of action studies

Many different inferences and conclusions can be drawn from the results of these mechanisms of action studies. In particular, drawing from both the results obtained in these studies and observations made by others, the following hypothetical sequence of events may occur in the short term after yeast cells have come into contact with tea tree oil. The first event is that the components of tea tree oil gain access to the PM by passively diffusing through the cell wall. Components then insert into the PM in a manner dependent on their chemical composition, structure and solubility. This results in a swelling and expansion of the PM and an increase in MF. This causes an increase in the passive movement of ions across the membrane and a loss of membrane polarity, effects consistent with the action of an ionophore. With increasing concentrations of tea tree oil, there is a loss of intracellular potassium ions and an influx of hydrogen ions which leads to acidification of the cell interior. An increase in the specific activity of the PM ATPase occurs to counter the passive movement of ions, and this consumes large amounts of intracellular ATP, leading to depletion of the intracellular ATP pool and a reduction in the rate of growth. The degree of ion movement eventually becomes too large for the PM ATPase to compensate for, leading to a complete loss of ion homeostasis. At some time, the components of tea tree oil gain entry to the cell interior, possibly again by passive diffusion, and depolarise mitochondrial membranes, inhibiting respiration. The functioning of the PM ATPase is then inhibited, either because of a lack of available ATP or conformational changes in the PM associated with the increased MF. Gross effects such as the leakage of large intracellular molecules occur which may coincide with cell death.

Although the mechanisms described above are largely concentrated on the structure and functioning of cell membranes, other effects on cells cannot be ruled out. Once the components of tea tree oil have gained entry to the cell interior, they may affect any number of intracellular functions such as the synthesis of macromolecules. It is also evident that the activities and different effects of tea tree oil and components depends on several factors, including concentration, individual water and membrane

solubilities, and possibly the shape of the molecules, including the presence of, or number of double bonds.

In conclusion, tea tree oil and components have a range of effects on yeast cells. Many, if not all of these effects relate to deleterious changes in membrane functioning caused by the intercalation of tea tree oil components between the phospholipid molecules of the lipid bilayer. At low concentrations, yeasts are able to compensate for these effects but at higher concentrations these effects cannot be overcome and cell death ensues. Although these studies have illustrated some of the factors contributing to either the survival or death of cells treated with tea tree oil, more studies are required to show additional aspects of cell survival or death, and to better understand those seen in this work.

5.3 Implications for in vivo efficacy and clinical trials

These studies have indicated that tea tree oil and some components have in vitro activity against yeasts, dermatophytes and other filamentous fungi. The aim now must be to achieve clinical success will tea tree oil.

With regard to *Candida* infections, only one clinical evaluation has been published and this describes a case series of patients with oral candidiasis (Jandourek *et al.*, 1998). The series included 13 patients who were HIV positive and had already failed treatment with a 14 day course of oral fluconazole. Patients were treated with 15 ml of tea tree solution four times a day for up to 28 days. After treatment of the 12 evaluable patients, two were cured, six were improved, four were unchanged and one patient had deteriorated. Overall, eight patients had a clinical response, and seven had a mycological response. These results show great promise for tea tree oil as a treatment for this infection, particularly since in these patients the infection was very difficult to treat. Another *Candida* infection that would be suited to tea tree oil treatment is vaginal candidiasis or thrush, however the effectiveness of tea tree oil in treating this infection remains to be seen.

Most of the trials describing the use of tea tree oil or tea tree oil products for dermatophyte infections (tinea pedis or onychomycosis) have shown only moderate success (Tong *et al.*, 1992; Buck *et al.*, 1994; Syed *et al.*, 1999). However, two trials were for onychomycosis which is believed to be unresponsive to topical treatment and a high rate of cure should not necessarily be expected (Weitzman & Summerbell, 1995b). A more recent trial investigating tinea pedis showed reasonable clinical efficacy with solutions of either 25% or 50% tea tree oil (Satchell *et al.*, 2002). This last trial is encouraging as it indicates that clinical success with tea tree oil can be achieved.

Chapter 6: Implications and recommendations

There is now sufficient data to support the use of tea tree oil as a topical antifungal agent. The next step would be to carefully formulate and evaluate tea tree oil products for the treatment of specific fungal infections. The sorts of infections or conditions that are associated with fungi and may be suitable for treatment with topical tea tree oil include oral or vaginal candidiasis (caused predominantly by *C. albicans*), tinea and ringworm (caused by dermatophytes) and dandruff and seborrhoeic dermatitis (caused by *Malassezia* yeasts). Obviously these different infections have different etiologies and in all likelihood will require different treatment regimes, which must be of prime consideration in the design of therapeutic products for each condition or infection.

Another use of tea tree oil supported by these data is the utilisation of tea tree oil products in air conditioning systems to reduce fungal loads in either the air handling system itself or in air. Whilst data from the present study indicate activity against the sorts of organisms commonly found as contaminants, experiments with aerosolised tea tree oil either in the laboratory or in real situations would be required to determine if fungal loads are reduced under these conditions.

The results of this study need to be disseminated and publicised by publication in scientific journals, industry newsletters and by presentations at scientific and industry meetings. To date, the following publications have resulted from this work;

Hammer KA, Carson CF & Riley TV. **2002**. In vitro activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi. Journal of Antimicrobial Chemotherapy **50:** 195-199

Hammer KA, Carson CF & Riley TV. 2002. Effects of sub-inhibitory concentrations of *Melaleuca alternifolia* (tea tree) oil on *Candida albicans.* In Program and abstracts of the Australian Society for Microbiology Annual Scientific Meeting and Exhibition, 29 Sept - 3 Oct 2002, Melbourne, Australia. Abstr. PP03.4

Hammer KA, Carson CF & Riley TV. **2001**. In vitro activity of *Melaleuca alternifolia* (tea tree) oil against filamentous fungi. *In* Program and Abstracts of the Australian Society for Antimicrobials, 5-7 April, 2001, Melbourne, Australia. Abstr. 07

Hammer KA, Carson CF & Riley TV. 2001. *Melaleuca alternifolia* (tea tree) oil and components alter the permeability of *Candida albicans*. In Program and abstracts of the Australian Society for Microbiology Annual Scientific Meeting and Exhibition, 30 Sept - 5 Oct 2001, Perth, Australia. Abstr. PP4.1, p. A75

The data contained within this report may be used in the development of new antifungal products, or expansion of existing ranges of products which may in turn benefit the tea tree oil industry commercially.

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