Antifungal activity of
Melaleuca alternifolia
(tea tree) oil

by

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This thesis is presented for the degree of
Doctor of Philosophy of the University of Western Australia

2002

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Summary

Natural medicines have become a popular alternative to conventional medications. In particular, the essential oil of *Melaleuca alternifolia*, also known as tea tree oil, has become accepted in the community, and increasingly by medical practitioners, as a legitimate alternative to conventional therapies. The growing acceptance of tea tree oil by healthcare professionals may be attributed in part, to the sound scientific data that has been published mainly on the antibacterial properties of the oil. Unfortunately, the antifungal properties of tea tree oil have not been thoroughly investigated. Therefore, the purpose of this thesis was to redress this imbalance and investigate the antifungal activity of tea tree oil. Studies were focused initially on determining the antifungal susceptibilities of a wide range of fungi to both tea tree oil and its components. Then further investigations were conducted to establish the mechanism of action of tea tree oil and components against fungi, in particular *Candida albicans*.

In vitro susceptibility testing using the microdilution method showed that yeasts from the genera *Candida, Cryptococcus, Malassezia, Rhodotorula, Saccharomyces* and *Trichosporon* (n = 207) had minimum inhibitory concentrations (MICs) in the range of 0.016 - 1.0% and minimum fungicidal concentrations (MFCs) in the range of 0.06 - 1% (v/v). Dermatophytes (n = 106) of the genera *Epidermophyton, Microsporum* and *Trichophyton* tested against tea tree oil and griseofulvin by the broth microdilution method showed MICs and MFCs of tea tree oil that ranged from 0.004 - 0.06% and <0.03 - 1% (v/v), respectively, and griseofulvin MICs that ranged from 0.25 - 2 µg/ml. Ranges of MICs and MFCs of tea tree oil for *Alternaria, Aspergillus, Cladosporium, Fusarium* and *Penicillium* spp. (n = 78) were 0.008 - 0.25% and 0.06 - 8% (v/v), respectively. Tea tree oil had the least fungicidal activity against isolates of *Aspergillus niger*, two of which had MFCs of 8%.

The activity of the components against fungi was investigated and, by the broth microdilution method, terpinen-4-ol and α-terpineol had the greatest activity against yeasts (n = 6), followed by 1,8 cineole. The components with poor activity were α-terpinene, γ-terpinene, terpinolene and ρ-cymene, and these 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 (n = 8), 1,8-
cineole and terpinolene had moderate activity, and \( \alpha \)-terpinene, \( \gamma \)-terpinene and \( p \)-cymene showed little activity, as determined by the broth microdilution assay. 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 in the microdilution assay, were considerably lower when determined by the macrodilution method. This indicated a degree of variation in results that was dependent on the method used.

Time kill experiments with tea tree oil at 4 \( \times \) MFC for dermatophytes and 1 \( \times \) MFC for filamentous fungi demonstrated a comparatively slow rate of kill, with three of the four test organisms still viable after 8 h treatment with tea tree oil. However, no organisms could be recovered after 24 h treatment. Comparison of the susceptibility to tea tree oil of germinated and non-germinated \( A. niger \) conidia showed that germinated conidia were significantly more susceptible than non-germinated conidia. Germinated conidia were not recovered from the 0.12% treatment whereas the viability of the non-germinated conidia remained unaffected at all concentrations up to and including 0.25%. Time kill studies with \( C. albicans \) showed that organisms were rapidly killed when treated with 0.5 and 1.0% tea tree oil, whereas 0.25% produced a slower kill and very little killing was evident with 0.12%. Similarly, negligible changes in cell permeability occurred when cells were treated with 0.12% tea tree oil, whereas moderate changes occurred with 0.25% and rapid changes occurred at 0.5 and 1%. Studies of permeability changes after treatment with tea tree oil components showed that terpinen-4-ol, \( \alpha \)-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.

The membrane fluidity of cells treated with tea tree oil or components for 10 and 30 min was significantly altered after 30 min treatment with all components except \( \gamma \)-terpinene. Membrane fluidity was increased after treatment with all components except \( \alpha \)-terpineol, which caused a decrease in fluidity. The only component causing a significant increase in membrane fluidity after 10 min was 1,8-cineole.
Tea tree oil inhibited glucose-induced acidification of the external medium of yeast cell suspensions at approximately MIC concentrations. Inhibition was dose-dependent and apparent within a relatively short time (10 - 20 min). Studies on cells that had been pre-treated with diethylstilboestrol to inhibit the plasma membrane ATPase, or carbonylcyanide m-chlorophenyl hydrazone to depolarise cell membranes, showed that these cells were acutely susceptible to tea tree oil compared to cells that were not pre-treated with either agent. These investigations suggest that tea tree oil affects cell membranes, and that both intact energy production and the plasma membrane ATPase are 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 was 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. Cells grown with tea tree oil did not accumulate trehalose, a disaccharide that has been shown to accumulate intracellularly in response to a range of external challenges. This result was unanticipated and it was 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.

The effects of tea tree oil on a proposed virulence factor of *C. albicans*, germ tube formation (GTF), were also investigated. GTF was inhibited in the presence of 0.03, 0.06, 0.012 and 0.25% tea tree oil but remained unaffected at the lower concentrations of 0.004, 0.008 and 0.016% tea tree oil. In further experiments, cells that were pre-treated with tea tree oil, washed and resuspended in fresh horse serum had a slowed rate of GTF, suggestive of a post-antifungal effect.

In conclusion, the work presented in this thesis shows that tea tree oil and several of its components have activity against a wide range of medically important fungi. Furthermore, the results suggest that some mechanisms of action may be related, either directly or indirectly, to alterations in membrane properties and functions. These in vitro data indicate that tea tree oil may be effective in the treatment of fungal infections such as oral or vaginal candidiasis, dandruff, seborrhoeic dermatitis and tinea. In vivo efficacy now needs to be confirmed by clinical trials.
Statement/Declaration

Except where duly acknowledged, all aspects of the work reported in this thesis have been performed by myself.

Katherine A. Hammer
Acknowledgments

The assistance of many people in the production of this thesis is gratefully acknowledged. I would like to specifically thank the following people;

- My supervisors Thomas and Christine for their encouragement, scientific input and guidance, and for fostering the scientist in me. Although not formally my supervisors, Kerry Carson and Brian Mee have also filled these roles on several occasions.
- Ian and Gracie from Mycology PathCentre for providing me with numerous fungi to work with, Dr Chris Heath and others from Mycology at RPH for proving yeast strains, Drew Randall in Special Chemistry, Clinical Biochemistry, PathCentre, for help with the fluorescence spectrophotometer and all other staff of UWA, PathCentre and other institutions who have been of assistance over the years.
- Although in all likelihood they will never read this acknowledgement or thesis, thanks go to all the people I’ve contacted with queries about their work (J. Leeming, P. Mayser, E. Gueho, R. Hancock and A. De Lucca, to name a few) because their informative, enthusiastic and encouraging responses reminded me that I belong to an extraordinary global scientific community.
- All fellow students and employees, for their friendship, companionship and scientific stimulation. Special thanks go to Christine and Josie for the friendship, laughs and multitude of in-jokes.
- My family and friends for just being around and being themselves. In particular, very special thanks go to Andrew, for his patience, gentleness and encouragement, and for being a rock in my life.
- Much of the work produced in this thesis was made possible by the funding provided by both Australian Bodycare Pty. Ltd. and the Rural Industries Research and Development Committee (RIRDC). In addition to these groups, thanks go to individuals from these, and other, tea tree oil industry bodies who have been supportive of this project.
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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BHIB</td>
<td>brain heart infusion broth</td>
</tr>
<tr>
<td>CBS</td>
<td>Centraalbureau voor Schimmelcultures</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonylcyanide (m)-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilboestrol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DPH</td>
<td>1,6-diphenyl-1,3,5-hexatriene</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FIC</td>
<td>fractional inhibitory concentration</td>
</tr>
<tr>
<td>g</td>
<td>force of gravity</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GC</td>
<td>germinated conidia</td>
</tr>
<tr>
<td>GTF</td>
<td>germ tube formation</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HS</td>
<td>horse serum</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IC(_{50})</td>
<td>concentration inhibitory to 50% of the population</td>
</tr>
<tr>
<td>ISO</td>
<td>International Standards Organisation</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>LD(_{50})</td>
<td>concentration lethal to 50% of the population</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>m</td>
<td>metre</td>
</tr>
<tr>
<td>MB</td>
<td>methylene blue</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MBC</td>
<td>minimum bactericidal concentration</td>
</tr>
<tr>
<td>MF</td>
<td>membrane fluidity</td>
</tr>
<tr>
<td>MFC</td>
<td>minimum fungicidal concentration</td>
</tr>
<tr>
<td>MFS</td>
<td>major facilitator superfamily</td>
</tr>
<tr>
<td>MHB</td>
<td>Mueller Hinton broth</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MOPS</td>
<td>morpholino propane sulfonic acid</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin resistance <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>NCCLS</td>
<td>National Committee for Clinical Laboratory Standards</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Type Culture Collection</td>
</tr>
<tr>
<td>NGC</td>
<td>non-germinated conidia</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Wales</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PCR-REA</td>
<td>PCR restriction endonuclease analysis</td>
</tr>
<tr>
<td>PDA</td>
<td>potato dextrose agar</td>
</tr>
<tr>
<td>PDB</td>
<td>potato dextrose broth</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>R</td>
<td>resistant</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SABC</td>
<td>Sabouraud dextrose agar with chloramphenicol</td>
</tr>
<tr>
<td>SDA</td>
<td>Sabouraud dextrose agar</td>
</tr>
<tr>
<td>SDB</td>
<td>Sabouraud dextrose broth</td>
</tr>
<tr>
<td>SDW</td>
<td>sterile distilled water</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
</tbody>
</table>
spp. species (plural)
T transmission
TMA-DPH 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate
TTO tea tree oil
U unit
v/v volume for volume
wt weight
w/v weight for volume
YEPPG yeast extract peptone glucose (broth)
Publications

**JOURNAL ARTICLES**


**CONFERENCE ABSTRACTS**


Chapter 1 - Literature review

1.1 Characteristics of Melaleuca alternifolia (tea tree) oil

The volatile essential oil of Melaleuca alternifolia (Maiden and Betche) Cheel is known as tea tree oil, or melaleuca oil. The Melaleuca genus belongs to the Myrtaceae family and contains approximately 230 species, almost all of which are native to Australia (Craven, 1999). M. alternifolia trees are also known as narrow-leaved paperbarks and are native to the swampy, sub-tropical, coastal areas of northern New South Wales and southern Queensland (Swords & Hunter, 1978). When left to grow naturally, M. alternifolia trees reach heights of approximately 5-8 metres (Colton & Murtagh, 1999). Trees older than three years flower typically in October and November (Lassak & McCarthy, 1983; Baker, 1999) and flowers are produced in loose, white to creamy coloured terminal spikes, which can give trees a 'fluffy' appearance (Weiss, 1997).

What is meant by the colloquial terms 'tea tree oil' or 'tea tree' can at times be unclear. In addition to referring to the essential oil of M. alternifolia, the name tea tree has also been used to refer to ornamental household plants such as members of the Leptospermum genus. Also, two essential oils from New Zealand, ‘kanuka oil’ from Kunzea ericoides and ‘manuka oil’ from Leptospermum scoparium have been referred to as tea tree oils (Christoph et al., 2000). Furthermore, the term 'tea tree oil' refers to a specific composition of oil which does not stipulate a particular botanical source (International Organisation for Standardisation, 1996). As such, commercial tea tree oil that meets the compositional requirements may be sourced from chemotypes of M. dissitiflora, M. linariifolia and M. uncinata (Murtagh, 1999). However, this is a relatively infrequent occurrence since most tea tree oil is now produced from large-scale plantations of M. alternifolia plants. Other Melaleuca oils, although not referred to as tea tree oils, are cajuput oil from M. cajuputi (Doran, 1999) and niaouli oil from M. quinquenervia (Trilles et al., 1999). In this thesis, the term tea tree oil will refer only to the oil of M. alternifolia.

1.1.1 Production and extraction

Originally, tea tree foliage was sourced by bush cutters who would cut the foliage from natural stands of trees. However, during the 1980s, large commercial plantations of M.
M. alternifolia trees were established which essentially replaced the bush cutting system with mechanical harvesting (Brophy et al., 1989; Johns et al., 1992).

Modern commercial plantations are typically established by planting seedlings in rows at high density. Newly planted seedlings are ready for harvesting after 1-3 years, depending on the climate and rate of tree growth. Harvesting involves cutting off the whole plant approximately 15 cm above ground level and then chopping the stems and foliage into lengths of 10 – 30 cm (Colton & Murtagh, 1999). These chopped sections are held within a large bin or hopper which is then used to transport the plant matter to the distillation area. The tree stumps left after harvesting coppice readily and vigorously with new shoots appearing as soon as a few weeks after harvesting (Colton & Murtagh, 1999). Trees are usually reharvested every 6 - 18 months, with the shorter harvest periods occurring in the warmer climates where plants grow more vigorously (Colton & Murtagh, 1999). It is not known how long trees can be harvested in this manner, however some trees in natural stands have been harvested regularly for more than 50 years (Williams & Home, 1988).

Commercial tea tree oil is extracted by steam distillation. This is achieved by passing steam through the closed container which holds the tree stems and foliage. During this process, the oil sacs which are borne on the undersides of the leaves burst, releasing the oil into the steam. The vapour is then passed through a condenser where the steam condenses to water and the oil condenses to liquid and is separated off (Johns et al., 1992). The average yield of oil may vary according to the season or month of harvest, temperature and other environmental conditions (Murtagh, 1999) but is typically in the range of 1-2% (volume of oil per weight fresh leaf) (Swords & Hunter, 1978; Johns et al., 1992). Tea tree oil is a clear to pale yellow liquid (International Organisation for Standardisation, 1996) that has been described as having a ‘strong greenish aroma’ (Kawakami et al., 1990).

### 1.1.2 Composition

There are several varieties, or chemotypes, of *M. alternifolia* that produce oils with distinct chemical compositions. Six chemotypes have been described as follows; terpinen-4-ol chemotype (1), terpinolene chemotype (2) and four types of 1,8-cineole chemotypes (3 - 6) (Homer et al., 2000). The terpinen-4-ol chemotype typically
contains levels of terpinen-4-ol of between 30-40% (Homer et al., 2000) and is the chemotype used in commercial tea tree oil plantations. This is the chemotype that will be discussed below.

*M. alternifolia* oil (terpinen-4-ol chemotype) contains more than 100 components which are mostly monoterpenes, sesquiterpenes and their related alcohols (Southwell, 1999). A comprehensive study which analysed the composition of 800 typical samples of *M. alternifolia* oil by gas chromatography and gas chromatography-mass spectrometry, identified 97 components (Brophy et al., 1989). This study has formed the basis of both the Australian Standard and International Standards, which stipulate *M. alternifolia* oil compositional range (Table 1.1). The two components terpinen-4-ol and 1,8-cineole are regarded as being of particular importance. A minimum level of 30% has been set for terpinen-4-ol as this has been thought of as the main antimicrobial component of the oil, and a maximum level of 15% has been stipulated for 1,8-cineole as it has had a reputation as a skin irritant, although this has now been discounted (Southwell et al., 1997).

### 1.1.2.1 Physical properties of *M. alternifolia* oil and components

Tea tree oil has a relative density of 0.885 – 0.906 (International Organisation for Standardisation, 1996), and is only sparingly soluble in water but is miscible with non-polar solvents. The insolvability in water is because the components that make up tea tree oil are largely non-polar hydrocarbons that have only relatively weak dispersion forces as their intermolecular forces. The major components of tea tree oil are terpenes or terpene alcohols. Terpenes are volatile, aromatic hydrocarbons and may be considered as polymers of isoprene, which has the formula C₅H₈ (Sharp, 1983). Monoterpenes such as terpinen-4-ol have the general formula of C₁₀H₁₆ (two isoprene units) and sesquiterpenes have the formula of C₁₅H₂₄ (three isoprene units). Terpenes may be open-chained compounds or may contain one or more C₆ rings (Sharp, 1983), as do the major components of tea tree oil. Some of the chemical and physical properties of tea tree oil components are shown in Table 1.2.

### 1.1.3 Medicinal properties

#### 1.1.3.1 History of medicinal use
<table>
<thead>
<tr>
<th>Component</th>
<th>ISO 4730 Range(^1)</th>
<th>Typical composition(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>terpinen-4-ol</td>
<td>≥ 30*</td>
<td>40.1</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>10 – 28</td>
<td>23.0</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>5 – 13</td>
<td>10.4</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>≤ 15*</td>
<td>5.1</td>
</tr>
<tr>
<td>terpinolene</td>
<td>1.5 – 5</td>
<td>3.1</td>
</tr>
<tr>
<td>ρ-cymene</td>
<td>0.5 – 12</td>
<td>2.9</td>
</tr>
<tr>
<td>α-pinene</td>
<td>1 – 6</td>
<td>2.6</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>1.5 – 8</td>
<td>2.4</td>
</tr>
<tr>
<td>aromadendrene</td>
<td>traces – 7</td>
<td>1.5</td>
</tr>
<tr>
<td>δ-cadinene</td>
<td>traces – 8</td>
<td>1.3</td>
</tr>
<tr>
<td>limonene</td>
<td>0.5 – 4</td>
<td>1.0</td>
</tr>
<tr>
<td>sabinene</td>
<td>traces - 3.5</td>
<td>0.2</td>
</tr>
<tr>
<td>globulol</td>
<td>traces - 3</td>
<td>0.2</td>
</tr>
<tr>
<td>viridiflorol</td>
<td>traces - 1.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* No upper or lower limit set; \(^1\) International Organisation for Standardisation, 1996; \(^2\) Brophy et al., 1989
### Table 1.2 Properties of tea tree oil components

<table>
<thead>
<tr>
<th>Component</th>
<th>Type of compound</th>
<th>Chemical formula</th>
<th>Solubility (ppm)</th>
<th>log $K_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>terpinen-4-ol</td>
<td>monocyclic terpene alcohol</td>
<td>$C_{10}H_{18}O$</td>
<td>1491</td>
<td>3.26</td>
</tr>
<tr>
<td>$\gamma$-terpine</td>
<td>monocyclic terpene</td>
<td>$C_{10}H_{16}$</td>
<td>1.0</td>
<td>4.36</td>
</tr>
<tr>
<td>$\alpha$-terpine</td>
<td>monocyclic terpene</td>
<td>$C_{10}H_{16}$</td>
<td>8.2</td>
<td>4.25</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>monocyclic terpene alcohol</td>
<td>$C_{10}H_{18}O$</td>
<td>907</td>
<td>2.84</td>
</tr>
<tr>
<td>$\alpha$-terpinolene</td>
<td>monocyclic terpene</td>
<td>$C_{10}H_{16}$</td>
<td>4.3</td>
<td>4.24</td>
</tr>
<tr>
<td>$\beta$-cymene</td>
<td>monocyclic terpene</td>
<td>$C_{10}H_{14}$</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>(+)-$\alpha$-pinene</td>
<td>dicyclic terpene</td>
<td>$C_{10}H_{16}$</td>
<td>0.57</td>
<td>4.44</td>
</tr>
<tr>
<td>$\alpha$-terpineol</td>
<td>monocyclic terpene alcohol</td>
<td>$C_{10}H_{18}O$</td>
<td>1827</td>
<td>3.28</td>
</tr>
<tr>
<td>aromadendrene</td>
<td>sesquiterpene</td>
<td>$C_{15}H_{24}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\delta$-cadinene</td>
<td>sesquiterpene</td>
<td>$C_{15}H_{24}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)-limonene</td>
<td>monocyclic terpene</td>
<td>$C_{10}H_{16}$</td>
<td>1.0</td>
<td>4.38</td>
</tr>
<tr>
<td>sabinene</td>
<td>dicyclic monoterpene</td>
<td>$C_{10}H_{16}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>globulol</td>
<td>sesquiterpene alcohol</td>
<td>$C_{15}H_{26}O$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Griffin et al., 1999b, 2 Griffin et al., 1999a
The earliest reported use of the *M. alternifolia* plant is the traditional use by the Bundjalung Aborigines of Northern New South Wales. Crushed leaves of 'tea trees' were inhaled to treat coughs and colds, or were sprinkled on wounds after which a poultice was applied (Shemesh & Mayo, 1991). In addition, tea tree leaves were soaked to make an infusion to treat sore throats or skin ailments (Low, 1991; Shemesh & Mayo, 1991). The Aborigines have also told of healing lakes which were lagoons into which *M. alternifolia* leaves had fallen and decayed over time (Altman, 1988).

Several reports appeared in medical journals in the 1930s describing the use of the oil in solutions for washing and dressing wounds, or for the treatment of conditions such as paronychia, ringworm, tinea, tonsillitis, gingivitis, thrush and sore throat (Humphery, 1930; Penfold & Morrison, 1937). Tea tree oil was apparently quite popular in the 1930s and 1940s, with Australian soldiers having the oil as part of their medical kits during World War II. During this time, production of the oil was ensured by making oil suppliers exempt from military service (Shemesh & Mayo, 1991).

The popularity and use of tea tree oil waned in the 1950s, with both the advent of antibiotics and a lack of consistent supply (Markham, 1999). Over the following two decades only a few reports were published describing the use of tea tree oil. The earliest of these was a study by Feinblatt (1960) investigating the treatment of furunculosis with neat *M. alternifolia* oil, which was painted over each sore 2 - 3 times daily. Results overall showed that sores treated with *M. alternifolia* oil healed more quickly than non-treated sores (Feinblatt, 1960). A study by Peña in 1962 investigated the use of *M. alternifolia* oil for treating trichomonal vaginitis, vaginal candidiasis, cervicitis and chronic endocervicitis and also found favourable outcomes (Peña, 1962). In 1972, Walker reported a series of cases of podiatric conditions including onychomycosis, inflamed corns and bunions, tinea pedis, cracked and peeling heels and bromhidrosis, all treated with *M. alternifolia* oil (at different concentrations for varying time periods) (Walker, 1972). While all of these studies produced mostly qualitative results, they indicated that interest in tea tree oil, primarily as a topical antimicrobial agent, was continuing.

With a resurgence of interest in alternative health care and natural medicines in the 1980s and 1990s, tea tree oil has again 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. Of all reported medicines, topically applied or inhaled tea tree oil was the most common, at 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.

1.1.3.2 Antimicrobial properties, *in vitro* and *in vivo*

**Assays for determining in vitro activity**

Several methods have been developed for assessing the antimicrobial activity of substances *in vitro*. These have largely been developed with conventional antibiotics in mind.

The disc or agar diffusion method involves the diffusion of a known amount of the test substance from a disc through an agar medium onto which the test organism has already been inoculated. After incubation a ‘zone of inhibition’ may be apparent around the disc and the size of this zone may be measured. This method is generally considered to be a quick and easy way of screening plant compounds for in vitro activity but is problematic in that many essential oils are hydrophobic and may not diffuse through the agar medium uniformly.

The broth and agar dilution methods involve diluting the substance of interest, usually in a two-fold step-wise fashion, in either a solid (agar) or liquid (broth) medium. The test organism is then inoculated either onto the agar surface or into the liquid medium and incubated. After a given time, the presence of growth is determined. The parameter that can be determined from these assays is the lowest concentration of the agent inhibiting growth, or the minimum inhibitory concentration (MIC). Also, the lowest concentration that is lethal to 99.9% of the inoculum, or the minimum bactericidal concentration (MBC), may be determined from the broth dilution assay by subculturing from each dilution.
Antibacterial activity

In the 1920s and 1930s, Dr A. R. Penfold and colleagues, of the Technological Museum in Sydney, published a series of papers characterising and describing the antimicrobial activity of tea tree oil, in addition to other Australian essential oils and essential oil components (Penfold & Grant, 1925; Penfold & Morrison, 1937). The antibacterial activity of the oils or components was assessed by determining the Rideal Walker coefficient, which compares the activity of each substance to phenol. The Rideal Walker coefficient for *M. alternifolia* oil was 11, meaning the oil was 11 times more active than phenol (Markham, 1999). This early work supported much of the subsequent use and promotion of the oil, particularly in Australia.

Only a few reports of the antibacterial activity of tea tree oil appear in the literature from 1940 to the 1980s. The earliest of these was published in 1955 by Atkinson and Brice (Atkinson & Brice, 1955), who assessed plants of the Myrtaceae family for antibacterial activity by both agar and broth dilution assays. Antibacterial titres (% v/v) as determined by the agar and broth dilution assays, respectively, were 0.63 and 0.31 for *Staphylococcus aureus*, 1.25 and 0.24 for *Salmonella typhi* and 0.31 and 0.10 for *Mycobacterium phlei* (Atkinson & Brice, 1955). Similarly, in 1974 Low and colleagues described the antibacterial activity of a number of essential oils from the Myrtaceae family. They used the agar dilution method of Atkinson and Brice and found MICs (% v/v) of 0.062 for *S. aureus* and 0.031 for *Salm. typhi*. They also used an assay where test organisms were exposed to each neat essential oil for 10 minutes only, after which viable organisms were recovered. With *M. alternifolia* oil, *S. aureus* could not be recovered whereas viable *Pseudomonas aeruginosa* were recovered (Low *et al.*, 1974).

In the study by Beylier in 1979, more than 100 oils were initially examined for antimicrobial activity, and 10 of these (including *M. alternifolia* oil) were selected for further investigation. The MIC (% v/v) ranges were 0.25 - 0.5 for *S. aureus*, 0.125 - 0.25 for *E. coli* and 4 for *P. aeruginosa* (Beylier, 1979). MICs for *Candida albicans* and *Aspergillus niger* were also determined in this study and these will be discussed in a later section.

In 1987, Walsh and Longstaff used both broth and agar dilution methods to assess 'Melasol', a product containing 40% tea tree oil, 13% isopropyl alcohol and 47% water
for activity against oral pathogens. MICs (% v/v) of Melasol were 0.08 for *S. aureus*, 0.16 for *Streptococcus faecalis*, 0.16 for *P. aeruginosa* and 0.08 for *Escherichia coli*, by the agar method (Walsh & Longstaff, 1987). These MICs are low compared to those obtained in the previous studies, especially considering that Melasol contains only 40% tea tree oil. The alcohol contained in the solution may account for this activity. A range of oral microorganisms such as *Actinomyces viscosus*, *Bacteroides gingivalis*, *Eikenella corroden* and *Strep. mutans* were also tested and MICs ranged from 0.02 - 0.08% (Walsh & Longstaff, 1987).

From the early 1990s onwards, many reports detailing the antimicrobial activity of tea tree oil appeared in the scientific literature. Although there was still a degree of discrepancy between the methods used in the different publications, often the MIC values reported were relatively similar. A summary of some of the published in vitro susceptibility data for bacteria is shown in Table 1.3. The majority of MICs and MBCs are in the range of 0.06% - 1.0%, however, MICs of more than 2% have been reported for some commensal skin staphylococci and micrococci, *Enterococcus faecalis* and *P. aeruginosa* (Hammer et al., 1996; Banes-Marshall et al., 2001). *P. aeruginosa* has been shown in several studies to have reduced susceptibility to tea tree oil, and this has been attributed to the outer membrane of this organism (Mann et al., 2000).

Resistance to tea tree oil *per se* has not been reported despite the medicinal use of the oil in Australia since the 1920s. However, Nelson (Nelson, 2000) reported that resistance in *S. aureus* can be induced in vitro. In this study, an initial screen of 100 methicillin resistant *S. aureus* (MRSA) isolates indicated that resistance was not occurring naturally in this population, with all isolates having tea tree oil MICs of <2.5%. The second part of the study examined resistance in five MRSA isolates which was experimentally induced by subculturing each isolate onto agar plates containing increments of tea tree oil up to 4.0%. MICs were then determined by a broth dilution method to verify susceptibility. All isolates had initial MICs of 0.25% whereas after induction of resistance MICs had increased to 1.0% for three isolates, and 2.0% and 16.0% for one isolate each (Nelson, 2000). In addition, Strains of *E. coli* displaying the multiple antibiotic resistance phenotype ('mar') have been shown to have reduced susceptibility to tea tree oil and components, although the relative reduction in susceptibility appears to be minimal (Gustafson et al., 2001). More data are needed to
Table 1.3 Susceptibility data for bacteria tested against *M. alternifolia* oil (% v/v)

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>MIC, MIC\textsubscript{range} or MIC\textsubscript{90}</th>
<th>MBC, MBC\textsubscript{range} or MBC\textsubscript{90}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>1.0^8</td>
<td>1.0^8</td>
</tr>
<tr>
<td>Actinomyces viscosus</td>
<td>0.6^6</td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>0.3^2</td>
<td></td>
</tr>
<tr>
<td>Bacteroides spp.</td>
<td>0.06^1, 0.5^1</td>
<td>0.06-0.12^1</td>
</tr>
<tr>
<td>Corynebacterium sp.</td>
<td>0.2-0.3^2, 2.0^8</td>
<td>2.0^8</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>0.5-0.75^2</td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis (vancomycin R)</td>
<td>0.5-1^4, &gt;8^10</td>
<td>0.5-1^4, &gt;8^10</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.25^3, 0.08^11</td>
<td>0.25^3, 0.08^11</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>&gt;0.6^6</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0.25^8, 0.3^2</td>
<td>0.25^8</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>2^1</td>
<td>2^1</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>0.06-0.5^8</td>
<td>0.25-6.0^8</td>
</tr>
<tr>
<td>Peptostreptococcus anaerobius</td>
<td>0.2^6, 0.25^1</td>
<td>0.03-0.12^1</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>0.11^6</td>
<td></td>
</tr>
<tr>
<td>Prevotella spp.</td>
<td>0.03^1, 0.25^1</td>
<td>0.03^1</td>
</tr>
<tr>
<td>Propionibacterium acnes</td>
<td>0.5^2, 0.31-0.63^5</td>
<td>0.5^13</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>0.08^11, 0.3^2, 2.0^10</td>
<td>4.0^10</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1-&gt;2^2, 1-8^10, 3.0^8</td>
<td>2-&gt;8^10, 3.0^8</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.63-1.25^5, 0.5^7, 10</td>
<td>1.0^10, 2.0^7</td>
</tr>
<tr>
<td>Staphylococcus aureus (methicillin R)</td>
<td>0.04^11, 0.25^4, 9</td>
<td>0.5^4, 0.5^9</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>0.63-1.25^5, 1.0^8</td>
<td>4.0^8</td>
</tr>
<tr>
<td>Staphylococcus hominis</td>
<td>0.5^8</td>
<td>4.0^8</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>0.12^12</td>
<td>0.25^12</td>
</tr>
</tbody>
</table>

corroborate these studies.

**Mechanism of antibacterial activity**

Tea tree oil at concentrations of 0.25 and 0.5% alters the permeability of cell membranes of *S. aureus* and *E. coli*, as evidenced by the leakage of intracellular potassium and 260 nm-absorbing material, and the uptake of propidium iodide in cells treated with tea tree oil (Cox et al., 1998; Cox et al., 2000; Cox et al., 2001b; Carson et al., 2002). Tea tree oil also inhibits respiration in the abovementioned organisms at concentrations of 0.25 to 0.5% (Cox et al., 2000).

Monoterpane compounds act on the cell membranes of bacteria and this is postulated as being due to the lipophilic nature of terpenes, meaning that these compounds preferentially partition into lipid-rich membranes (Sikkema et al., 1994; Cox et al., 2001b). The partitioning of these compounds into cell membranes over time causes an expansion of the membrane and an increase in membrane fluidity. These effects result in a loss of membrane integrity leading to the loss of intracellular compounds (Sikkema et al., 1994). Many of the inferences about the mechanism of action of tea tree oil can be drawn from studies investigating related compounds.

**Clinical data**

Despite the increasing amount of in vitro data for bacteria, few in vivo (or clinical) investigations have been performed. Clinical studies investigating the effects of tea tree oil treatment on acne, dental plaque formation and the elimination of MRSA colonisation have been published. An earlier study investigating furunculosis (Feinblatt, 1960) has been discussed in a previous section.

In an investigation of acne treatment, Bassett et al. (1990) compared the efficacy of 5% tea tree oil and 5% benzoyl peroxide for therapy, with 58 and 61 evaluable patients in each treatment group, respectively (Bassett et al., 1990). Patients were assessed at commencement, and at 1, 2, and 3 months. Parameters assessed were the numbers of inflamed and non-inflamed lesions, and a grade was given for oiliness, erythema, scaling, pruritis and dryness. The major findings of the study were that both treatments reduced the numbers of inflamed lesions, although benzoyl peroxide performed significantly better than tea tree oil. The benzoyl peroxide group also showed significantly less oiliness than the tea tree group, however the tea tree group showed
significantly less scaling, pruritis and dryness. Erythema did not differ between groups. Interestingly, significantly fewer overall side effects were reported by the tea tree oil group (27 of 61 patients) than the benzoyl peroxide group (50 of 63 patients).

A study comparing the effects of mouthwashes containing either approximately 0.34% tea tree oil, 0.1% chlorhexidine or placebo on plaque formation and vitality was performed using eight volunteers (Arweiler et al., 2000). On day zero, volunteers had their teeth professionally cleaned, and for the next four days they rinsed twice daily with one of the treatments and did not clean their teeth in any other manner. Teeth were clinically evaluated on days 1, 2, 3 and 4. Each mouthwash was evaluated in this manner, with a wash-out period of 10 days between the end of one treatment and the beginning of the next. The plaque index and plaque vitality from the tea tree oil mouthwash treatment did not differ from placebo mouthwash on any day, whereas the chlorhexidine mouthwash differed significantly on all days. Thus the tea tree oil treatment was considered ineffective at reducing plaque regrowth or the vitality of plaque organisms (Arweiler et al., 2000).

A pilot study conducted by Caelli et al. (2000) examined the effectiveness of a 4% tea tree oil nasal ointment and a 5% tea tree oil body wash for the eradication of MRSA carriage, as compared to conventional treatment of mupirocin nasal ointment and triclosan body wash. Of the 15 patients receiving conventional treatment, two were cleared and eight were chronic carriers at the end of therapy, compared to the tea tree group where five were cleared and three were chronic carriers. In addition, five patients from the conventional treatment group and seven from the tea tree oil group did not complete therapy. Unfortunately, due to the low patient numbers these differences were not statistically significant, although they indicate that tea tree oil therapy may be effective in decolonising MRSA carriers.

In addition to these clinical studies, there is a single case report of a woman who treated herself successfully with a 5 day course of tea tree oil pessaries after having been clinically diagnosed with bacterial vaginosis (Blackwell, 1991). Of the three studies described above, two are limited by low numbers of patients and all have some ambiguous or equivocal outcomes, indicating that much remains unknown about optimising tea tree oil efficacy in vivo.
Antiviral properties

The few studies that have investigated the antiviral properties of tea tree oil support the anecdotal notion that tea tree oil has antiviral properties. One study has indicated that tea tree oil can reduce the infectivity of tobacco mosaic virus on tobacco plants (Bishop, 1995). A field trial was conducted in which *Nicotiana glutinosa* plants were sprayed with 100, 250 or 500 ppm tea tree oil or control solutions, and all plants were then experimentally infected with tobacco mosaic virus. After 10 days, there were significantly fewer lesions per square centimetre of leaf of plants treated with tea tree oil as compared to controls (Bishop, 1995).

Schnitzler and colleagues investigated the activity of tea tree and eucalyptus oils against herpes simplex virus (HSV) (Schnitzler *et al.*, 2001). Briefly, the activity of tea tree oil was determined by incubating virus with varying concentrations of tea tree oil, and then using these treated viruses to infect cell monolayers. After 4 days the numbers of plaques formed by virus treated with tea tree oil, or untreated control virus, were determined and compared. The concentration of tea tree oil inhibiting 50% of plaque formation, as compared to controls, was 0.0009% for HSV1 and 0.0008% for HSV2. These studies also showed that at the higher concentration of 0.003%, tea tree oil reduced HSV1 titres by 98.2% and HSV2 titres by 93.0%. Also, by applying tea tree oil at different stages in the virus replicative cycle, tea tree oil was shown to have the greatest effect on free virus (prior to infecting cells) although when tea tree oil was applied during the adsorption period a reduction in plaque formation was seen also.

Further evidence for antiviral activity comes from a pilot study investigating the treatment of recurrent herpes labialis (cold sores) with a 6% tea tree oil gel or a placebo gel without tea tree oil (Carson *et al.*, 2001). Comparison of each patient group (both containing nine evaluable patients) at the end of the study showed that re-epithelialisation after treatment occurred after 9 days for the tea tree group and after 12.5 days for the placebo group. Other measures such as duration of virus positivity by culture or polymerase chain reaction (PCR), viral titres and time to crust formation were not significantly different, possibly due to small patient numbers.

The results of these studies indicate that tea tree oil may act against viruses in several ways. In addition to lethal effects directly on virus particles, tea tree oil can also affect
the way virus adsorbs to tissue culture cells and can cause a reduced rate of infection in tobacco plants.

**Antifungal properties**

The antifungal properties of tea tree oil are discussed comprehensively in section 1.3.

### 1.1.3.3 Other

In addition to antimicrobial properties, tea tree oil has been credited with activity against protozoa, dust mites, head lice (Downs *et al*., 2000) and scabies mites (*Sarcoptes scabiei*) (Walton *et al*., 2000), and also with analgesic (Markham, 1999) and anti-inflammatory activity (Hart *et al*., 2000; Brand *et al*., 2001; Brand *et al*., 2002).

Data regarding antiprotozoal activity is limited to two publications. Tea tree oil caused a 50% reduction in growth (as compared to controls) of the protozoa *Leishmania major* and *Trypanosoma brucei* at concentrations of 403 µg/ml and 0.5 µg/ml, respectively (Mikus *et al*., 2000). In a different study, tea tree oil at 300 µg/ml killed all cells of *Trichomonas vaginalis* (Viollon *et al*., 1996).

The activity of tea tree oil against house dust mites has also been shown in two studies. In the first study, the activity of several essential oils (including tea tree oil) against house dust mites was compared to that of benzyl benzoate, which is a standard treatment. Oils of citronella and tea tree were as effective as 0.5% benzyl benzoate and tea tree oil at a concentration of 0.8% killed 79% of mites after a 10 min exposure time (McDonald & Tovey, 1993). In the second study, tea tree oil was the most effective at killing the house dust mite *Dermatophagoides pteronyssinus*, when compared to lavender and lemon essential oils (Priestley *et al*., 1998). Tea tree oil at a concentration of 10% caused 100% immobility after 30 min and 100% mortality after 2 h.

Recent work investigating the anti-inflammatory properties of tea tree oil has demonstrated that terpinen-4-ol can inhibit the production of several inflammatory mediators (such as interleukins) by human blood peripheral monocytes (Hart *et al*., 2000). This suggests a mechanism by which tea tree oil may reduce the normal inflammatory response. Terpinen-4-ol also suppresses superoxide production by agonist-stimulated monocytes, but not neutrophils (Brand *et al*., 2001).
Interestingly, terpenes are used as transdermal penetration enhancers to increase the percutaneous penetration of topically applied drugs. Studies investigating the modes of action of these penetration enhancers have revealed the ways in which terpenes interact with both the stratum corneum and lipid membranes (Cal et al., 2001).

1.1.4 Toxicology of *M. alternifolia* oil and components

The toxicity of tea tree oil can be considered in two major areas; toxicity from ingestion and from topical application. Topical or dermal toxicity can be further divided into allergic and irritant types of reaction.

1.1.4.1 Oral toxicity

Tea tree is categorised as a Schedule 6 poison in Australia. According to the Drugs, Poisons and Controlled Substances Act 1981, substances classed within this category have “a moderate potential for causing harm, the extent of which can be reduced through the use of distinctive packaging with strong warnings and safety directions on the label”. To this end, neat tea tree oil is labelled that it must be kept out of the reach of children, is packaged with a childproof cap and is labelled ‘not to be taken internally’.

Tea tree oil can be toxic if ingested, as evidenced by studies with animals and from cases of human poisoning. An established laboratory method for measuring the toxicity of a substance is to determine the LD$_{50}$, which is the ingested dose that is lethal to 50% of a test population. This is expressed as units of toxic substance per kilogram of body weight. The LD$_{50}$ for tea tree oil in a rat model is 1.9 – 2.6 ml/kg (Russell, 1999). Although values determined in animal models are not necessarily directly related to human toxicity, the animal model data indicate that tea tree oil is orally toxic and therefore not suitable for internal use.

Several incidences of oral poisoning in humans have been reported in the literature. Such occurrences tend to be more dramatic in children because of their low body weight as compared to an adult. One such case report involves a 23 month old child who drank approximately 10 ml of tea tree oil. After a nap of approximately 30 min, the child was unsteady on his feet and appeared as if ‘drunk’. The child was taken to a hospital and treated with activated charcoal and sorbitol via a naso-gastric tube, and approximately 5
hours later he appeared to be asymptomatic. All other signs (such as respiratory rate, oxygen saturation, pupil reactivity, electrolytes and blood glucose) were normal throughout (Jacobs & Hornfeldt, 1994). The authors attribute the clinical symptoms to a central nervous system depression caused by the ingested tea tree oil.

A case of poisoning in an adult occurred when a patient drank approximately half a tea cup of tea tree oil corresponding to a dose of approximately 0.5-1.0 ml/kg body weight (Seawright, 1993). The patient was comatose for 12 hours, and was semi-conscious and hallucinatory for the following 36 hours. Symptoms of abdominal pain and diarrhoea continued for approximately 6 weeks after this. In another incident, a 60 year old man who swallowed one and a half teaspoonfuls of tea tree oil as a preventative for a cold presented with a red rash which covered his feet, knees, upper body and arms including his palms and elbows (Elliott, 1993). His hands, feet and face were also swollen. The rash and other symptoms gradually disappeared and approximately one week later he had more or less recovered.

1.1.4.2 Dermal toxicity

Systemic effects from topical tea tree oil application in humans or other animals appear to be very rare, judging by published reports. The topical application of significant quantities of eucalyptus oil (containing approximately 80% 1,8-cineole) to a 6 year old girl caused systemic effects, including slurred speech, drowsiness, vomiting, ataxia and unconsciousness, although the girl recovered fully within approximately 6 h (Darben et al., 1998). Severe systemic effects following dermal application of tea tree oil to cats have been reported (Bischoff & Guale, 1998). Three cats with shaved but intact skin had approximately 120 ml of neat tea tree oil applied to them topically as a flea repellant. Within 5 h all three cats were experiencing symptoms such as hypothermia, uncoordination, dehydration and trembling, and one was comatose (Bischoff & Guale, 1998). All cats were treated by a veterinarian and two recovered after 24 and 48 h, respectively, but the third cat was found dead 3 days after admission.

Irritant reactions

Irritant reactions are an inflammatory type of response caused when an irritating substance comes into contact with a body surface, usually the skin. Importantly, these reactions are often concentration dependant, but are not dependant on previous exposure
to the irritating agent. The irritant capacity of tea tree oil has been evaluated in both animal models and human trials, however only the human data will be discussed below.

The irritant capacity of tea tree oil has been investigated using an occlusive patch test method with Finn chambers (Southwell et al., 1997). Tea tree oil was prepared in white soft paraffin at a concentration of 25% and this mixture was applied in patch tests on the backs or upper arms of volunteers. After 24 h, patches were removed and the skin was checked for any reactions. A new chamber was then applied to the same area, and checked again 24 h later. This was repeated at subsequent 24 h intervals for a total of 21 days. None of the 25 participants produced an irritant reaction from these tests. However, three of the original 28 participants showed distinct allergic reactions and were withdrawn from the trial. The tea tree oil component 1,8-cineole, which has a reputation as a skin irritant, was also tested at concentrations up to and including 28% and did not produce any irritant reactions in the 25 (non-allergic) participants (Southwell et al., 1997). Another study similarly found that of 20 patients patch-tested with 1% tea tree oil, none had irritant reactions (Knight & Hausen, 1994). This study also showed that tea tree oil is a ‘weak sensitiser’ after attempts were made to experimentally sensitise guinea pigs to tea tree oil. Subsequent experiments have confirmed that newly distilled tea tree oil has a relatively low sensitising capacity whereas tea tree oil that had been exposed to light, oxygen, warmth and moisture, and was considered ‘degraded’, was a moderate to strong sensitiser (Hausen et al., 1999).

Contact allergy

Contact allergy is defined as a cutaneous reaction caused by direct contact with an allergen to which the patient has become sensitised (Hensyl, 1990). A series of seven such patients were described in a report by Knight & Hausen (1994). All patients reacted to 1% tea tree oil when tested by patch testing using Finn chambers. In addition, these patients also reacted to one or more of the components d-limonene, α-terpinene, aromadendrene, terpinen-4-ol and α-phellandrene at 1, 5 or 10%. In the study by Southwell discussed above, the three participants having allergic type reactions to 25% tea tree oil were tested against tea tree oil components and reacted mostly to the sesquiterpenoid fractions but not the pure monoterpenes (Southwell et al., 1997). These studies indicate that contact allergy to tea tree oil can occur, although the rate of occurrence is still not known.
1.1.4.3 Toxicity against cell lines in vitro

The testing of human or animal cells in vitro is seen as a modern alternative to animal testing to determine toxicity. Several studies have investigated the toxic effects of tea tree oil and/or components on (human) cell lines in vitro. The amounts of tea tree oil that reduced the growth of cells by 50% as compared to controls (IC50) after 24 h, ranged from 20 to 2700 μg/ml for HeLa, K562, CTVR-1, Molt-4 and Hep G2 cells (Hayes et al., 1997). IC50 values determined in other studies were 43.0 μg/ml for human HL-60 cells (Mikus et al., 2000), 0.006% for RC-37 cells (Schnitzler et al., 2001), 575 μg/ml for human fibroblasts and about 450 μg/ml for human epithelial cells (Söderberg et al., 1996). In addition, tea tree oil produced toxic effects against human monocytes at concentrations of ≥0.004% (Hart et al., 2000) or ≥0.016% (Brand et al., 2001) and at ≥0.016% against human neutrophils (Brand et al., 2001).

1.2 Fungi of medical importance

Fungi are capable of causing a range of both superficial and systemic infections. Since tea tree oil is suitable only for topical application (which includes mucous membranes such as the vaginal or oral mucosa), only superficial fungal infections amenable to topical treatment will be discussed. 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 briefly.

1.2.1 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). However, 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). The yeast genera discussed below are of interest because of their involvement in superficial infections.
1.2.1.1 *Malassezia* spp.

The genus *Malassezia* Baillon comprises the lipophilic yeasts found as commensal organisms on the skin of humans and other animals (Guého *et al.*, 1996). All *Malassezia* species, with the exception of *M. pachydermatis*, have an absolute growth requirement for long-chain fatty acids, a characteristic that hampered the in vitro study of these yeasts for many years (Leeming & Notman, 1987; Guého *et al.*, 1996). There have been numerous changes in the taxonomy of *Malassezia* since the first description of the organism in 1846, with these yeasts being ascribed to the *Pityrosporum* genus for many years. With the advent of molecular techniques, *Malassezia* taxonomy has changed again. The genus is currently comprised of seven species; *M. furfur*, *M. pachydermatis*, *M. restricta*, *M. globosa*, *M. sympodialis*, *M. obtusa* and *M. slooffiae*, although another species, *M. dermatis*, has more recently been proposed (Sugita *et al.*, 2002). Whilst isolates can be identified to the species level based on molecular techniques, they can also be characterised by their ability to utilise different lipid sources. The lipid sources commonly used are the polyoxyethylene sorbitan esters or Tween surfactants (C12 - C16 fatty acid chain lengths), lauric acid (C12) and cremophor EL, which is PEG-35 castor oil. Of the current *Malassezia* species, *M. pachydermatis* is not usually associated with human skin (Leeming & Notman, 1987). The remaining *Malassezia* yeasts are most commonly found on the sebum-rich areas of the body such as the upper body, face and scalp (Leeming & Notman, 1987) and colonisation occurs during puberty, coinciding with the onset of sebum production.

**Pityriasis versicolor**

Pityriasis versicolor is characterised by scaling hypo- or hyper-pigmented skin lesions, located typically on the upper body, neck and upper arms. Microscopy of scrapings from lesions reveals a characteristic mixture of budding yeast cells and short hyphae (Richardson & Warnock, 1997). Although pityriasis versicolor can be treated successfully with topical or oral therapies, the condition has a high recurrence rate and is often referred to as chronic.

**Seborrhoeic dermatitis and dandruff**

Seborrhoeic dermatitis is characterised by scaling and inflammation in sebum-rich areas like the face, scalp and upper trunk (Guého *et al.*, 1994). Dandruff is characterised by excessive scaling of the scalp and is thought by some to be a milder form of seborrhoeic
dermatitis (Ingham & Cunningham, 1993). *Malassezia* yeasts have been implicated in these conditions, largely because of the excellent clinical response of both conditions to treatment with antifungal agents (Ingham & Cunningham, 1993).

**Other *Malassezia*-related conditions**

*Malassezia* yeasts can cause *Malassezia* folliculitis, a condition closely resembling acne. The infection is located primarily on the upper body, neck and arms and is characterised by pruritic follicular lesions (Faergemann, 1994). Very rarely, *Malassezia* yeasts have caused fungaemia in low birth weight infants receiving lipid-rich intravenous infusions (Leeming *et al.*, 1995), and *M. pachydermatis* has been reported as causing an outbreak in a neonatal care nursery (Chang *et al.*, 1998).

**1.2.1.2 Candida spp.**

Of the *Candida* yeasts, *Candida albicans* is the most commonly isolated pathogen, followed by *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). *C. albicans* is the species most frequently isolated from both normal oral cavities and the female genital tract, and 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 commonly responsible for superficial infections, such as vaginal or oral candidiasis, and onychomycosis (Richardson & Warnock, 1997).

**Vaginal candidiasis**

Vaginal candidiasis, or thrush, is caused by the overgrowth of *Candida* in the vagina (Nyirjesy *et al.*, 1995). Approximately 80-90% of vaginal candidiasis is caused by *C. albicans* with the remainder caused by *C. glabrata, C. tropicalis* and *C. parapsilosis* (Horowitz *et al.*, 1992). It is a relatively common infection, with up to 75% of women experiencing at least one episode in their lifetime (Richardson & Warnock, 1997; Eckert *et al.*, 1998). Clinically, the infection is characterised by vulval erythema and fissuring, accompanied by a thick, white, creamy discharge (Working Group of the British Society for Medical Mycology, 1995). Patients with this infection complain of intense vulval and vaginal pruritis and burning (Richardson & Warnock, 1997). Factors that
may predispose women to an episode of vaginal candidiasis include pregnancy and previous antibiotic use (Working Group of the British Society for Medical Mycology, 1995). Treatment with topical therapy such as nystatin or clotrimazole is usually effective (Working Group of the British Society for Medical Mycology, 1995).

**Oral candidiasis**

Oral candidiasis or candidosis is broadly defined as an infection of the oral cavity by *Candida* yeasts. Several different clinical forms have been defined, the most common of which is pseudomembranous candidiasis (Richardson & Warnock, 1997). This infection is characterised by white or creamy patches on the surface of the tongue, gums or oral mucosa which may form confluent plaques at later stages (Cannon et al., 1995; Richardson & Warnock, 1997). Factors that predispose individuals to this infection include age (neonates and the elderly), systemic antibiotic or steroid use, chemotherapy and AIDS (Cannon et al., 1995). From these factors it is apparent that oral candidiasis is an infection of individuals with compromised or immature immune systems. Oral candidiasis can be one of the first signs that an individual is HIV infected and this infection can be a persistent problem in these individuals. The standard treatment of oral candidiasis is with agents such as fluconazole, nystatin or amphotericin B, administered topically or systemically (Cannon et al., 1995). Treatment may not be as straightforward in HIV positive patients as relapse is common in these individuals (Richardson & Warnock, 1997).

**Onychomycosis caused by Candida**

The term onychomycosis refers to fungal infection of the nails, of which approximately 10% are caused by *Candida* yeasts (Richardson & Warnock, 1997). *Candida* onychomycosis affects the nail, nail folds and/or nail bed, occurs more commonly in women than men and usually involves fingernails rather than toes (Richardson & Warnock, 1997). Nail folds typically become swollen, painful and erythematous (Richardson & Warnock, 1997).

**Virulence factors**

Several characteristics of *C. albicans* are thought to contribute to the ability of this organism to cause disease, although the exact role or contribution made by these characteristics is not well understood. Factors thought to play important roles in the virulence of *C. albicans* include the ability to adhere to mucosal sites, form hyphae,
evade the host immune system and secrete hydrolytic enzymes (Cannon et al., 1995).

1.2.1.3 Other yeasts causing superficial infections

There are several further genera of yeasts that cause superficial fungal infections although these infections are not very common. These yeasts in some instances are considered to be emerging yeast pathogens because of the increasing frequency of infections caused by these usually uncommon organisms (Hazen, 1995).

*Trichosporon* yeasts can be found as part of the normal human mycoflora (Guého et al., 1994) and are also the causative organism of white piedra. This is an asymptomatic infection of the hair of the scalp and pubic or facial hair and, of these, the beard or moustache are most commonly affected. The infection is characterised by white to grey nodules forming along the length of the hair shafts (Richardson & Warnock, 1997) and is treated by shaving or clipping the infected hairs. A topical imidazole cream is also usually applied.

Yeasts of the genera *Rhodotorula* and *Saccharomyces* are rarely involved in human disease, although both are capable of causing serious disease in immunocompromised patients (Gyaurgieva et al., 1996) and *S. cerevisiae* has been reported as causing vaginitis (Sobel et al., 1993).

1.2.2 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 pedis, unguium and corporis are discussed below because these infections are generally amenable to topical treatment and both tinea pedis and unguium have been the subject of clinical trials using tea tree oil.
1.2.2.1 Tinea pedis

Dermatophytic infections of the feet are called tinea pedis or athlete’s foot. The infected areas are mainly the interdigital toe-spaces but more severe cases (so-called moccasin type tinea pedis) may affect the whole sole of the foot (Richardson & Warnock, 1997). A typical infection appears as peeling, maceration and fissuring, particularly between the two last toes (Weitzman & Summerbell, 1995b). The causative organisms are most often *T. rubrum* and *T. mentagrophytes* var *interdigitale* although this varies with geographic location (Rogers *et al.*, 1996). Treatment with agents such as the imidazoles or terbinafine applied topically for four or two weeks respectively, is often successful (Richardson & Warnock, 1997). Oral treatment may be necessary for more extensive infections.

1.2.2.2 Tinea corporis

Tinea corporis, or ringworm, refers to dermatophytic infections of the body - meaning the trunk, arms and legs, but excluding the hands, feet and groin. Infections are most common in tropical and subtropical areas (Richardson & Warnock, 1997) and are associated with contact with an infected domestic or farm animal. For example, tinea corporis caused by *M. canis* is commonly due to contact with an infected cat (Richardson & Warnock, 1997). The preferred treatment is with topical imidazoles or allylamines applied twice daily for 2-4 weeks (Richardson & Warnock, 1997).

1.2.2.3 Tinea unguium

Tinea unguium refers to dermatophytic infections of the finger or toe nail plate. The infections usually appear at the free end of the nail as white or yellow lesions with irregular edges. Lesions typically spread slowly across the nail and this may lead to the eventual lifting of the nail from the nail bed (Richardson & Warnock, 1997). Toenails are more commonly affected than fingernails and the toenail infection is often secondary to tinea pedis (Richardson & Warnock, 1997). The most commonly isolated dermatophyte is *T. rubrum* (Bradley *et al.*, 1999). Topical therapy for tinea unguium is generally ineffective thus systemic therapy with itraconazole or terbinafine may be necessary (Meis & Verweij, 2001). Organisms other than the dermatophytes, such as *Candida* or other filamentous fungi can also cause nail infections although these infections are referred to as onychomycosis.
1.2.3 Filamentous fungi associated with air or air conditioning systems

Of the filamentous fungi discussed below, 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, air conditioning systems, and the potential health risks associated with this.

1.2.3.1 Indoor air

Many species of fungi can be commonly found in indoor air. The most prevalent of these are Aspergillus, Alternaria, Cladosporium, Penicillium, Eurotium and Wallemia (Maroni et al., 1995). 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).

1.2.3.2 Air handling units

Fungi are commonly found in air handling units in buildings that have air-conditioning or heating systems. Growth of fungi is 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, ways of controlling fungi within these systems are desirable but are hampered by the high moisture within these systems which favours fungal growth.

1.3 Antifungal activity of tea tree oil

Published studies investigating the antifungal activity of tea tree oil have focussed on assessing either the in vitro activity of the oil against medically relevant fungi, or the use of tea tree oil to treat human fungal infections. These studies will be discussed in the following two sections and studies assessing tea tree oil for non-medical applications will be considered in section 1.3.3.

1.3.1 In vitro activity

The development of protocols for evaluating the susceptibility of fungi to antifungal agents has lagged behind similar methods that have been developed for bacteria and
only recently have standard methods been published for evaluating the in vitro activity of antifungal agents (Rex et al., 2001). Prior to the publication of these standard methods, researchers have used a variety of different assays to assess in vitro activity, which means that data from these studies is often difficult to compare. Another limitation of some of these published studies is that very often only one isolate of a given species is tested in any particular investigation, meaning that generalisations about susceptibility are limited.

1.3.1.1 Yeasts

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\textsubscript{90}S that have been reported for C. albicans, by either the broth or agar dilution assay 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 minimum fungicidal concentrations (MFCs) ranged from 0.5 to 1.0% (Vazquez et al., 2000; Banes-Marshall et al., 2001; D'Auria et al., 2001). Malassezia yeasts also appear 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 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).

1.3.1.2 Dermatophytes

Two studies have 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 \(\mu\)l of neat tea tree oil, using isolates of Epidermophyton floccosum, M. audouini, M. canis, T. mentagrophytes, T. rubrum and T. tonsurans (Ánséhn, 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 have shown 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 and 0.25 – 1.0% for T. rubrum (D'Auria et al., 2001).

1.3.1.3 Other filamentous fungi
Similar to studies performed with the dermatophytes, several methods have been used to investigate the activity of tea tree oil against other filamentous fungi. 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, Penicillium spp., Rhizopus spp. 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). Fungicidal data for these organisms have not been published.

1.3.2 Clinical trials
A small number of trials has been published investigating the efficacy of tea tree oil for fungal infections. The earliest of these was by Walker (Walker, 1972), who published a series of his observations of patients treated with a tea tree oil solution for a range of foot problems, including tinea pedis and onychomycosis. More recently, two comparative trials investigating onychomycosis (Buck et al., 1994; Syed et al., 1999) and one investigating tinea pedis (Tong et al., 1992) have been published. In the first of the onychomycosis trials (Buck et al., 1994), patients were treated twice daily with either neat tea tree oil or 1% clotrimazole solution for a total of 6 months of treatment. After this time, of 64 patients treated with tea tree oil, 18% were culture negative with a total of 60% of participants having full or partial resolution. This compared to the clotrimazole treatment group (n = 53) of whom 11% were culture negative and 61% had full or partial resolution. Overall, there were no statistically significant differences
between the two treatment groups. The second onychomycosis trial (Syed et al., 1999) compared two creams, one containing 5% tea tree oil alone and the other containing 5% tea tree oil and 2% butenafine, both applied 3 times daily for 8 weeks. At completion of treatment the overall cure rate in patients treated with 5% tea tree oil was 0%, compared to 80% for patients treated with both butenafine and tea tree oil. In the trial investigating tinea pedis, patients were treated with 10% tea tree oil in sorbolene, 1% tolnaftate or placebo, applied twice daily for 4 weeks (Tong et al., 1992). At completion of treatment, patients treated with tea tree oil had mycological cure and clinical improvement rates of 30% and 65%, respectively. This compares to mycological cure rates of 21% in patients receiving placebo and 85% in patients receiving tolnaftate. Similarly, clinical improvement was seen in 41% of patients receiving placebo and 68% of patients receiving tolnaftate. Lastly, a case series of patients using tea tree oil mouthwash for oropharyngeal candidiasis has been published (Jandourek et al., 1998). The 13 patients included in the series were HIV positive patients who had already failed treatment with a 14 day course of oral fluconazole. Patients were treated with 15ml 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. In summarising the outcomes of these trials it seems apparent that treatment with tea tree oil does not elicit a high rate of infection cure. This is most likely due to many factors such as length and frequency of treatment and the formulation of the trial product. In addition, it is believed that onychomycosis is unresponsive to topical treatment therefore a high rate of cure should not be expected (Weitzman & Summerbell, 1995b).

1.3.3 Non-medical applications of essential oils with antifungal activity

The antifungal activity of essential oils has been investigated for potential uses outside the medical setting. Such applications include the use of essential oils to protect food crops against fungi or insects, or the use of essential oils in air conditioning systems to reduce or eliminate environmental fungi.

Bishop & Thornton (1997) investigated the in vitro activity of tea tree oil and Monarda citriodora var citriodora essential oil against 15 fungal pathogens that commonly cause post harvest disease in a wide range of crops. A disc diffusion assay was used, with 20
µl of oil per disc, and at days 3, 5, 7 and 9 post-inoculation all isolates had reduced growth as compared to controls. A later study by the same group investigated the effectiveness of tea tree oil in controlling the fungus *Botrytis cinerea* on stored cabbages (Bishop & Reagan, 1998). Discs of cabbage leaf were dipped in either a tea tree oil solution or a standard fungicide and were then inoculated with *B. cinerea*. After incubation, the area of cabbage leaf infected was determined by microscopy. The treatment containing 3.2% tea tree oil was as effective as the standard fungicides but the treatments containing less tea tree oil were not as effective. Washington *et al.* (1999) compared the effects of several agents, including a 1.5% tea tree oil spray, on both the yield and rot of strawberries. These trials used areas on six different commercial strawberry farms and each area was sprayed at weekly intervals with the allocated treatment. The use of the tea tree oil spray resulted in a modest reduction in total fruit rot in each of the three field trials but did not result in increased fruit yield (Washington *et al.*, 1999).

### 1.4 Research aims

The antifungal activity of tea tree oil has not been extensively investigated and comprehensive in vitro susceptibility data is still lacking. This data may impact on the way that tea tree oil could be used in the treatment of superficial fungal infections.

Therefore the aims of this research project were to:

- Determine the in vitro susceptibility of a wide range of 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*
2.1 Microbial isolates

2.1.1 Reference isolates

Reference isolates were obtained from the culture collections of the Department of Microbiology at The University of Western Australia (UWA) and the Division of Microbiology and Infectious Diseases at the Western Australian Centre for Pathology and Medical Research (PathCentre). *Malassezia* reference isolates were purchased from the Yeast Collection of the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. Reference isolates are given in Table 2.1.

2.1.2 Other isolates

Yeasts, dermatophytes and other filamentous fungi were obtained from the Division of Microbiology and Infectious Diseases at PathCentre. A number of yeast isolates were obtained from the Mycology Section of the Department of Microbiology at Royal Perth Hospital. A collection of 19 *Malassezia* spp. was obtained from Ms Siew Lee Thoo, previously a student in the Department of Microbiology at UWA, and an isolate of *M. sympodialis* was obtained from Dr Chris Heath at Royal Perth Hospital. Additional commensal and clinical isolates were isolated and identified as described in the following sections.

2.1.2.1 Non-*Malassezia* yeasts

Yeasts from skin were obtained by swabbing the foreheads of volunteers with a cotton-tipped swab pre-moistened with 0.85% saline. Vaginal yeast isolates were recovered from vaginal swabs submitted to PathCentre. All swabs were inoculated onto either Sabouraud Dextrose agar (SDA) or SDA with chloramphenicol (SABC) and plates were incubated for 2 - 7 d at 32°C. Yeast isolates were subcultured onto SDA to obtain pure cultures.

**Identification**

All isolates resembling *Candida* were tested for the formation of germ tubes in horse serum according to the method of Fromtling (Fromtling, 1991). Briefly, part of a colony
<table>
<thead>
<tr>
<th>Organism</th>
<th>ATCC\textsuperscript{1}</th>
<th>NCTC\textsuperscript{2}</th>
<th>CBS\textsuperscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>10231</td>
<td></td>
<td>6431</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>90028</td>
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<tr>
<td>Candida albicans</td>
<td>90029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida glabrata</td>
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<tr>
<td>Candida glabrata</td>
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<td></td>
</tr>
<tr>
<td>Candida parapsilosis</td>
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</tr>
<tr>
<td>Malassezia globosa</td>
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<td>7966</td>
</tr>
<tr>
<td>Malassezia slooffiae</td>
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<td>7956</td>
</tr>
<tr>
<td>Malassezia furfur</td>
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<td></td>
<td>1878</td>
</tr>
<tr>
<td>Malassezia obtusa</td>
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<td></td>
<td>7876</td>
</tr>
<tr>
<td>Malassezia sympodialis</td>
<td>96803</td>
<td></td>
<td>7222</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>9763</td>
<td>10716</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} American Type Culture Collection; \textsuperscript{2} National Collections of Type Cultures; \textsuperscript{3} Centraalbureau voor Schimmelcultures
was emulsified in 0.5 ml of horse serum (HS) and incubated for 2 - 4 h at 37°C. Cells were then examined microscopically for the formation of germ tubes. Isolates that produced germ tubes were identified as *C. albicans*. Isolates not producing germ tubes were further identified with API 32C strips, used according to the manufacturer’s instructions. All isolates showing a distinctive pink colony colour on SDA were given a preliminary identification of *Rhodotorula* spp., which was confirmed using API 32C strips.

**2.1.2.2 Malassezia yeasts**

*Malassezia* isolates were obtained from clinical specimens by culturing skin scrapings from patients with a clinical diagnosis of pityriasis versicolor onto Medium A (Leeming & Notman, 1987) or Dixon's agar (van Abbe, 1964). Isolates from clinically normal skin were obtained by swabbing the forehead of each volunteer with a sterile cotton-tipped swab premoistened with 0.85% saline. The swab was then spread across Medium A or Dixon's agar. All plates were incubated for 7 - 14 d at 32°C in plastic bags to reduce moisture loss. Plates were then examined and colonies resembling *Malassezia* spp. were subcultured onto Dixon's agar for further investigation. Where more than one isolate was obtained from a single specimen or person, each was numbered or lettered in the following manner. Clinical isolates were designated a number followed by a letter (eg. 1a) and commensal isolates were designated a capital letter followed by a numeral (eg. A1). Multiple isolates from the same specimen or person were maintained as different if one or more of the characteristics used for identification to the species level were consistently different.

**Identification**

*Malassezia* isolates were identified to the species level according to the results of the following tests: absolute requirement for lipids, catalase production, pattern of Tween utilisation, and esculin hydrolysis. If required, the following additional tests were performed; growth on laurate agar (Leeming et al., 1997), utilisation of Tweens and cremophor EL as determined by agar diffusion (Guillot et al., 1996; Mayser et al., 1997b) and microscopy. Assays are described below and reference isolates were included in all assays.
Absolute requirement for lipids
Lipid requirement was determined by streaking isolates for single colonies onto a non-lipid containing medium such as SDA and incubating plates at 32°C for 7 d. If no substantial growth was seen, isolates were deemed lipid dependent.

Catalase production (Hendrickson & Krenz, 1991)
Catalase production was determined by emulsifying 1 – 2 colonies of the test organism in a drop of 3% hydrogen peroxide on a glass slide. The appearance of bubbles indicated a positive result.

Utilisation of Tweens and laurate, determined by agar incorporation
The utilisation of Tween surfactants as lipid sources was determined according to the method of Guého et al. (1996). Basal medium consisted of (per litre distilled water) glucose 20 g, bacteriological peptone 10 g and agar 15 g. After autoclaving, individual Tweens were added to sterile basal medium to give the following final concentrations: Tween 20 5%, Tween 40 0.5%, Tween 60 0.5% and Tween 80 0.1% and agar was dispensed as stated below. Laurate agar contained (per litre distilled water) lauric acid 2.5 g, bacteriological peptone 10 g, ox bile 4 g and agar 10 g (Leeming et al., 1997). After the addition of distilled water, all ingredients were dissolved and the pH was adjusted to 7.0 before autoclaving. All media were dispensed in amounts of approximately 20 ml into 90 mm petri dishes. Agar plates were dried for approximately 30 min prior to inoculation.

Inocula were prepared as described in section 2.4.2.2 and two spots of 1-3 µl of each isolate containing approximately 10³ cfu were stamp inoculated onto plates as described in section 2.4.4. Plates were incubated at 32°C for 7 d after which time growth was recorded as positive, negative or scanty. Isolates were tested for utilisation of each lipid source on at least two separate occasions.

Determination of lipid utilisation by agar diffusion
Utilisation of lipid sources by the agar diffusion method was based on methods published by Mayser et al. (1997b) and Guillot et al. (1996). Basal medium consisted of (per litre distilled water) bacteriological peptone 10 g, glucose 10 g and agar 12.5 g (Mayser et al., 1997b). After autoclaving, agar was dispensed in 16 ml volumes in glass
McCartney bottles and was allowed to cool to 50°C. Organisms were prepared by suspending each isolate in 0.85% saline and adjusting to approximately $5 \times 10^6$ cfu/ml as described in section 2.4.2.2. One ml of the suspension was added to 16 ml of basal medium, which was mixed and dispensed immediately into a 90 mm plastic petri dish. After the agar had set, one hole of 6 mm and three of 2 mm were punched in each agar plate. The 6 mm hole was filled with 50 µl of cremophor EL (Mayser et al., 1997b) and the 2 mm holes were filled with 5 µl of Tween 20, 40 and 80, respectively (Guillot et al., 1996). Plates were incubated at 32°C for 10 d. After this time, the presence of growth around each well was recorded. Assays were repeated at least twice per isolate.

**Esculin hydrolysis (detection of β-glucosidase)**

Esculin test medium consisted of (per litre distilled water) esculin 1.0 g, ferric citrate 0.5 g and heart infusion agar 40 g (Nash & Krenz, 1991). After autoclaving, agar was dispensed in 3 ml volumes into plastic screw-capped vials which were used within 48 h. Tubes were stab inoculated with colonies from 3 d growth on Dixon's agar and then incubated for 5 d at 32°C. Complete darkening of the vial was recorded as esculin hydrolysis positive, no colour change was recorded as negative and any partial or slight darkening was recorded as 'slight'. Each isolate was tested on at least three separate occasions.

**Production of precipitate on Dixon's agar**

Precipitate production on Dixon's agar was examined by streaking isolates for single colonies on Dixon's agar. Plates were incubated at 32°C for 72 h, after which time the presence of precipitate was recorded. Each isolate was tested on at least three separate occasions. In addition, 25 of the isolates were re-incubated for an additional 11 d at 32°C. These isolates were *M. furfur* (n = 9), *M. sympodialis* (n = 13), *M. globosa* (n = 4) and *M. obtusa* (n = 1) and this extended incubation assay was conducted once only. Plates were observed at 24 h intervals and the day on which precipitate first became visible was noted.

**2.1.3 Storage of isolates**

Yeast isolates were stored in 2% brain heart infusion broth (BHIB) with 20% glycerol, in 1 – 2 ml cryotubes stored at −80°C. Dermatophytes and other filamentous fungi were stored on PDA slopes at room temperature.
2.2 Materials

2.2.1 Chemicals, reagents and desiccated media

Chemicals, reagents and desiccated media are listed in Table 2.2.

2.2.1.1 *Melaleuca alternifolia* (tea tree) oil and components

Tea tree oil was supplied as a single batch (971) by Australian Plantations Pty. Ltd., Wyrallah, NSW, Australia. The composition of this batch was determined by gas chromatography-mass spectrometry, performed by the Wollongbar Agricultural Institute, Wollongbar, NSW, Australia, and is shown in Table 2.3. Levels of all components stipulated in the ISO Standard 4730 were determined, as were levels of ledene. The oil complied with ISO 4730.

The eight components studied were terpinen-4-ol, 1,8-cineole, γ-terpinene, α-terpinene, terpinolene, α-terpineol, α-pinene, and p-cymene. Solutions of tea tree oil or components were prepared throughout these studies as % v/v solutions. A solution of 1% (v/v) is equivalent to 10,000 ppm or, based on a specific gravity of between 0.885 and 0.906, approximately 9 mg/ml.

2.2.2 Buffers, solutions and stains

Buffers and solutions were prepared in distilled water, sterilised by autoclaving for 15 min at 121°C and were stored at room temperature, unless stated otherwise.

**0.85% saline**

<table>
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<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
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<tr>
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<tr>
<td>Distilled water</td>
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</tr>
<tr>
<td>Chemical, reagent or medium</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------</td>
<td>----------</td>
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<td>Bacteriological agar</td>
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</tr>
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<td>Bacteriological peptone</td>
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<tr>
<td>BHIB with 20% glycerol</td>
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<tr>
<td>Glycerol α-monoooleate</td>
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<tr>
<td>Glycerol α-monostearate</td>
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Table 2.2 Cont.

<table>
<thead>
<tr>
<th>Chemical, reagent or medium</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Griseofulvin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Heart infusion agar</td>
<td>Gibco Diagnostics, Madison, WIS, USA</td>
</tr>
<tr>
<td>Horse serum</td>
<td>Excel Laboratory Products</td>
</tr>
<tr>
<td>Hydrogen peroxide, 30% w/v</td>
<td>BDH</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Janssen Biotechnology, Olen, Belgium</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>BDH</td>
</tr>
<tr>
<td>hexahydrate</td>
<td></td>
</tr>
<tr>
<td>Malt extract</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Methanol</td>
<td>BDH</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>Searle Diagnostics, High Wycombe, Bucks, England</td>
</tr>
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<td>Miconazole</td>
<td>Sigma</td>
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<tr>
<td>Morpholinopropane-sulfonic</td>
<td>Sigma</td>
</tr>
<tr>
<td>acid</td>
<td></td>
</tr>
<tr>
<td>Mueller Hinton broth</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Mycological peptone</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Nystatin</td>
<td>Pharmacy Dept., Sir Charles Gairdner Hospital, Nedlands, WA, Australia</td>
</tr>
<tr>
<td>Ox bile</td>
<td>Oxoid</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>Sigma</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>BDH</td>
</tr>
<tr>
<td>Potassium dihydrogen</td>
<td>BDH</td>
</tr>
<tr>
<td>orthophosphate</td>
<td></td>
</tr>
<tr>
<td>Potato dextrose agar</td>
<td>Oxoid</td>
</tr>
<tr>
<td>RPMI Medium 1640</td>
<td>Gibco BRL, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Sabouraud dextrose agar</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>BDH</td>
</tr>
<tr>
<td>di-Sodium hydrogen</td>
<td>BDH</td>
</tr>
<tr>
<td>orthophosphate</td>
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Table 2.2 Cont.

<table>
<thead>
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<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide</td>
<td>David Brown Scientific, Osborne Park, WA, Australia</td>
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<tr>
<td>Succinic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>BDH</td>
</tr>
<tr>
<td>α-Terpinene</td>
<td>Sigma</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>Aldrich</td>
</tr>
<tr>
<td>(+)-Terpinen-4-ol</td>
<td>Fluka</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>Fluka</td>
</tr>
<tr>
<td>Trehalase</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tween 20, 40, 60 and 80</td>
<td>Sigma</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Whole fat cows' milk</td>
<td>Bonlac Foods Ltd. Melbourne, VIC, Australia</td>
</tr>
</tbody>
</table>
Table 2.3 Composition of *Melaleuca alternifolia* (tea tree) oil batch 971

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition (%)</th>
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<tbody>
<tr>
<td>terpinen-4-ol</td>
<td>41.5</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>21.2</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>10.2</td>
</tr>
<tr>
<td>terpinolene</td>
<td>3.5</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>2.9</td>
</tr>
<tr>
<td>α-pinene</td>
<td>2.5</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>2.1</td>
</tr>
<tr>
<td>ρ-cymene</td>
<td>1.5</td>
</tr>
<tr>
<td>δ-cadinene</td>
<td>1.0</td>
</tr>
<tr>
<td>aromadendrene</td>
<td>1.0</td>
</tr>
<tr>
<td>limonene</td>
<td>0.9</td>
</tr>
<tr>
<td>ledene</td>
<td>0.9</td>
</tr>
<tr>
<td>globulol</td>
<td>0.6</td>
</tr>
<tr>
<td>sabinene</td>
<td>0.4</td>
</tr>
<tr>
<td>viridiflorol</td>
<td>0.3</td>
</tr>
<tr>
<td>other</td>
<td>9.5</td>
</tr>
</tbody>
</table>
Phosphate buffered saline (pH 7.4) (Hendrickson & Krenz, 1991)

NaCl 8 g
KCl 0.2 g
KH₂PO₄ 0.12 g
Na₂HPO₄ 0.91 g
Distilled water 1 litre

Ingredients were dissolved in distilled water and the pH was adjusted to 7.4 before sterilising.

Succinate buffer (pH 6.0) (Dawson et al., 1986)

0.2M succinic acid 250 ml
0.2M NaOH 435 ml
Distilled water 315 ml

All solutions were mixed and the pH was adjusted to 6 with NaOH before sterilising.

Loeffler's methylene blue (Finegold & Baron, 1986)

Methylene blue 0.3 g
Ethanol 30 ml
Distilled water 100 ml

Methylene blue (0.05%) stain (Boyum & Guidotti, 1997)

Methylene blue 0.05 g
Distilled water 100 ml

2.2.3 Culture media

Blood agar and SABC were supplied by Excel Laboratory Products, Belmont, Western Australia.

Potato dextrose agar, SDA and Mueller Hinton broth were purchased as powders and prepared according to the manufacturers' instructions.

The following media were prepared according to the instructions given in each cited publication. Broth media were the same formulation without the agar powder. Unless
stated otherwise, all ingredients were added to distilled water, heated to dissolve and media were sterilised by autoclaving at 121°C for 15 min.

**Potato dextrose broth**

Potato dextrose broth was prepared by mixing potato dextrose agar powder with distilled water and then passing the mixture through a paper filter before sterilising.

**Dixon's agar** (van Abbe, 1964)

- Malt extract: 36 g
- Mycological peptone: 6 g
- Ox bile (desiccated): 20 g
- Tween 40: 10 ml
- Glycerol α mono-oleate: 2.5 ml
- Bacteriological agar: 15 g
- Distilled water: 1 litre

Distilled water was added to the dry ingredients which were heated to dissolve. The Tween 40 and glycerol α mono-oleate were then added and agar was autoclaved.

**Medium A agar** (Leeming & Notman, 1987)

- Bacteriological peptone: 10 g
- Glucose: 5 g
- Yeast extract: 0.1 g
- Ox bile (desiccated): 4 g
- Glycerol: 1 ml
- Glycerol mono-stearate: 0.5 g
- Tween 60: 0.5 ml
- Whole fat cows' milk (ultra heat treated): 10 ml
- Bacteriological agar: 12 g
- Distilled water: 1 litre

All ingredients except milk were added to the distilled water, mixed and heated to dissolve. After autoclaving, the agar was cooled to 55°C, the milk was added and the agar was poured immediately. The chloramphenicol (50 µg/ml) and cycloheximide (200 µg/ml) included in the original formulation were omitted in the present study. Medium A broth contained all of the ingredients listed above except the bacteriological agar.
**Sabouraud dextrose broth**

Glucose 40 g
Mycological peptone 10 g
Distilled water 1 litre

**RPMI Medium 1640**

RPMI Medium 1640 powder 5.2 g
(with L-glutamine, without sodium bicarbonate)
Morpholinopropane-sulfonic acid (MOPS) 0.165 M
Distilled water 1 litre

MOPS buffer was dissolved in 900 ml of distilled water and the pH was adjusted to 7 with sodium hydroxide. The RPMI 1640 powder was then added and the pH readjusted to 6.7 - 6.8. The medium was filter sterilised, which in itself caused the pH to rise by 0.2 - 0.3, and was stored at 4°C.

**Yeast extract peptone glucose broth (YPEG)**

Glucose 20 g
Bacteriological peptone 20 g
Yeast extract 10 g
Distilled water 1 litre

**2.2.4 Other consumables**

Other consumables are listed with their supplier in Table 2.4. Disposable plasticware such as pipette tips, transfer pipettes and cryotubes were manufactured by Greiner bio-One, Samco Scientific Co., Robbins Scientific Co., Nunc, Elkay and Eppendorf. Disposable centrifuge tubes (10 ml) were supplied by Sarstedt Australia Pty. Ltd. and 500 ml centrifuge tubes were manufactured by Nalgene and supplied by Sigma, Castle Hill, NSW.

**2.3 General methods**

**2.3.1 Standardisation of inocula**

Inocula were standardised by diluting fungal suspensions using a nephelometer (Hach Company, Loveland, Colorado, USA). Suspensions were adjusted to the opacity of
### Table 2.4 Other consumables

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>API 32C Analytical Profile Index strips</td>
<td>BioMerieux sa, Geneva, Switzerland</td>
</tr>
<tr>
<td>Microcuvettes #1938 for spectrophotometer</td>
<td>Kartell, Noviglio, Italy</td>
</tr>
<tr>
<td>Microcuvettes #1961 for fluorescence spectrophotometer</td>
<td>Kartell, Noviglio, Italy</td>
</tr>
<tr>
<td>Microplates (96-well, UV transparent) for use with the SpectraMax 250 spectrophotometer</td>
<td>Molecular Devices, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Microtitre trays (96-well, polystyrene) for in vitro susceptibility testing</td>
<td>Nalge Nunc International, Roskilde, Denmark</td>
</tr>
<tr>
<td>Plastic petri dishes, 90 mm</td>
<td>Biolab, East Tamaki, New Zealand</td>
</tr>
<tr>
<td>Sterile syringe filter, Acrodisc 0.45µm</td>
<td>Pall Gelman Laboratory, MI, USA</td>
</tr>
</tbody>
</table>
either a known McFarland Standard or to a percentage transmission value (T), and the cfu/ml contained within these suspensions was confirmed by viable counts, as shown in Table 2.5. McFarland Standards of 0.5, 1, 2 and 3 corresponded to %T values of 80 – 88, 67 – 77, 46 – 56 and 27 – 37, respectively.

2.3.2 Viable counts

Viable counts were performed by diluting each sample in a series of 10-fold dilutions using the diluent stated for each particular assay, which was either sterile distilled water (SDW), 0.85% saline or buffer. As there are no known neutralisers for tea tree oil, samples containing tea tree oil were diluted at least 1 in 2 (for assay described in section 2.7), or more commonly at least 1 in 10 before enumerating colonies. Colonies were then enumerated on agar as described below. For the Miles-Misra and spread plate methods, agar plates were surface dried for approximately 30 min prior to inoculation.

Miles-Misra

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

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 $3 \times 10^3$ cfu/ml.

Pour-plates

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 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. After plates had set, they were incubated for up to 72 h at 35°C. Counts were calculated where one or both
### Table 2.5 Standardisation of inocula

<table>
<thead>
<tr>
<th>Organism</th>
<th>% Transmission</th>
<th>Corresponding range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(McFarland)</td>
<td>(cfu/ml)</td>
</tr>
<tr>
<td><strong>Alternaria spp.</strong></td>
<td>70 (1)</td>
<td>$2.5 \times 10^4 - 7.9 \times 10^5$</td>
</tr>
<tr>
<td><strong>Aspergillus flavus</strong></td>
<td>70 (1)</td>
<td>$1.3 \times 10^5 - 2.4 \times 10^6$</td>
</tr>
<tr>
<td><strong>Aspergillus fumigatus</strong></td>
<td>70 (1)</td>
<td>$1.7 \times 10^5 - 6.6 \times 10^6$</td>
</tr>
<tr>
<td><strong>Aspergillus niger</strong></td>
<td>70 (1)</td>
<td>$8.5 \times 10^5 - 1.6 \times 10^6$</td>
</tr>
<tr>
<td><strong>Candida spp.</strong></td>
<td>70 (1)</td>
<td>$1.1 \times 10^7 - 2.0 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>50 (2)</td>
<td>$1.0 \times 10^8 - 2.0 \times 10^8$</td>
</tr>
<tr>
<td><strong>Cladosporium spp.</strong></td>
<td>70 (1)</td>
<td>$1.9 \times 10^5 - 1.6 \times 10^6$</td>
</tr>
<tr>
<td><strong>Epidermophyton floccosum</strong></td>
<td>55-65</td>
<td>$2.5 \times 10^4 - 1.0 \times 10^5$</td>
</tr>
<tr>
<td><strong>Fusarium spp.</strong></td>
<td>70 (1)</td>
<td>$4.7 \times 10^5 - 2.2 \times 10^6$</td>
</tr>
<tr>
<td><strong>Malassezia spp.</strong></td>
<td>60</td>
<td>$4.5 \times 10^6 - 1.6 \times 10^7$</td>
</tr>
<tr>
<td><strong>Microsporum canis</strong></td>
<td>60-70</td>
<td>$2.5 \times 10^4 - 2.8 \times 10^4$</td>
</tr>
<tr>
<td><strong>Microsporum gypseum</strong></td>
<td>50-60</td>
<td>$2.0 \times 10^5 - 4.8 \times 10^5$</td>
</tr>
<tr>
<td><strong>Penicillium spp.</strong></td>
<td>70 (1)</td>
<td>$1.2 \times 10^5 - 6.6 \times 10^6$</td>
</tr>
<tr>
<td><strong>Trichophyton mentagrophytes var interdigitale</strong></td>
<td>50-60</td>
<td>$3.0 \times 10^6 - 7.5 \times 10^6$</td>
</tr>
<tr>
<td><strong>Trichophyton mentagrophytes var mentagrophytes</strong></td>
<td>50 (2)</td>
<td>$1.25 \times 10^6 - 5.5 \times 10^6$</td>
</tr>
<tr>
<td><strong>Trichophyton rubrum</strong></td>
<td>60-70</td>
<td>$1.7 \times 10^6 - 2.5 \times 10^6$</td>
</tr>
<tr>
<td><strong>Trichophyton tonsurans</strong></td>
<td>50-60</td>
<td>$3.5 \times 10^5 - 2.5 \times 10^6$</td>
</tr>
<tr>
<td><strong>Other yeasts (not Candida or Malassezia)</strong></td>
<td>70 (1)</td>
<td>$2.5 \times 10^6 - 6.5 \times 10^6$</td>
</tr>
</tbody>
</table>
plates had between 30 and 300 colonies. The lower limit of detection, based on 30 colonies per plate, was 300 cfu/ml.

2.3.2.1 Yeasts

Unless stated otherwise, the diluent for yeast viable counts was SDW and colonies were enumerated using Dixon's media for *Malassezia* spp. or SDA for all other yeasts. Plates were incubated at 32°C for 2 - 7 d for *Malassezia* spp., or at 35°C for 24 - 72 h for all other yeasts.

2.3.2.2 Dermatophytes

Viable counts of dermatophytes were performed using SDW as the diluent and colonies were enumerated on SDA using the Miles-Misra method. Plates were incubated at 30°C for 2 – 7 d. These counts were performed for time-kill assays and to confirm inoculum concentrations for in vitro susceptibility tests.

2.3.2.3 Other filamentous fungi

Viable counts for the other filamentous fungi were performed using SDW as the diluent and colonies were enumerated on PDA using spread plates of 30 μl, not 100 μl. Times and temperatures of incubation were specific to the growth characteristics of each genera, as follows; *Aspergillus* spp. were incubated for 18 - 24 h at 35°C, *Penicillium* spp. were incubated for 48 h at 35°C, *Fusarium* were incubated for 2 - 4 d at 35°C and, *Cladosporium* and *Alternaria* were incubated at 30°C for 2 - 4 d.

2.3.3 Preparation of Candida cells

2.3.3.1 Cells for time-kill, methylene blue, germ tube and other assays

*Candida* cells were prepared by inoculating approximately 8 ml of SDB 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 $6 \times 10^6 - 1.3 \times 10^7$ cfu/ml (60% T). Cells for the germ tube assay were washed and resuspended in PBS, not SDW.
2.3.3.2 Cells for assays determining leakage of 260 nm-absorbing material

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 $1.2 \times 10^9$ cfu/ml for *C. albicans* and $2.4 \times 10^9$ cfu/ml for *C. glabrata*. All centrifugation was conducted at 4°C at 8670 x $g$ 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.

Where the influence of cations on the leakage of 260 nm-absorbing material was examined, cells were prepared as above, except that after the initial collection step, the cells were washed with approximately 150 ml of 10 mM ethylene diamine tetraacetic acid (EDTA), then twice in 50 mM succinate buffer. The cells were finally resuspended in succinate buffer to 0.2 g wet weight cells/ml.

2.3.4 Dry weight determinations

Cellular dry weights were determined by drying empty crucibles for 24 h at 100°C. Crucibles were transferred to a desiccator for approximately 30 min and then weighed. The optical densities of an overnight culture (neat), and a 1 in 2 dilution in broth of the overnight culture were determined at 600 nm. Volumes of 10 ml of overnight culture of each test organism were washed twice, then resuspended in SDW to the original volume. Aliquots of 5 ml of this neat suspension and also 5 ml of the 1 in 2 dilution of the suspension were transferred to crucibles and dried at 100°C for 48 h. Crucibles were weighed as described above. Optical density (OD) was plotted against dry weight per ml of culture and a standard curve fitted (two data points per organism).

2.4 In vitro susceptibility assays

2.4.1 Preparation of antimicrobial agents

Stock solutions of tea tree oil or component were prepared at twice the highest required concentration in the relevant test medium (% v/v). Stock solutions were prepared and used on the same day.
Stock solutions of econazole, miconazole, ketoconazole, griseofulvin and nystatin were prepared by dissolving powders in dimethylsulfoxide (DMSO) to final concentrations of 160 mg/ml, 80 mg/ml, 4 mg/ml, 6.4 mg/ml and 5 mg/ml (w/v), respectively. Stock solutions were stored at -20°C. The highest concentration of DMSO in the susceptibility assays was 2.5% (v/v). The stock solution of boric acid was prepared in distilled water (w/v), sterilised by filtration and stored at 4°C.

2.4.2 Broth microdilution assays

Broth microdilution assays were based on two methods recommended by the National Committee for Clinical Laboratory Standards: Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved Standard M27-A (National Committee for Clinical Laboratory Standards, 1997) and Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi; Proposed Standard M38-P (National Committee for Clinical Laboratory Standards, 1998). Modifications for particular fungi are described below. All isolates were tested on at least two separate occasions and re-tested if resultant MIC or MFC values differed. Modal values for each isolate were then selected.

2.4.2.1 Preparation of the microdilution tray

RPMI Medium 1640 was the test medium for all fungi, with the exception of Malassezia spp., which were tested using Medium A broth. Mueller Hinton broth (MHB) was also used occasionally as a comparison to RPMI Medium 1640.

Doubling dilutions of the test agent were prepared in 100 μl volumes of medium in a 96-well microdilution tray. Where tea tree oil or a component was the test agent, a final concentration of 0.001% (v/v) Tween 80 was included to enhance tea tree oil/component solubility. Concentrations of tea tree oil/component ranged from 8% to 0.004% (v/v). Test trays were prepared and inoculated on the same day. All trays were inoculated with 100 μl volumes of inocula prepared as described below.
2.4.2.2 Inoculum preparation, incubation conditions and other test procedures

*Candida, Saccharomyces, Rhodotorula, Cryptococcus and Trichosporon* spp.

Isolates were grown on SDA for 24 - 48 h at 35°C and growth from these plates was suspended in approximately 2 ml of 0.85% saline or SDW. The density of this suspension was adjusted to 1 McFarland in SDW. This was serially diluted in SDW as necessary to correspond to a final inoculum concentration range of 1.5 - 3.0 × 10^3 cfu/ml for the broth microdilution assay, and these were confirmed by Miles-Misra viable counts.

Microtitre trays were inoculated by adding 100 µl volumes of inocula to each well and trays were then incubated for 48 h at 35°C. Prior to subculturing, all wells were mixed by aspirating the well contents several times after which 10 µl volumes were spot inoculated onto SDA. Subcultures were incubated for 24 - 48 h at 35°C, and then all growth was recorded. MICs and MFCs were determined according to the criteria in section 2.4.2.3.

**Comparison of NCCLS methods M27-A and M7-A5**

The in vitro susceptibility of yeasts to tea tree oil as determined by the NCCLS method M27-A for yeasts was compared to the NCCLS method M7-A5 for bacteria which uses a different method of inocula preparation, inocula concentration and test medium. Each isolate was inoculated into approximately 5 ml of MHB and incubated overnight at 35°C with shaking. Cultures were adjusted to 1 McFarland and were diluted in SDW to twice the desired final inoculum concentration, which for this assay was 5 × 10^5 cfu/ml. Final inocula concentrations were confirmed by Miles-Misra viable counts. Microdilution trays were prepared as described in section 2.4.2.1 except that the growth medium was MHB. The incubation and subculture of microtitre trays was conducted as described above.

*Malassezia* spp.

Isolates were subcultured onto Dixon's agar and incubated for 3 d at 32°C. Colonies were suspended in 1 - 2 ml of 0.85% saline and mixed using a vortex mixer. This suspension was diluted using 0.85% saline to approximately 5 × 10^6 cfu/ml (60% T),
then further diluted as necessary in SDW to twice the desired final inoculum concentration, which was approximately 1.5 - 3.0 × 10³ cfu/ml. Final inocula concentrations were confirmed by Miles-Misra viable counts. After inoculation, test trays were incubated at 32°C for 48 h and then mixed as described above. Aliquots of 5 µl were removed and spot inoculated onto Dixon's agar. The surfactant components of both Medium A and Dixon's agar meant that larger subculture volumes were not feasible. Subcultures were incubated for 2 - 7 d at 32°C, after which growth was recorded and MICs and MFCs determined. Preliminary experiments showed that isolates of *M. obtusa* and *M. globosa* did not produce sufficient growth in this assay, thus microdilution testing of these isolates was not pursued.

**Dermatophytes**

Inocula were prepared by subculturing isolates onto 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 using a vortex mixer. After the settling of larger particles, suspensions were adjusted as shown in Table 2.5. Inocula were diluted in SDW to correspond to the required final inocula concentrations of approximately 2.5 × 10³ - 2.5 × 10⁴ cfu/ml (Hazen, 1998) as confirmed by Miles-Misra viable counts. After inoculation, microtitre trays were incubated for 96 h at 30°C (Norris *et al.*, 1999). MICs were then determined visually with the aid of a reading mirror, according to NCCLS guidelines (National Committee for Clinical Laboratory Standards, 1998). Briefly, growth in each agent-containing well was compared to growth in the control well for each organism. Wells were scored as follows: 4, no reduction in growth; 3, approximately 25% reduction in growth; 2, approximately 50% reduction in growth; 1, approximately 75% reduction in growth; 0, optically clear. MFCs of tea tree oil were determined by subculturing 10 µl from wells that were not visibly turbid and spot inoculating onto SDA plates (Aguilar *et al.*, 1999). Subcultures were incubated for up to 7 d at 30°C, after which time MFCs were determined.

**Other filamentous fungi**

Inocula were prepared as described above, with the following modifications. Isolates of *Cladosporium* and *Alternaria* were grown on PDA slopes incubated 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 - 72 h at 35°C and then at approximately 28°C for the remaining 4 - 5 d. Slopes were flooded with PBS containing 0.05% Tween 80 (Del Poeta *et al.*, 1997) and suspensions were adjusted and diluted as described above, and according to the data shown in Table 2.5. Final inoculum concentrations were 0.4 - 5.0 x 10⁴ cfu/ml (National Committee for Clinical Laboratory Standards, 1998) as confirmed by spread-plate viable counts. After inoculation, microtitre trays were incubated for 48 h at 35°C for *Aspergillus*, *Penicillium* and *Fusarium*, and at 30°C for *Alternaria* (48 h) and *Cladosporium* (72 h). MICs and MFCs were determined as described above for the dermatophytes except that subcultures were grown at the temperatures stated above for each genus.

### 2.4.2.3 Criteria for determining MICs and MFCs

For yeasts, MICs were determined from broth dilution assays as the lowest concentration of tea tree oil resulting in the maintenance or reduction of the inoculum. MFCs were determined as the lowest concentration of tea tree oil resulting in the death of 99.9% of the inoculum. MICs and MFCs were determined by comparing colony numbers in subcultures to original inocula concentrations.

For the dermatophytes and filamentous fungi MICs were determined at the lowest concentration of agent resulting in a 75% reduction in growth as compared to control growth, determined visually. MFCs were determined as the lowest concentration resulting in no growth upon subculture.

### 2.4.3 Chequerboard microdilution assays

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 and then diluting a stock solution of tea tree oil in RPMI Medium across the 12 columns of the microtitre tray, 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 and subcultured as described in section 2.4.2.2 and the
MIC of each agent alone was determined as described in section 2.4.2.3. Synergy or
antagonism between agents was determined by establishing the fractional inhibitory
concentration (FIC) for each combination. The FIC for each agent was calculated by
dividing the MIC of each agent in combination by the MIC of that agent alone, and then
adding the FIC values for the two agents together. Considering two agents A and B, this
can be written as $\text{MIC A combination}/\text{MIC A alone} + \text{MIC B combination}/\text{MIC B alone} = \text{FIC A} + \text{FIC B} = \sum \text{FIC}$. 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.

### 2.4.4 Broth macrodilution assays

The broth macrodilution method was compared to the broth microdilution assay with *C.
albicans* ATCC 10231 as the test organism. Doubling dilutions of tea tree oil or
component were prepared in RPMI Medium in 0.5 ml volumes in glass McCartney
bottles, with final concentrations of either 0.001% or 0.1% Tween 80. The range of tea
tree oil or component tested was 2% - 0.06%. Inocula were prepared as described in
section 2.4.2.2 and 0.5 ml volumes of inocula were added to each dilution of oil or
component. Macrodilution bottles were incubated at 35°C, either statically or with
shaking. After 24 h and 48 h incubation, 10 μl aliquots were removed from each
dilution and spot inoculated onto SDA. Plates were incubated for 24 - 48 h after which
time growth was recorded. Criteria for MICs and MFCs are described above.

### 2.4.5 Agar dilution assay for Malassezia

A series of two-fold dilutions of tea tree oil, ketoconazole, econazole or miconazole was
prepared in 20 ml volumes of Medium A agar (Leeming & Notman, 1987). Final
concentration ranges were as follows: tea tree oil, 1 - 0.008%; ketoconazole, 0.5 - 0.001
μg/ml; miconazole, 32 - 0.015 μg/ml and econazole, 32 - 0.03 μg/ml. For dilutions with
tea tree oil, a final concentration of 0.5% Tween 20 was incorporated into the agar to
enhance oil solubility (Hammer et al., 1999b). Control plates both with and without
0.5% Tween 20 were also poured. Plates were surface dried for 30 min prior to
inoculation.
Inocula were prepared as described in section 2.4.2.2. Inocula were diluted to correspond to approximately $10^6$ cfu/ml and volumes of each were then aliquoted into a 52-well inoculum block. Agar plates were stamp inoculated using a multipoint replicator (Mast Laboratories Ltd., Liverpool, UK) which delivered inocula spots of 1 - 3 µl, containing approximately $10^3$ cfu per spot. Inoculated plates were then incubated for 7 d at 32°C.

For the agar dilution assay, MICs were determined as the lowest concentration of the agent preventing the growth of the isolate, disregarding one or two colonies. Each isolate was tested on at least two separate occasions. If MIC values differed, isolates were re-tested and modal MIC values were selected.

2.5 *Candida albicans* growth curves with or without 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.

Tea tree oil treatments containing 0, 0.016, 0.03, 0.06 and 0.12% were prepared in 30 ml volumes in 150 ml conical flasks, all in SDB with a final concentration of 0.001% Tween 80. To start the experiment, 0.1 ml of the 18 h culture was added to each flask. Samples were taken immediately for OD$_{540}$ 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 OD$_{540}$ measurements and at 8 h and 24 h for viable counts. Viable counts were performed using the Miles-Misra method.

The OD$_{540}$ was 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 concentrations of tea tree oil greater than 0.016% 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 measurements below 1.0.
The log_{10} value for each OD measurement was calculated and the data were plotted on a logarithmic scale. The mean generation time, or time required for the population to double, was calculated using data points from the exponential phase of growth according to the following formula:

\[ g = \frac{t}{3.3 (\log B_t - \log B_0)} \]

where \( g \) is the generation time, \( t \) is the elapsed time period of growth, \( \log B_t \) is the \( \log_{10} \) of the absorbance value at time \( t \) (the end time), \( \log B_0 \) is the \( \log_{10} \) of the absorbance value at the start time (Atlas, 1989).

After 24 h, wet cell weight was determined. Cells were collected by centrifuging exactly 20 ml of culture for 5 min at 3000 rpm (1300 \( \times \) 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 before and after cells were collected and the mass of cells was determined. The wet weight of cells per ml of culture was then calculated. Growth experiments were repeated 3 - 4 times.

2.5.1 Growth curves of cells pre-conditioned with tea tree oil

Assays were performed as described above with a few modifications. Cells were prepared by inoculating 1 – 2 colonies of \( C. \) \( albicans \) 10231 into approximately 10 ml of SDB with 0.001% Tween 80 either with or without 0.062% 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 non pre-conditioned 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.
2.6 Time kill assays

2.6.1 *Candida albicans*

A series of treatments containing tea tree oil or components, at one or more concentrations ranging from 1 to 0.25% was prepared in 1 ml volumes of PBS with 0.002% Tween 80. On occasion, where larger sample volumes were required for viable counts, treatments were prepared in 1.5 ml volumes and in these instances a 1.5 ml volume of inoculum was used. Controls contained PBS with a final concentration of 0.001% Tween 80.

Inocula were prepared as described in section 2.3.3.1 and starting inoculum concentrations were approximately $5 \times 10^6$ cfu/ml. At 1 min intervals, 1 ml of inocula was added to each tea tree oil or component treatment and mixed for 20 s. Samples were then taken by removing 80 μl from each treatment and adding this to 0.72 ml PBS for viable counts. All treatments were incubated with shaking at 35°C. Additional samples were taken at 30 min and at 1, 2, 3, 4 and 6 h. Initial viable counts were performed by the Miles-Misra method. However, where required, spread and pour plates were also used. Assays were repeated at least twice and mean and standard error values were calculated from viable count data.

2.6.2 *Trichophyton* and *Aspergillus* spp.

Time kill studies were performed against an isolate each of *T. rubrum*, *T. mentagrophytes* var. *interdigitale*, *A. niger* and *A. fumigatus*. Inocula were prepared as described in section 2.4.2.2 except that dermatophyte inocula were suspended and diluted in PBS, and *Aspergillus* spp. were suspended and diluted in PBS with 0.02% (v/v) Tween 80. Starting inocula concentrations were approximately $10^6$ cfu/ml for dermatophytes and *A. fumigatus*, and $10^5$ cfu/ml for *A. niger*. Tea tree oil concentrations were chosen from preliminary experiments and corresponded to $4 \times$ MFC for dermatophytes and $1 \times$ MFC for *Aspergillus* spp. Tea tree oil treatments were prepared in 1 ml volumes at twice the desired final concentrations in PBS, with final concentrations of 0.001% Tween 80 for dermatophytes or 0.02% Tween 80 for *Aspergillus* spp. Controls contained PBS with the relevant concentration of Tween 80.
Treatments and controls were inoculated with 1 ml volumes of inocula and a 100 μl sample was taken immediately from each control for viability counts. Treatments and controls were incubated at 35°C with shaking. Further samples were taken at 2, 4, 6, 8 and 24 h for viable counts which were performed using SDA and with SDW as the diluent. Limits of detection were calculated from a minimum of 30 cfu from the $10^{-1}$ dilution, taking into account different spread-plating volumes for each organism. Detection limits were $7.5 \times 10^3$ cfu/ml for dermatophytes and $3.0 \times 10^3$ for *Aspergillus* spp. Assays were performed 2 - 6 times. Colony count data for each experiment were converted to values relative to the colony count at time zero to 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, using a log scale.

2.7 Comparative susceptibility of germinated and non-germinated *Aspergillus niger* conidia to tea tree oil

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.

2.7.1 Preparation of conidia

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 potato dextrose broth (PDB) and gently probing the growth. Conidial suspensions were adjusted as described previously (section 2.3.3.1) to approximately $10^6$ conidia/ml, and then diluted 1/100 in PDB to approximately $10^4$ conidia/ml. Part of this suspension was used immediately (non-germinated conidia) and part was incubated for 8 h at 30°C to produce germinated conidia. Attempts were made to also germinate *A. fumigatus* conidia, however, these were unsuccessful.

2.7.2 Tea tree oil treatments

Tea tree oil treatments ranged from 0.25 - 0.03% (final concentrations) and were prepared in PDB with Tween 80 at a final concentration of 0.001%. 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 each by half and then 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.

2.8 Assays to determine alterations in cell permeability

2.8.1 Methylene blue dye exclusion assay

Inocula were prepared as described in section 2.3.3.1. Treatments containing tea tree oil/component were prepared as described in section 2.6.1 since the methylene blue (MB) assays were performed concurrently with time-kill assays.

At 1 min intervals, 1 ml of inocula 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% MB for staining. This was mixed well and left for 5 min at room temperature. A wet mount was then prepared and the cells were examined microscopically using a final magnification of × 400. 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.

2.8.2 Leakage of 260 nm-absorbing material

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 & Michel (1989). Test organisms were C. albicans 10231 and C. glabrata ATCC 15545. C. albicans was used in all assays whereas C. glabrata was tested only against 0.25, 0.5 and 1.0% tea tree oil.
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. In initial experiments results for the component terpinolene were not reproducible with a final concentration of 0.001% Tween 80, thus subsequent treatments containing terpinolene, \( \gamma \)-terpinene and \( \alpha \)-terpinene were prepared in 0.2% Tween 80. Treatments and controls were inoculated with 2 ml of the suspension of organisms as prepared in section 2.3.3.2. Solutions were mixed for approximately 10 s, and 20 s after the addition of the inoculum, and a 150 \( \mu \)l sample was taken from each treatment and diluted 1/10 by adding to 1.35 ml PBS with 0.001% Tween 80. These solutions were then filtered through a 0.45 \( \mu \)m 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 filtered as described above. The absorbance of the blanks was measured by dispensing four 200 \( \mu \)l 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, California, USA). After the OD\(_{260}\) of blanks was determined, they were emptied from the tray and then test filtrates were dispensed in the same manner into the corresponding wells of the microplate. The OD\(_{260}\) values of the test filtrates were then determined. The microplate reader took six individual measurements for each well and calculated the average.

The OD\(_{260}\) of all test filtrates was 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. The mean, standard deviation and standard error was determined and plotted.

2.8.2.1 Modifications for leakage experiments with cations

The influence of calcium and magnesium ions on the leakage of 260 nm-absorbing material caused by tea tree oil was investigated. Tea tree oil was assessed at final
concentrations of 0.5 and 1.0% only and treatments were prepared as described in section 2.8.1 except that succinate buffer with 0.001% Tween 80 was used in all instances instead of PBS. Also, treatments contained calcium or magnesium ions, which were added from 1 M solutions of CaCl₂.2H₂O and MgCl₂.6H₂O that had been prepared in distilled water and sterilised by autoclaving, at final concentrations of 10 mM and 50 mM. Controls were succinate buffer with 0.001% Tween 80 and succinate buffer containing 10 mM and 50 mM concentrations of both cations. Assays were performed as described above in section 2.8.2 except that samples were taken at 0 h and 6 h only.

2.9 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 (2000), with some modifications. Cells of *S. cerevisiae* NCTC 10716, *C. albicans* ATCC 10231 and *C. glabrata* ATCC 15545 were prepared by inoculating 1 - 2 colonies into 30 ml of YEPG in a 150 ml flask, and growing for 14 - 16 h with shaking at 125 rpm. The growth temperatures for *S. cerevisiae* and *Candida* spp. were 30°C and 35°C, respectively.

Cells were collected and washed twice in cold SDW. The cells were then resuspended in cold SDW with 0.001% Tween 80 to approximately 10⁷ cfu/ml, and were kept on ice. Volumes of cells were aliquoted and amounts of a 10% tea tree oil stock solution were added 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, then 1 ml of a 20% (w/v) glucose solution was added to each control or treatment at timed intervals. This resulted in a final concentration of 2% glucose. 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 after 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. To compensate for this effect 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. In addition, changes in pH were analysed by subtracting the pH value at time zero from pH values at subsequent time points, for each control or tea tree oil treatment.

2.10 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.

C. albicans cells were prepared as described in section 2.3.3.1 and cells were resuspended and adjusted to approximately $10^7$ cfu/ml (60% T) in either PBS or succinate buffer.

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$_2$O 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.

### 2.11 Trehalose accumulation during treatment with tea tree oil

#### 2.11.1 Preparation of cells

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 and treated as described below.

#### 2.11.2 Tea tree oil and heat shock treatments

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 in section 2.7.1.1 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).

The OD₆₀₀ 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).

2.11.3 Stationary phase cells

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 \textit{S. cerevisiae} or at 35°C for \textit{C. albicans} for 48 h with shaking at 125 rpm. Cells were then collected, washed and stored as described above.

2.11.4 Extraction and assay of trehalose

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 was 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 H₂SO₄. The OD of each cell supernatant was then read against the reagent blank at 540 nm.
Attempts were made to quantify the amount of protein in each sample using the Bradford method (Technical Bulletin, Sigma Chemical Co., St Louis, Mo., USA), however, this was unsuccessful due to turbidity of samples. Therefore, 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.

2.12 Effects of tea tree oil on C. albicans germ tube formation

The reference isolate C. albicans ATCC 10231 and nine clinical isolates (from patients diagnosed with vaginal candidiasis) were used for these studies. Screening of isolates showed a range of percentage germ tube formation (GTF) in horse serum, therefore the isolates chosen for this assay were those where approximately 100% of blastospores formed germ tubes after 4 h incubation at 37°C in horse serum (HS).

2.12.1 Induction of germ tubes in the presence of tea tree oil

Cells were prepared as described in section 2.3.3.1. After resuspension in PBS, cells were diluted 1 in 10 in HS, and then used in the assay described below. Two isolates (ATCC 10231 and clinical isolate 88E) were tested against the entire range of tea tree oil concentrations, sampled at each time point. Based on these results, the assay was repeated with a further eight isolates with the 0.03% tea tree oil treatment only, sampled at 4 h only.

Tea tree oil treatments were prepared in 0.5 ml volumes in HS in glass Bijou bottles and concentrations after inoculation ranged from 0.25 – 0.04%. Controls and treatments were inoculated with 0.5 ml of cell suspensions in HS and were mixed thoroughly. A sample was removed immediately from each control for viable counts and microscopy. A sample was also removed from the 0.25% tea tree oil treatment for a time zero viable count. All treatments were then incubated at 37°C, without shaking, and sampled at 1, 2, 3 and 4 h. Prior to each subsequent sampling bottles were mixed thoroughly. For some experiments, additional samples were removed from the 0.12% and 0.25% tea tree oil treatments at each time point for viable counts. Viable counts were performed by the Miles-Misra method, using SDA and PBS as the diluent. Experiments were repeated 2 - 4 times per isolate.
2.12.2 Induction of germ tubes after 1 h pre-treatment with subinhibitory concentrations of tea tree oil

Cells of *C. albicans* ATCC 10231 and 88E were prepared as described in section 2.3.3.1 and, after washing, resuspended in RPMI Medium. Tea tree oil treatments were prepared in 2 ml volumes of RPMI Medium with 0.002% Tween 80, at twice the desired final concentration (%v/v) as follows: 0.5, 0.25, 0.125, 0.031 and 0. These concentrations were halved when controls and treatments were inoculated with equal volumes of the cell suspensions in RPMI Medium. Controls and treatments were incubated for 1 h at 35°C with shaking. Cells were collected by centrifugation for 3 min (1300 × g), and washed twice with PBS with 0.5% Tween 80. Cells were finally resuspended in PBS without Tween 80. Each cell suspension was adjusted to approximately 8 × 10⁶ cfu/ml and 50 μl of each suspension was added to 0.95 ml HS, mixed thoroughly and incubated at 37°C, without shaking. A time zero sample was taken from each control (0% tea tree oil) for microscopy. Additional samples were removed for microscopy at 1, 2, 3 and 4 h, and at 5 and 6 h for selected treatments only. For some experiments, samples were also removed from the 0.12% and 0.25% treatments at each time point for viable counts. Assays were repeated 2 – 4 times per isolate.

2.12.3 Microscopy

Cells were prepared for microscopy by adding 50 μl of each sample to an equal volume of 1% glutaraldehyde in PBS (pH 7.4) (Ghannoum *et al.*, 1990). After mixing well with the pipette tip, a portion was removed immediately and spread onto a glass slide. Slides were air-dried, fixed with methanol and stained with Loeffler's methylene blue. Cells were examined using bright field microscopy, under oil immersion (final magnification × 1000). Fifty sequential cells from each slide were examined (Warnock *et al.*, 1989) and scored morphologically according to the following definitions. A germ tube was defined as a cell bearing a rounded outgrowth with a length ≥ the diameter of the parent cell, not constricted at the base (Chaffin, 1985; Lynch *et al.*, 1993). The presence of septae was noted. A protuberance was defined as a cell bearing a rounded outgrowth ≤ the parent cell diameter, not constricted at the base (Hubbard *et al.*, 1985). A bud was defined as being as large as the parent cell and/or fully septated (Lynch *et al.*, 1993). Singly budding cells were defined as a parent attached to a single daughter cell and
multiply budding cells were defined as a parent cell attached to two or more daughter cells. Cells bearing pseudohyphae (a marked constriction at the site of emergence) were disregarded (Ellepola & Samaranayake, 1998b). The percentage of cells bearing each morphology type was then calculated.

2.13 Effects of tea tree oil and components on membrane fluidity

2.13.1 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 2.5, except that the medium was YEPG instead of SDB. 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.

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

Cells of *C. albicans* ATCC 10231 were prepared by inoculating 1 - 2 colonies into 30 ml of YEPG in a 150 ml flask and growing for 24 h at 35°C with shaking. Cells were collected, washed twice in PBS and resuspended in PBS with 0.001% Tween 80 to a concentration of approximately $1 \times 10^8$ cfu/ml (50% T). Aliquots of 9.75 ml or 15 ml of this suspension were dispensed for each treatment or control, respectively.

Stock solutions of tea tree oil or components were prepared as 10% (v/v) solutions in PBS with 0.001% Tween 80 and 0.25 ml of each stock solution was added to the dispensed cells. Stock solutions were mixed thoroughly prior to their addition to cell suspensions and cells suspensions were mixed thoroughly after the addition of tea tree oil or component. 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.
2.13.3 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 in all instances 20 μl of this stock solution was added to 4 ml volumes of cells to result in a final probe concentration of 2 μM (Ansari et al., 1993a). Stock solutions were stored at room temperature protected from light. 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 fluorescence intensity every 6 s 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, and for the assay investigating immediate changes in membrane fluidity these were from the time zero time point. 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.

2.14 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.

In addition, statistical analyses of the results of the germ tube assays were performed using the computer program GraphPad Prism 2.01 (1996), from GraphPad Software Inc. GTF in the presence of tea tree oil, and after pre-treatment with tea tree oil, and also viable count data, were compared to controls using analysis of variance (ANOVA). Differences between the treatment groups were compared using Dunnett's Multiple Comparison Test.
3.1 Introduction

The Malassezia yeasts are important organisms, both as inhabitants of normal human skin and as agents associated with skin conditions such as pityriasis versicolor, seborrhoeic dermatitis and dandruff. The taxonomy of the genus has recently undergone revision and the genus now contains the six lipid-dependent species; *M. furfur, M. sympodialis, M. slooffiae, M. globosa, M. obtusa* and *M. restricta*, and the non-lipid dependant species *M. pachydermatis*. However, very little data exist on the in vitro susceptibility of these new species to antifungal agents. The aims of this Chapter were to firstly obtain a range of Malassezia isolates from both normal and diseased skin and to identify these isolates to species level using available biochemical and physiological tests. The second aim was to determine the in vitro susceptibility of these species to tea tree oil, ketoconazole, miconazole and econazole and to compare the susceptibility of the various Malassezia species to each antifungal agent.

3.2 Results

3.2.1 Isolation and Identification

A total of 30 Malassezia isolates was obtained in the present study: 24 isolates were obtained from clinical specimens and six (two *M. furfur* and four *M. sympodialis*) were isolated from clinically normal skin. Multiple isolates from clinical specimens were 23a (*M. sympodialis*) and 23c (*M. slooffiae*), 25a and 25c (both *M. sympodialis*), 28a and 28b (*M. sympodialis*), 31a and 31b (*M. furfur*) and 31c (*M. sympodialis*), 36a (*M. obtusa*) and 36d (*M. globosa*), and 40a and 40b (*M. sympodialis*). Multiple isolates from normal skin were D1 (*M. furfur*) and D2 (*M. sympodialis*).

Results of the growth characteristics and biochemical tests for the entire collection of Malassezia isolates (*n* = 55) are shown in Table 3.1. Several tests were used to identify isolates to the species level. The use of Tween agar incorporation plates alone was not
Table 3.1. Results of biochemical and growth tests for *Malassezia* spp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate No.</th>
<th>n</th>
<th>precipitate*</th>
<th>esculin hydrolysis</th>
<th>0.5% Tw40</th>
<th>0.5% Tw60</th>
<th>0.1% Tw80</th>
<th>5% Tw20</th>
<th>laurate agar</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. furfur</em></td>
<td>A1, 1, 7, 9, 31a, D1, CBS 1878, 42</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>11, 18</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>sc</td>
</tr>
<tr>
<td></td>
<td>31b</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>sc</td>
<td>sc</td>
</tr>
<tr>
<td><em>M. sympodialis</em></td>
<td>4, 5, 6, 10, 20, 21, 22a, 23a, 29a, 30a, 31c, 37a, 40a</td>
<td>13</td>
<td>+</td>
<td>s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B1, 27a, 28b, 32b, D2, 39a, 25c</td>
<td>7</td>
<td>+</td>
<td>s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>sc</td>
</tr>
<tr>
<td></td>
<td>25a, 28a, C1</td>
<td>3</td>
<td>+</td>
<td>s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>41, 2</td>
<td>2</td>
<td>+</td>
<td>s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>sc</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>26, 40b</td>
<td>2</td>
<td>+</td>
<td>+ / s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>sc</td>
</tr>
<tr>
<td>Species</td>
<td>Isolate No.</td>
<td>Reaction or growth characteristic</td>
<td>n</td>
<td>precipitate*</td>
<td>esculin</td>
<td>0.5% Tw40</td>
<td>0.5% Tw60</td>
<td>0.1% Tw80</td>
<td>5% Tw20</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------</td>
<td>-----------------------------------</td>
<td>----</td>
<td>--------------</td>
<td>---------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td><em>M. sympodialis</em></td>
<td>13</td>
<td></td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CBS 7222</td>
<td></td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td></td>
<td>1</td>
<td>+</td>
<td>s</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>sc</td>
</tr>
<tr>
<td></td>
<td>MsyRPH</td>
<td></td>
<td>1</td>
<td>+</td>
<td>s</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. globosa</em></td>
<td>16, 17, 19, 38a</td>
<td></td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>36d, CBS 7966</td>
<td></td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>sc</td>
<td>sc</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. obtusa</em></td>
<td>36a, CBS 7876</td>
<td></td>
<td>2</td>
<td>-</td>
<td>s</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. slooffiae</em></td>
<td>CBS 7956, 23c</td>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td></td>
<td>1</td>
<td>-</td>
<td>s</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* = precipitate on Dixon's agar, s = slight reaction, sc = scanty growth
sufficient to identify isolates to the species level and therefore further tests were employed. Eleven isolates were identified as *M. furfur*, 30 as *M. sympodialis*, five as *M. globosa*, one as *M. obtusa* and two as *M. slooffiae*. In addition, isolates within each species were grouped according to similar results for each of the tests, resulting in four *M. furfur* groups, 10 *M. sympodialis* groups, two *M. globosa* groups, one *M. obtusa* group and two *M. slooffiae* groups.

3.2.1.1 Tween agar incorporation method

A final concentration of 5% Tween 20 was used in the present study as 10% Tween 20, as recommended by Gueho *et al.* (1996), prevented the agar from solidifying.

Of 12 *M. furfur* isolates, three did not grow on 5% Tween 20 and one produced only scanty growth (Table 3.1). The remaining eight isolates (including CBS 1878) were positive for growth on 5% Tween 20. All 12 isolates grew on 0.5% Tween 40 and 60, and 0.1% Tween 80. All 32 *M. sympodialis* isolates grew on the Tween 40 and 60 plates. The reference isolate *M. sympodialis* CBS 7222 grew on 5% Tween 20, as did isolates 13, 26 and 40b. Only four isolates did not grow on the 0.1% Tween 80 plates, and an additional two produced only scanty growth. Of the three *M. slooffiae* isolates, all grew on the Tween 40 and 60 plates, and two grew on 0.1% Tween 80 agar (including CBS 7956). These two isolates were also positive for growth on 5% Tween 20 agar. Two of the six *M. globosa* isolates (including CBS 7966) produced scanty growth on the 0.5% Tween 40 and 0.5% Tween 60 plates. No growth was seen on the other plates.

3.2.1.2 Esculin hydrolysis

*M. globosa* and *M. slooffiae* were consistently esculin hydrolysis negative and the two *M. obtusa* isolates were consistently positive. Of the *M. furfur* isolates, isolates 1, 7 and reference isolate CBS 1878 each showed a slight reaction in one out of three test repeats, however these isolates were designated as esculin hydrolysis negative. All except two isolates of *M. sympodialis* gave positive or slight reactions. The esculin hydrolysis negative isolates were 13 and 24. One *M. slooffiae* isolate gave a slight reaction.
3.2.1.3 Laurate agar

Ten *M. furfur* isolates grew on laurate agar, including the eight isolates that grew on the 5% Tween 20 agar. The two remaining *M. furfur* isolates showed scanty growth on laurate agar. In addition, three isolates of *M. sympodialis* produced growth on laurate agar, whilst an additional 11 isolates showed scanty growth. No other isolates or species showed growth on laurate agar.

3.2.1.4 Agar diffusion assay for Tweens and cremophor EL

A total of 27 isolates (including three reference isolates) were tested for utilisation of Tweens and cremophor EL by the agar diffusion method (Table 3.2). Of the eight *M. furfur* isolates tested, only the reference strain CBS 1878 showed solid growth around cremophor EL. *M. furfur* isolates 7 and 42 showed hazy growth and a further six *M. furfur* isolates did not grow. All eight *M. furfur* isolates tested (A1, 1, 7, 9, 11, 31a, 31b and CBS 1878), and four *M. sympodialis* isolates (26, 40b, 29a and CBS 7222) showed solid zones of growth around Tween 20, 40 and 80. *M. slooffiae* isolates CBS 7956 and 23c also showed this pattern except that the zone around Tween 80 was markedly reduced in comparison. Nine of the *M. sympodialis* isolates (20, 24, 25a, 25c, 28a, 28b, 31c, 32b and 40a) showed solid growth around Tween 40 and 80 and a halo of growth around Tween 20. *M. sympodialis* isolate 13 showed solid growth around Tween 40, a halo around Tween 20, and faint growth around Tween 80, as did *M. slooffiae* isolate 14.

3.2.1.5 Production of precipitate on Dixon's agar

Of 12 *M. furfur* isolates, two *M. obtusa* isolates and three *M. slooffiae* isolates, none produced precipitate on Dixon’s agar (Fig 3.1). All 32 *M. sympodialis* isolates and six *M. globosa* isolates produced precipitate. Isolates were either consistently positive or negative for precipitate on repeated tests. Of the isolates observed on Dixon’s agar for 14 d, at no time did precipitate appear for any isolate of *M. furfur* or *M. obtusa*. Precipitate appeared after days 1 and 2 for *M. sympodialis* and days 2 and 3 for *M. globosa*. Over the 14 d incubation, precipitate became more pronounced and granular, and a zone of clearing was seen directly adjacent to microbial growth.
Table 3.2 Utilisation of Tweens and Cremophor EL by the agar diffusion assay

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Species</th>
<th>Isolate No.</th>
<th>Utilisation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><em>M. furfur</em></td>
<td>1878</td>
<td>+   +   +   +   +</td>
</tr>
<tr>
<td>II</td>
<td><em>M. furfur</em></td>
<td>42</td>
<td>H   +   +   +   +</td>
</tr>
<tr>
<td>III</td>
<td><em>M. furfur, M. sympodialis</em></td>
<td>A1, 1, 7, 11, 26, 29, 31a, 31b, 40b, 7222</td>
<td>-   +   +   +   +</td>
</tr>
<tr>
<td>IV</td>
<td><em>M. sympodialis</em></td>
<td>20, 24, 25a, 25c, 28a, 28b, 31c, 32, 40a</td>
<td>-   H   +   +   +</td>
</tr>
<tr>
<td>V</td>
<td><em>M. slooffiae</em></td>
<td>7956, 23c</td>
<td>-   +   SM  +   +</td>
</tr>
<tr>
<td>VI</td>
<td><em>M. slooffiae, M. sympodialis</em></td>
<td>13, 14</td>
<td>-   H   SM  +   +</td>
</tr>
</tbody>
</table>

H: haze, SM: small zone
Fig 3.1 Precipitate production on Dixon's agar after 5 d at 32°C. (A) *M. furfur* CBS 1878. (B) *M. slooffiae* CBS 7956. (C) *M. obtusa* CBS 7876. (D) *M. sympodialis* CBS 7222. (E) *M. sympodialis* (isolated from pityriasis versicolor skin scrape) (F) *M. globosa* CBS 7966.
3.2.2 In vitro susceptibility data

A total of 54 isolates was tested against tea tree oil, ketoconazole, econazole and miconazole by the agar dilution method, while 10 isolates each of *M. furfur* and *M. sympodialis* were tested using the broth dilution method.

3.2.2.1 Agar dilution data

MICs of tea tree oil ranged from 0.016 – 0.25% for all species (Table 3.3). MICs of ketoconazole ranged from 0.03 – 0.25 μg/ml for *M. furfur* and 0.008 – 0.016 μg/ml for the remaining species. MICs of econazole and miconazole ranged from 4 – 16 μg/ml for *M. furfur* and from 0.12 – 4 μg/ml for the remaining species. Ketoconazole was the most active of the imidazoles, followed by miconazole and econazole which were similar in activity. *M. furfur* was the species least susceptible to imidazoles: the remaining species showed susceptibility.

3.2.2.2 Broth dilution data

Tea tree oil was inhibitory to *M. furfur* and *M. sympodialis* at similar concentrations, each species having an MIC₉₀ of 0.12 and 0.06%, respectively (Table 3.4). MFCs differed slightly, with *M. furfur* and *M. sympodialis* having MFC₉₀ values of 1.0% and 0.12%, respectively. Ketoconazole was also the most active of the imidazoles in the broth dilution assay. Miconazole and econazole showed similar activity against each species but demonstrated differences in activity between species. *M. sympodialis* was more susceptible than *M. furfur* with all MIC₉₀ and MFC₉₀ values lower than those obtained for *M. furfur*. For ketoconazole, MIC and MFC values were equivalent or one dilution apart. For miconazole and econazole, MIC and MFC values differed by several dilutions, for both species.

3.3 Discussion

3.3.1 Introduction

These *Malassezia* yeasts are of interest because of their involvements in human disease, although these diseases are usually not life-threatening. Also, the taxonomy, specific disease involvement and in vitro susceptibility to antifungal agents of *Malassezia* species are still very new areas of research. Therefore it was of interest in the present Chapter to identify a range of clinical and commensal *Malassezia* isolates to the species
Table 3.3 In vitro susceptibilities of 54 Malassezia spp. as determined by the agar dilution assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Agent</th>
<th>MIC (% v/v or μg/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td><strong>M. furfur (12)</strong></td>
<td>Tea tree oil</td>
<td>0.12-0.25</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.03-0.25</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>4-16</td>
</tr>
<tr>
<td></td>
<td>Econazole</td>
<td>4-16</td>
</tr>
<tr>
<td><strong>M. sympodialis (32)</strong></td>
<td>Tea tree oil</td>
<td>0.016-0.25</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.008-0.03</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>0.12-0.5</td>
</tr>
<tr>
<td></td>
<td>Econazole</td>
<td>0.12-1</td>
</tr>
<tr>
<td><strong>M. slooffiae (3)</strong></td>
<td>Tea tree oil</td>
<td>0.12-0.25</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.008-0.03</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>2-4</td>
</tr>
<tr>
<td></td>
<td>Econazole</td>
<td>0.25-0.5</td>
</tr>
<tr>
<td><strong>M. globosa (5)</strong></td>
<td>Tea tree oil</td>
<td>0.03-0.12</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.008-0.016</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>0.12-2</td>
</tr>
<tr>
<td></td>
<td>Econazole</td>
<td>0.25-0.5</td>
</tr>
<tr>
<td><strong>M. obtusa (2)</strong></td>
<td>Tea tree oil</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.008-0.016</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>0.5-2</td>
</tr>
<tr>
<td></td>
<td>Econazole</td>
<td>0.5-4</td>
</tr>
</tbody>
</table>

<sup>a</sup> % v/v for tea tree oil; μg/ml for ketoconazole, miconazole and econazole.
Table 3.4 In vitro susceptibilities of *M. furfur* and *M. sympodialis* as determined by the broth dilution assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Agent</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MFC&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Range 90%</td>
<td>Range 90%</td>
</tr>
<tr>
<td><em>M. furfur</em> (10)</td>
<td>Tea tree oil</td>
<td>0.03-0.12 0.12</td>
<td>0.5-1.0 1.0</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.06-0.25 0.25</td>
<td>0.06-0.5 0.25</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>8-128 128</td>
<td>16-&gt;256 &gt;256</td>
</tr>
<tr>
<td></td>
<td>Econazole</td>
<td>8-128 64</td>
<td>16-&gt;256 &gt;256</td>
</tr>
<tr>
<td><em>M. sympodialis</em> (10)</td>
<td>Tea tree oil</td>
<td>0.016-0.12 0.06</td>
<td>0.06-0.12 0.12</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.008-0.03 0.016</td>
<td>0.008-0.06 0.03</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>0.25-4 1</td>
<td>0.5-&gt;8 &gt;8</td>
</tr>
<tr>
<td></td>
<td>Econazole</td>
<td>0.25-4 1</td>
<td>1-32 16</td>
</tr>
</tbody>
</table>

<sup>a</sup>%v/v for tea tree oil; μg/ml for ketoconazole, miconazole and econazole.
level, using mostly biochemical tests and to then assess the in vitro susceptibility of these isolates to tea tree oil and several other antifungal agents.

### 3.3.2 Isolation and identification of Malassezia spp.

Identification of Malassezia isolates obtained from clinical specimens showed that *M. furfur* (*n* = 9), *M. sympodialis* (*n* = 26), *M. globosa* (*n* = 5), *M. obtusa* (*n* = 1) and *M. slooffiae* (*n* = 2) were recovered. In addition, *M. furfur* (*n* = 2) and *M. sympodialis* (*n* = 4) were recovered from normal skin. A study of Malassezia yeasts on the skin of both diseased and non-diseased individuals showed that the species most commonly recovered were *M. sympodialis* and *M. globosa*, regardless of disease state (Gupta *et al.*, 2001b), a trend similar to that seen in the present study. The lack of isolation of *M. restricta* in the present study may be due to the fastidious nature of this species. In addition, *M. restricta* has been associated with scalp colonisation and is therefore less likely to be isolated with skin scrapings from areas affected by pityriasis versicolor or the forehead swabs that were used in the present study (Gupta *et al.*, 2001a). That only one isolate of *M. obtusa* was recovered may be due to the relatively low frequency of occurrence of this species (Gupta *et al.*, 2001b).

Since several of the Malassezia species have only recently been described (Simmons & Guého, 1990; Guého *et al.*, 1996), methods for their identification are still being developed and published. In addition to the more conventional identification methods such as biochemical and physiological tests that were used in the current study, molecular identification methods have been described. These include karyotyping (Boekhout & Bosboom, 1994) and Polymerase Chain Reaction-Restriction Endonuclease Analysis (PCR-REA) of the internal transcribed spacer and/or the large subunit of the ribosomal ribonucleic acid gene (Guillot *et al.*, 2000; Gupta *et al.*, 2000b), although these methods will not be discussed here.

The Tween incorporation method described by Guého *et al.* (1996) was easy to perform and it provided relatively straightforward results for specific lipid utilisation patterns of individual Malassezia isolates. However, some of the results differed from those obtained in their study (Guého *et al.*, 1996). For example, not all *M. furfur* isolates from the present study would grow on 5% Tween 20 agar and not all isolates of *M. sympodialis* and *M. slooffiae* grew on 0.1% Tween 80 agar. Another study also found
M. slooffiae isolates that did not grow on 0.1% Tween 80 (Midgley, 2000). Although the results of this assay should allow most Malassezia isolates to be identified to the species level, some isolates may require further characterisation.

Using the agar diffusion assay, Mayser et al. (1997b) showed that five isolates of M. furfur were able to use cremophor EL. Of the M. furfur isolates tested in the present study, only three showed appreciable growth whilst the remaining five showed no growth. This discrepancy cannot be easily explained, although Midgley (Midgley, 2000) has also noted M. furfur isolates that are unable to use cremophor EL.

Analysis of Tween use by the agar diffusion method showed that the majority of isolates gave patterns of growth that correlated with those described previously (Guillot et al., 1996). However, several M. sympodialis isolates showed solid zones of growth around Tween 20, a characteristic supposedly indicative of only M. furfur or M. slooffiae (Guillot et al., 1996). A possible explanation is the suggestion that Tween 20 is only inhibitory to M. sympodialis and that after prolonged incubation these inhibitory effects may not persist (Guillot et al., 1996). Of four M. furfur isolates, five M. sympodialis isolates and two M. slooffiae isolates tested for Tween 20 utilisation by both the agar incorporation and agar diffusion methods, the only discrepant results were an isolate each of M. furfur and M. sympodialis which were both positive by the diffusion method but negative by the incorporation method. The fact that most results were in agreement suggests that the method used does not greatly influence results. Isolates 13 and 14 gave patterns of growth unlike any shown by Guillot et al. (1996), however, the patterns resembled most closely that of M. sympodialis.

As part of an identification scheme devised by Leeming et al. (1997) M. furfur isolates showed enhanced growth on laurate plates, in contrast to M. sympodialis which showed scanty or inhibited growth. The results seen in the present study were similar, apart from three isolates of M. sympodialis that showed frank growth on the laurate plates. The use of lauric acid instead of Tween 20 as a lipid source potentially avoids problems encountered with the use of Tween surfactants, since Tweens generally do not contain only one fatty acid and the presence of the other fatty acids in each Tween has the potential to confound results. Laurate agar appears to be useful tool in identifying
Malassezia species, particularly for distinguishing *M. slooffiae* isolates from *M. furfur* and *M. sympodialis*.

There was some variability in β-glucosidase activity within individual species in the present study. Two of the 32 *M. sympodialis* strains tested did not appear to hydrolyse esculin. This contrasts three previous studies which have shown *M. sympodialis* to be positive for esculin hydrolysis, using two (Mayser et al., 1997b), 10 (Leeming et al., 1997) and 26 (Aspiroz et al., 1999) isolates, respectively. *M. furfur* isolates were negative for esculin hydrolysis, a finding similar to that of Mayser et al. (1997b), who showed that fresh cultures of *M. furfur* were able to cause only a slight darkening of the medium indicating esculin hydrolysis. However, Leeming et al. (1997) showed that a single isolate of *M. furfur* was positive for esculin hydrolysis. Isolates of *M. slooffiae* have been shown to be both positive (Leeming et al., 1997) and negative (Leeming et al., 1997; Mayser et al., 1997b) for esculin hydrolysis, a finding similar to the present study. *M. globosa* and *M. restricta* have been shown previously to be esculin negative (Leeming et al., 1997). Although this assay appears to be useful for aiding in the identification of *Malassezia* isolates, more data are needed to better describe expected reactions for each species.

Some of the *Malassezia* species produce a distinct precipitate after incubation on Dixon’s agar. Other authors have noted either clearing or production of precipitates by *Malassezia* isolates (including *M. pachydermatis*) after growth on various lipid-containing media. Cunningham et al. (1990) noted that on Leeming–Notman agar (also known as medium A), serovar A (which corresponds to *M. sympodialis*), showed both clearing and opacity, *M. globosa* showed clearing but not opacity and *M. restricta* showed opacity but no clearing. Bond and Anthony (1995) observed that both typical and atypical *M. pachydermatis* isolates were surrounded by precipitates on Dixon’s agar after 5 d incubation. This finding is unusual, since this species is considered non-lipid dependent, and perhaps reflects the ability of *M. pachydermatis* to use external lipid sources, whilst not having an absolute requirement for lipids *in vitro*.

It is likely that the appearance of precipitate may be due to production of insoluble free fatty acids (FFAs) from lipid sources contained in Dixon’s agar. Furthermore, it is possible that as the isolate continues to grow, some of these precipitated FFAs are
utilised, thus explaining the zone of clearing that is seen directly adjacent to microbial growth. This observed difference in precipitate production may be most useful for distinguishing *M. furfur* and *M. sympodialis*. Although this test alone cannot differentiate *M. slooffiae* from *M. furfur*, it may aid in the identification of *M. slooffiae*, a species that can easily be misidentified as either *M. furfur* or *M. sympodialis* (Guého et al., 1998).

Of the isolates examined in the present study, a few did not fulfill all the identification criteria for a particular species. These included isolates 13 and 24 (esculin negative *M. sympodialis* isolates), and isolate 14, a *M. slooffiae* which gave a very different pattern of growth compared to *M. slooffiae* 7956 and 23c by the agar diffusion assay. Isolates 13 and 24 have characteristics suggestive of *M. sympodialis*, apart from being esculin hydrolysis negative. The most likely alternative identification for these isolates is *M. slooffiae*, since their ability to use Tween 40 and 60 preclude their identification as *M. globosa*, *M. obtusa* or *M. restricta*. However, neither isolate fulfilled the identification criteria for *M. slooffiae* (isolate 13 was Tween 80 negative and isolate 24 was Tween 20 negative) and they were identified as *M. sympodialis* on the basis of precipitate production.

Similarly, isolate 14 was identified as *M. slooffiae* although the growth patterns were different from the remaining two *M. slooffiae* isolates. The other possible identification for this isolate would be *M. sympodialis*. However, most *M. sympodialis* isolates (28 of 32) showed scantly or frank growth on 0.1% Tween 80 whereas this isolate did not, and all other *M. sympodialis* isolates were precipitate positive whereas this isolate was negative. It has been previously noted that most ambiguities in *Malassezia* identification were in differentiating *M. sympodialis* from *M. furfur* and *M. slooffiae* (Gupta et al., 2001b).

The above three isolates were identified as the species they best fit with, given the available biochemical and growth characteristics available. Isolates that do not exactly fit the criteria for a given species are to be expected, given that the new *Malassezia* species were described relatively recently. It is even possible that these isolates may represent further new species or subspecies, and one such species, *M. dermatis*, has recently been described (Sugita et al., 2002). Gupta et al. (2000b) analysed 78
Malassezia isolates by PCR-REA and found that a ‘small number of isolates’ had ambiguous status after analysis, highlighting the possibility that not all isolates will fall into existing species designations. In addition, Aspiroz et al. (1999) found that of 120 Malassezia isolates from normal human skin, 62 were M. globosa, 26 were M. sympodialis, 27 were M. restricta and 5 were non-typable (Aspiroz et al., 1999).

The differences in the ways that Malassezia species are able to utilise lipids have not been well characterised, as few studies have been performed with the newer Malassezia species. However, it seems likely that these differences are due to variations in the enzymatic capabilities of the different species, some of which have been shown in previous studies. One study has shown that when different Malassezia species were grown with olive oil as the lipid source, M. sympodialis, M. globosa, M. obtusa, M. restricta and M. slooffiae produced large amounts of FFAs whereas M. furfur produced only minimal amounts (Mayser et al., 1998). Whilst all organisms showed visible growth, very different physiological or biochemical processes were taking place (Mayser et al., 1998). This was interpreted as demonstrating that M. sympodialis must first hydrolyse triglycerides to produce FFAs for growth whereas M. furfur is able to take up triglycerides unchanged (Mayser et al., 1998). In another study, 120 Malassezia strains were examined for the presence of a lipase (C14) and M. globosa strains were positive for the enzyme whereas the other species analysed (M. sympodialis and M. restricta) were negative (Aspiroz et al., 1999).

More work is required to characterise Malassezia species, both in terms of their uniqueness as part of the normal human skin flora and as potential pathogens.

3.3.2 In vitro susceptibility of Malassezia spp. to tea tree oil and imidazoles

Comparison of published in vitro susceptibility data is complicated by the fact that most data have been obtained for ‘Malassezia furfur’ or ‘Pityrosporum’ species. Based on the recent taxonomic changes, these older names may represent as many as six different species.

There have been few reports of the susceptibility of Malassezia yeasts to tea tree oil. Nenoff et al. (1996) reported that 22 strains of M. furfur sensu lato had MICs ranging
from 0.06% - 0.44%, obtained by an agar dilution method. An MIC of 0.02% for an isolate of *Pityrosporum ovales* was reported by Griffin & Markham (2000), also by an agar dilution method. These previously reported MIC values are very similar to the data obtained in the present study by the agar dilution method, despite variations in test conditions such as test medium and both the temperature and duration of incubation.

Data obtained by the agar dilution method in the present study showed no obvious inter-species variation in susceptibility to tea tree oil, although data are limited by the relatively few isolates of *M. slooffiae*, *M. globosa* and *M. obtusa* that were available for testing. In contrast, some differences in tea tree oil susceptibilities between species were seen by the broth dilution method. In the present study, 10 isolates each of *M. furfur* and *M. sympodialis* were tested by the broth microdilution method and, whilst MIC ranges were essentially identical, MFC ranges differed. The MFC range was 0.5 - 1.0% for *M. furfur* and was 0.06 – 0.12% for *M. sympodialis*. Analysis of the differences between inhibitory and fungicidal concentrations for each isolate showed that the mean number of dilutions between inhibitory and fungicidal concentrations was 3.3 for *M. furfur* and only 1.4 for *M. sympodialis*.

Several other authors have reported MICs for ketoconazole against *Malassezia* spp. These include MIC ranges of 0.025 – 0.4 (Marcon *et al.*, 1987), 0.05 – 0.1 (Petranyi *et al.*, 1987), < 0.06 – 0.13 (Schmidt & Ruhl-Horster, 1996), 0.001 – 1 (Van Cutsem *et al.*, 1990) and 0.1 – 1.6 µg/ml (Van Gerven & Odds, 1995). MIC<sub>90</sub>s that have been reported include 0.06 (Schmidt & Ruhl-Horster, 1996), 0.1 (Nenoff & Haustein, 1994), 1.6 (Van Gerven & Odds, 1995) and 2.9 µg/ml (Strippoli *et al.*, 1997). These values are similar to those obtained in the present study, with the exception of the MIC<sub>90</sub>s of Van Gerven & Odds (1995) and Strippoli *et al.* (1997), which are higher.

MFC<sub>90</sub>s of ketoconazole determined in the present study were 0.25 µg/ml for *M. furfur* and 0.03 µg/ml for *M. sympodialis*. Ketoconazole showed fungicidal activity as MIC and MFC values for both species were largely identical. To the best of our knowledge, this is only the second reporting of MFC values, the others reported by Van Cutsem *et al.* (1990), who showed an MFC range of 0.01 – 10 µg/ml with an MFC<sub>90</sub> of 10 µg/ml. The differences between the values obtained in the present study and those published previously may be attributable to methodological differences since the study by Van
Cutsem used Dixon's broth incubated for 14 d at 32°C, compared to the present study which used Medium A broth, also incubated at 32°C but for only 48 h.

MICs for miconazole that have been previously reported include ranges of 0.4 – 1.5 μg/ml (Marcon et al., 1987), 0.05 – 50 μg/ml (Faergemann & Bernander, 1979) and 0.8 - >100 μg/ml (Van Gerven & Odds, 1995). MIC90s that have been reported for miconazole are 5.2 μg/ml (Strippoli et al., 1997) and 100 μg/ml (Van Gerven & Odds, 1995). Similar to ketoconazole, some of these previously reported values are higher than those reported in the present study.

Values previously reported for econazole include an MIC90 of 4.8 μg/ml (Strippoli et al., 1997) and an MIC range of 0.05 – 12.5 μg/ml (Faergemann & Bernander, 1979). These values are similar to the overall range of 0.12 - 16 μg/ml seen in the present study.

Data from the present study indicated that ketoconazole was more active than both miconazole and econazole. Similarly, Nenoff & Haustein (1994) found ketoconazole to have the lowest MICs as compared to fluconazole, itraconazole, tioconazole, clotrimazole and bifonazole. In another study, the testing of six azoles in vitro showed ketoconazole to have the lowest MIC50 and MIC90 values (Van Gerven & Odds, 1995). A relatively small difference between inhibitory and fungicidal values was seen for ketoconazole, but not for econazole or miconazole. Van Cutsem et al. (1990) showed a similar effect for ketoconazole against 10 strains of P. ovale. This indicates a primarily fungicidal mode of action for ketoconazole whereas econazole and miconazole appear to be primarily fungistatic.

*M. furfur* was the least susceptible of the *Malassezia* species. Mayser et al. (1997a) also found that *M. furfur* isolates were the least susceptible species, having polidocanol MICs that were more than 10-fold higher than the remaining *Malassezia* species. In contrast, Leeming et al. (1997) reported that while *M. sympodialis* was the species most susceptible to terbinafine, the remaining species, including *M. furfur*, were very similar in susceptibility. Faergemann & Bernander (1979) reported MICs for 19 strains of *P. orbiculare*. Miconazole MICs ranged from 0.05 – 50 μg/ml and econazole MICs ranged from 0.05 – 12.5 μg/ml. Three of the 19 strains showed markedly reduced susceptibility to all four antifungal agents used in their study. Although MICs as high as 50μg/ml
were not seen in the present study, it is interesting to speculate as to whether the three less susceptible strains in their study were strains of the species now designated *M. furfur*.

Gupta *et al.* (2000a) tested all seven *Malassezia* species against ketoconazole, voriconazole, itraconazole and terbinafine. Unfortunately, most strains were susceptible to ketoconazole at concentrations below the lowest concentration tested, which was 0.03 μg/ml. Results for terbinafine showed *M. sympodialis* to be the most susceptible while the remaining species were less susceptible with MICs ranging from < 0.03 - 64 μg/ml. Their data did not show any obvious trend as to the comparative susceptibility of *Malassezia* species, except that *M. sympodialis* appeared to be the most susceptible species.

Nakamura *et al.* (Nakamura *et al.*, 2000) published a urea broth microdilution method and used this method to determine the susceptibilities of all seven *Malassezia* species to bifonazole, itraconazole, amorolfin and terbinafine. Comparison of the susceptibility data obtained for all species against each agent showed that *M. furfur* had the highest mean MICs for bifonazole, both *M. furfur* and *M. restricta* showed the highest mean MICs for itraconazole and terbinafine, and *M. pachydermatis* had the highest mean MICs for amorolfin (25 μg/ml), followed by *M. furfur* and *M. restricta* (12.5 μg/ml) (Nakamura *et al.*, 2000). In addition, they divided *Malassezia* species into two groups according to susceptibility: more susceptible were *M. sympodialis*, *M. slooffiae* and *M. obtusa*. Less susceptible were *M. restricta*, *M. globosa*, *M. pachydermatis* and *M. furfur*. Although some data indicate *M. furfur* to be the species least susceptible to antifungal agents in vitro, more data are required to substantiate this observation. This may have implications for antifungal therapy.

### 3.4 Conclusions

*Malassezia* yeasts can be isolated relatively frequently from both clinically normal and *Malassezia*-affected skin. Methods have been developed for identifying *Malassezia* isolates to the species level, however, some isolates aren't readily identified by these methods. In particular, isolates of *M. furfur*, *M. sympodialis* and *M. slooffiae* may be confused. Alternatively, some of these troublesome isolates may represent new subspecies or species.
In vitro susceptibility testing of *Malassezia* species showed isolates to be uniformly susceptible to tea tree oil with MICs ranging from 0.03 - 0.25%. Ketoconazole was the most active of the azoles and of all the *Malassezia* species tested, *M. furfur* appeared to be the least susceptible.
CHAPTER 4 - EFFECTS OF TEA TREE OIL AND COMPONENTS ON DERMATOPHYTES AND OTHER FILAMENTOUS FUNGI IN VITRO

4.1 Introduction

Few in vitro studies have been published investigating the effects of tea tree oil on dermatophytic and other filamentous fungi. Comprehensive investigations of the activity of tea tree oil against these organisms are an essential element of establishing the spectrum of antifungal activity of tea tree oil, and the suitability of the oil as a topical antifungal agent. The aim of this Chapter was to investigate the activity of tea tree oil and components against a range of filamentous fungi, including dermatophytes. Whilst in vitro susceptibility data provide some information about the susceptibility of organisms to a given antimicrobial agent, often additional studies are required to expand on these data. Therefore, time-kill dynamics and the comparative susceptibility of germinated and non-germinated A. niger conidia were investigated also. For ease of discussion in this Chapter, the dermatophytic fungi will be referred to as the dermatophytes and the remaining fungi will be termed the filamentous fungi.

4.2 Results

4.2.1 In vitro susceptibility data

4.2.1.1 Tea tree oil

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% (Tables 4.1 and 4.2). Generally, MIC90 and MFC90 values were lower for dermatophytes, as compared to the filamentous fungi.

MICs of 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 MIC90s of 0.03%, except for T. tonsurans which had a lower MIC90 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.
Table 4.1 In vitro susceptibility of dermatophytes to tea tree oil and griseofulvin determined by the broth microdilution method

<table>
<thead>
<tr>
<th>Organism (n)</th>
<th>Tea tree oil (% v/v)</th>
<th>Griseofulvin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
</tr>
<tr>
<td></td>
<td>Range 50% 90%</td>
<td>Range 50% 90%</td>
</tr>
<tr>
<td><em>E. floccosum</em> (15)</td>
<td>0.008 - 0.03 0.016 0.03</td>
<td>0.12 - 0.25 0.25 0.25</td>
</tr>
<tr>
<td><em>M. canis</em> (16)</td>
<td>0.004 - 0.03 0.016 0.03</td>
<td>0.06 - 0.25 0.25 0.25</td>
</tr>
<tr>
<td><em>M. gypseum</em> (6)</td>
<td>0.016 - 0.03 0.016 0.03</td>
<td>0.25 - 0.5</td>
</tr>
<tr>
<td><em>T. interdigitale</em> (21)</td>
<td>0.008 - 0.03 0.016 0.03</td>
<td>0.25 - 1 0.5 1</td>
</tr>
<tr>
<td><em>T. mentagrophytes</em> (14)</td>
<td>0.008 - 0.06 0.016 0.03</td>
<td>0.25 - 1 0.5 0.5</td>
</tr>
<tr>
<td><em>T. rubrum</em> (19)</td>
<td>0.008 - 0.03 0.016 0.03</td>
<td>&lt;0.03 - 0.25 0.12 0.25</td>
</tr>
<tr>
<td><em>T. tonsurans</em> (15)</td>
<td>0.004 - 0.016 0.008 0.016</td>
<td>0.12 - 0.5 0.25 0.5</td>
</tr>
</tbody>
</table>

*aT. mentagrophytes var. interdigitale; bT. mentagrophytes var. mentagrophytes*
<table>
<thead>
<tr>
<th>Organism (n)</th>
<th>MIC Range</th>
<th>Tea tree oil (%)</th>
<th>50%</th>
<th>90%</th>
<th>MFC</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria spp. (10)</td>
<td>0.016 - 0.12</td>
<td>0.06 - 0.12</td>
<td>0.06 - 2</td>
<td>0.12 - 2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Aspergillus flavus (12)</td>
<td>0.06 - 0.12</td>
<td>0.06 - 0.12</td>
<td>0.12 - 2</td>
<td>0.12 - 4</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A. fumigatus (12)</td>
<td>0.06 - 0.12</td>
<td>0.06 - 0.12</td>
<td>0.12 - 2</td>
<td>0.12 - 4</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A. niger (14)</td>
<td>0.008 - 0.12</td>
<td>0.06 - 0.12</td>
<td>0.12 - 2</td>
<td>0.12 - 4</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cladosporium spp. (10)</td>
<td>0.008 - 0.25</td>
<td>0.06 - 0.12</td>
<td>0.12 - 2</td>
<td>0.25 - 2</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Fusarium spp. (10)</td>
<td>0.03 - 0.06</td>
<td>0.03 - 0.06</td>
<td>0.06 - 2</td>
<td>0.06 - 2</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Penicillium spp. (10)</td>
<td>0.03 - 0.06</td>
<td>0.03 - 0.06</td>
<td>0.06 - 2</td>
<td>0.06 - 2</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
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*. MFC\(_{90}\)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.

### 4.2.1.2 Components

Terpinen-4-ol and \( \alpha \)-terpineol gave MIC values similar to, or a few dilutions lower than, those of tea tree oil for each of the test isolates (Table 4.3). MFCs were also similar or a few dilutions lower than those for tea tree oil. Preliminary MICs and MFCs for \( \alpha \)-pinene against dermatophytes were very low, with values of \(<0.004\%\), thus the testing of \( \alpha \)-pinene against dermatophytes was not pursued. For the filamentous fungi, MICs of \( \alpha \)-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 \( \alpha \)-terpinene and \( \gamma \)-terpinene showed moderate inhibitory and fungicidal activity against the dermatophytes but not the filamentous fungi. \( \rho \)-Cymene showed slight inhibitory activity against the dermatophytes only. Terpinolene showed moderate activity against both dermatophytes and filamentous fungi. 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.

### 4.2.1.3 Griseofulvin

MICs of griseofulvin for dermatophytes ranged from 0.25 to 2.0 \( \mu \)g/ml, with MIC\(_{90}\)s of 1 \( \mu \)g/ml for all species except *T. mentagrophytes var interdigitale*, which had an MIC\(_{90}\) of 0.5 \( \mu \)g/ml.
### Table 4.3 In vitro susceptibility of eight fungal isolates to tea tree oil and components

<table>
<thead>
<tr>
<th>Organism</th>
<th>Tea tree oil</th>
<th>Terpinen-4-ol</th>
<th>α-Terpinol</th>
<th>1,8-Cineole</th>
<th>α-Terpine</th>
<th>γ-Terpine</th>
<th>β-Cymene</th>
<th>Terpinolene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
<td>MFC</td>
</tr>
<tr>
<td><em>E. floccosum</em></td>
<td>0.03</td>
<td>0.25</td>
<td>0.008</td>
<td>0.016</td>
<td>0.016</td>
<td>ND</td>
<td>0.06</td>
<td>0.5</td>
</tr>
<tr>
<td><em>M. canis</em></td>
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<td>0.25</td>
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<td>4</td>
</tr>
<tr>
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<td>0.5</td>
<td>0.016</td>
<td>0.25</td>
<td>0.008</td>
<td>0.12</td>
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</tr>
<tr>
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<td>0.12</td>
<td>0.016</td>
<td>0.06</td>
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<td>0.5</td>
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<tr>
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<td>0.5</td>
<td>0.016</td>
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<td>0.016</td>
<td>0.5</td>
<td>4</td>
<td>&gt;8</td>
</tr>
</tbody>
</table>

<sup>a</sup>*T. mentagrophytes* var. *interdigitale*; <sup>b</sup>MFCs were not reproducible thus no value is given; <sup>c</sup>Isolates used in the time-kill assays.
4.2.2 Time-kill curves

4.2.2.1 *Trichophyton* spp.

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 4.1). 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\textsubscript{10} difference in viable count between treatment and control by 60 min, however, a >3 log\textsubscript{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.

4.2.2.2 *Aspergillus* spp.

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\textsubscript{10} until between 6 and 8 h of incubation with tea tree oil. At 24 h, numbers of control organisms had not changed significantly as compared to 0 h, 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.

4.2.3 Susceptibility of non-germinated and germinated *A. niger* conidia to tea tree oil

Germinated *A. niger* conidia were significantly more susceptible to tea tree oil than non-germinated conidia (NGC). The lowest concentration of tea tree oil tested was 0.03%, and the viability of germinated conidia (GC) treated with this concentration differed significantly from control (untreated) 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
Fig. 4.1 Time kill curves for dermatophytes. *Trichophyton rubrum* isolate 25 (A) and *Trichophyton mentagrophytes* var *interdigitale* isolate 8 (B) were incubated with 4 × MFC of tea tree oil and viable counts were performed at various time points. *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 (n = 3) plotted against time.*
Fig. 4.2 Time kill curves for *Aspergillus* spp. *Aspergillus niger* (A) and *Aspergillus fumigatus* (B) were incubated with 1 × MFC of tea tree oil and viable counts were performed at various time points. *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 (n = 3) plotted against time.*
Fig 4.3 Susceptibilities of germinated and non-germinated *Aspergillus niger* conidia to several concentrations of tea tree oil. Germinated (*shaded bars*) and non-germinated (*plain bars*) conidia of isolate 1 (A) and isolate 2 (B) were treated with tea tree oil for 30 min at 30°C after which time viable counts were performed. *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 (n = 3) are plotted.
not affected, compared to control NGC ($P > 0.05$). Significantly less control GC were recovered than control NGC ($P = 3.3 \times 10^{-5}$).

4.3 Discussion

4.3.1 Introduction

Tea tree oil and components have inhibitory and fungicidal activity against dermatophytes and filamentous fungi as shown by in vitro susceptibility assays, time kill methods and by investigating the susceptibility of germinated and non-germinated conidia to tea tree oil.

4.3.2 In vitro susceptibility data

4.3.2.1 Tea tree oil

The dermatophytes were alike in their susceptibility to tea tree oil, as were the other filamentous fungi, with the notable exception of *A. niger*, which had an MFC$_{90}$ of 8%.

Few previous studies have comprehensively investigated the activity of tea tree oil against dermatophytes and filamentous fungi, and only one other, by Vazquez *et al.* (2000), has used a method similar to the NCCLS method used in the current study.

Dermatophytes were both inhibited and killed by tea tree oil. Data from the current study can be compared to previously published data, except in some instances where methodological differences are too great. Several previously published reports used the disc diffusion method, which gives a qualitative indication of the anti-dermatophytic activity of tea tree oil but does not give specific, quantitative data, such as MICs.

Ansehn (Ansehn, 1990) tested 27 dermatophytes including *T. rubrum*, *T. mentagrophytes*, *E. floccosum*, *M. audouinii* and *M. canis* against 10 μl of tea tree oil in a disc diffusion method. All isolates showed zones of inhibition of greater than 3.5 cm. Similarly, Concha *et al.* (1998) showed zones of inhibition for 29 out of 30 dermatophytes including *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *E. floccosum* and *M. gypseum*, when using 20 μl of oil in a disc diffusion method. The organism not inhibited was an isolate of *E. floccosum*.
MICs obtained by an agar dilution method are more comparable to the data obtained in the present study than disc diffusion data. Rushton et al. (1997) inoculated isolates of *T. rubrum*, *T. mentagrophytes*, *E. floccosum* and *M. audouinii* onto agar plates containing 0, 0.1 and 1% tea tree oil. Plates were observed after 8 weeks incubation. All isolates showed growth on the 0.1% plate but none showed growth on the 1% plate, meaning all isolates had MICs of between 0.1 and 1%. Nenoff et al. (1996) used an agar dilution method and found MICs of 0.11% for *M. canis*, a range of 0.11 - 0.44% for *T. mentagrophytes* (*n* = 8) and a range of 0.11 - 0.22% for *T. rubrum* (*n* = 17). MICs obtained by the agar dilution method from other studies were 0.08% (Inouye et al., 2001b), 0.3 - 0.4% (Griffin & Markham, 2000), 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*. The overall range of MICs from the above studies was 0.04 to 1%, and these are mostly higher than the MICs obtained in the current study. However, it has been noted in previous studies that 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). A similar effect was seen when MICs of *Cymbopogon nardus* oil were determined by both agar and broth methods, although the test organism they used was *A. niger* (de Billerbeck et al., 2001).

In addition to the studies mentioned above, two have used a broth dilution method to determine the in vitro susceptibility of dermatophytes to tea tree oil. The first of these used SDB containing 0.5% Tween 80 in a macrodilution method (Christoph et al., 2000). Inoculated tests were incubated at 37°C for 7 d. MICs were 0.7% for *E. floccosum* and 0.6% (Christoph et al., 2000) for *T. rubrum*. The second study used a broth microdilution method, also with SDB. Tests were incubated for 2 - 7 d at 35°C after which time MICs were determined (D’Auria et al., 2001). MIC₉₀₈ were 0.25% and MFC₉₀₈ were 0.5% for *M. canis*, *T. mentagrophytes* and *T. rubrum*. 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. 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, or interpretation, than MICs.

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%. However, even less in vitro data have been published for the filamentous fungi compared to the dermatophytes.

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 an 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.

Earlier reports using a broth microdilution method showed MICs of >0.08% (Inouye *et al.*, 2001a) and >2% (Vazquez *et al.*, 2000) for *A. fumigatus*, whereas in the present study MICs for *A. fumigatus* ranged from 0.06 – 0.12%. In the study by Vazquez *et al.* (2000), the methodology was very similar to that used in the present study, but results were dissimilar. One difference between the two methods that may have had a significant influence on the results related to the final inoculum concentration. This was $0.4 \times 10^4 - 5.0 \times 10^4$ in the present study whereas in the study by Vazquez *et al.* (2000) the final inoculum concentration was $10^6$ conidia/ml. 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.

*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. Growth inhibition assays showed that tea tree oil inhibited the growth of two isolates each of *Fusarium* and *Alternaria* (Bishop & Thornton, 1997).
4.3.2.2 Components

There were great differences in the in vitro activity of the components of tea tree oil against both dermatophytes and filamentous fungi, with components such as terpinen-4-ol and α-terpineol showing considerably more in vitro activity than ρ-cymene and γ-terpinene. Unfortunately, comparison to previous data is difficult since many studies tested only one or two components of a particular essential oil and filamentous or dermatophytic fungi are not often chosen as test organisms. MIC data that have been reported 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. Again, this is most likely to be due to differences in test methods. This problem will be discussed in depth in Chapter 5.

4.3.2.3 Griseofulvin

Griseofulvin was included in the study as a comparator and to determine if any in vitro resistance to griseofulvin was developing. Previously published MICs of griseofulvin against dermatophytes were 0.25 - 4 μg/ml (Jessup *et al.*, 2000) and 2 - 4 μg/ml (Niewerth *et al.*, 1998), similar to the values obtained in this study. No isolates with elevated MICs were seen.

4.3.2.4 Methodological considerations for in vitro susceptibility assays

The broth microdilution method was chosen above other methods such as disc diffusion and agar dilution because much work has gone into optimising the assay to ensure reproducibility and it is widely accepted as a standardised method for determining in vitro susceptibility. Also, both MICs and MFCs can be readily determined (Espinel-Ingroff *et al.*, 1997). However, many factors such as time and temperature of incubation, and test medium, can affect the outcome of the broth dilution assay (Gehrt *et al.*, 1995). Also, the definition of and criteria for determining the MIC may influence results. Although RPMI 1640 is recommended as the test medium for antifungal
susceptibility testing because it is completely defined, and therefore not subject to the compositional variations seen with non-chemically defined media, it may allow comparatively poor growth of some fungi (Meletiadis et al., 2001). This is a significant disadvantage since sufficient growth of the test organism is one of the most basic requirements of an in vitro susceptibility test.

Another consideration for in vitro susceptibility assays is that inocula are prepared as conidial suspensions and therefore the outcome of the assay depends on how these conidia respond during the incubation period. During incubation of the test, the conidia must first germinate and then grow to produce growth visible to the naked eye. The absence of visible growth could be because the conidia are killed outright, the conidia are prevented from germinating, or only a few conidia germinate but are either killed after germination or cannot grow enough to produce visible growth (Manavathu et al., 1999a).

NCCLS methods are widely accepted for determining in vitro susceptibilities of both bacteria and fungi to antimicrobial agents. These methods have been designed largely for evaluating the activity of antibacterial or antifungal agents for systemic use in humans, so exactly how well essential oils fit within these protocols is debatable. Assays other than the NCCLS methods may better illustrate the activity of essential oils. Many essential oils are volatile, or contain volatile components, and some researchers have used the volatile rather than liquid phase to assess antifungal activity. Inouye et al. (2000) showed that the rate of elongation of hyphae could be slowed or stopped in the presence of essential oil vapours. Also, Espinosa-Garcia & Langenheim (1991) reported that γ-terpinene inhibited fungal growth at concentrations of 0.125 mg per ml air, whereas the results obtained by the broth microdilution method in the present study did not show γ-terpinene to have noteworthy activity. This exemplifies the different results that may be obtained by different methods.

4.3.3 Time kill experiments

The rate at which each of the four test organisms was killed by tea tree oil was not particularly rapid, despite the use of fungicidal concentrations of tea tree oil. More rapid rates of kill may be achievable with higher concentrations of tea tree oil, although this would partly depend on the mechanism of fungicidal action of tea tree oil.
The observation that the dermatophytes were more rapidly killed than the two *Aspergillus* isolates is most likely due to the differences between the concentrations of tea tree oil used for each pair of organisms. However, in studies assessing the fungicidal activity of disinfectants, Terleckyj & Axler (1987) found that *A. niger* and *A. fumigatus* were the most resistant organisms, surviving for longer times than the other test isolates. Although very few time kill studies have been performed with antifungal agents and filamentous fungi, one other study has used antifungal agents in excess of MIC concentrations. Manavathu *et al.* (1999a) performed time-kill studies with amphotericin B, voriconazole, itraconazole and SCH56592 against *A. fumigatus*, and amounts of agents from 2.5 to 20-fold higher than the MICs were required to produce a 95-100% kill by 24 h.

### 4.3.4 Comparative susceptibility of germinated and non-germinated *A. niger* conidia to tea tree oil

The present study showed that GC are more susceptible to tea tree oil than NGC. The viability of GC was affected at concentrations as low as 0.06%, whereas NGC remained unaffected at concentrations up to 0.25%. This difference in susceptibility may be because the GC lack the intact protective outer layers of the conidia that are thought to normally confer resistance to external insult. The two isolates examined demonstrated similar patterns of susceptibility suggesting that little strain-to-strain variation occurs with this particular assay.

Several other studies have assessed differences in the antifungal susceptibility of conidia, germinated conidia and hyphae, however, these studies did not agree about which fungal forms were most susceptible to the antifungal agent tested. In general, the assays with comparatively long incubation times such as 24 h (Manavathu *et al.*, 1999a) or 48 h (Pujol *et al.*, 2001) reported no significant differences in susceptibility, whereas the studies using shorter incubation times, such as the present study, as well as others (De Lucca *et al.*, 1997), have shown differences. This suggests that the time of exposure may be a critical parameter for the outcome of these assays, where the longer incubation time allows the agent to act on the non-germinated conidia or the germinated conidia have a greater chance to regrow after initial injury. Also, the test agents assessed in each
of these studies had different mechanisms of antifungal action, and therefore the assay results may simply be reflecting these differences.

4.3.5 General discussion

Using several different methods, the present study has shown that dermatophytes and other filamentous fungi are susceptible to tea tree oil. Most isolates showed a difference of several concentrations between inhibitory and cidal values indicating that, although tea tree oil is fungicidal, at particular concentrations it is fungistatic only (Tables 4.1 and 4.2). Unfortunately, very few studies have also determined both inhibitory and fungicidal concentrations of tea tree oil therefore comparisons to similar studies are limited.

The in vitro susceptibility assays used in the present study represent one way of evaluating the activity of antifungal agents, including essential oils. However, there are many different ways to evaluate the activity of oils against fungi and some assays have been developed to mimic particular applications, mostly agricultural. Bishop & Thornton (1997) assessed the antifungal activity of the vapour phase of tea tree oil since their interest was in tea tree oil as a crop fumigant. They found that the growth of a range of 15 post-harvest pathogens was inhibited by 0 to 85% in the presence of tea tree oil vapours, as compared to controls (Bishop & Thornton, 1997). Further studies by the same researchers showed that the treatment of discs of cabbage leaf with 3.2% tea tree oil prior to experimental infection with *Botrytis cinerea* prevented infection to approximately the same degree as a standard fungicide (Bishop & Reagan, 1998).

Several studies have investigated the inhibition of hyphal elongation of *Trichophyton* and *Aspergillus* spp. by the vapour phase of essential oils, including tea tree oil (Inouye *et al.*, 1998; Inouye *et al.*, 2000; Inouye *et al.*, 2001a; Inouye *et al.*, 2001b). While *T. mentagrophytes* and *T. rubrum* were inhibited by 800 μg/ml or 400 μg/ml tea tree oil, respectively, in a standard agar dilution assay, both organisms were inhibited by only 10 μg/ml by vapour contact (Inouye *et al.*, 2001b). Related studies with *A. fumigatus* showed that hyphae exposed to vapourised tea tree oil at 6.3 μg/ml continued to grow, whereas the growth of hyphae exposed to 63 μg/ml was stopped. When hyphae were removed from the 63 μg/ml vapour treatment, hyphae again grew at a normal rate. It was theorised from these studies that the vapours of essential oils cause inhibition of
growth by firstly adsorbing onto the hyphal surfaces and then accumulating inside the hyphae (Inouye et al., 2000). These studies illustrate the antifungal activity of tea tree oil in unusual but still valid ways.

One of the intriguing results from the present study is the observation that the conidia of A. niger appear to be comparatively less susceptible to tea tree oil than germinated conidia and most other species of fungi, including yeast cells. Although it is tempting to presume that fungal spores are resistant to antimicrobial agents in the same manner as bacterial spores, there is no evidence to suggest that this is a valid assumption. In fact, it has been reported that fungi are often more susceptible than bacterial spores, however, are less susceptible than vegetative bacterial cells (Russell, 1999). The reduced susceptibility of conidia to antifungal agents may be due to both the thickness and density of the conidial wall (Cheng & Levin, 1970). However, the thickness of the conidial wall is no greater than that of hyphae (Pujol et al., 2001) or a yeast cell, so the decreased susceptibility is more likely due to the composition and density of the conidial wall. It has also been reported that the relative distance from the outermost edge of the conidial wall to the cell membrane is much greater in conidia than in yeast cells or hyphae, suggesting that antifungal agents need to travel a greater distance in conidia to gain entry to the cell interior (Cheng & Levin, 1970). Also, conidia have a very hydrophobic surface which may impede interactions between the drug and conidia (Bundgaard-Nielsen & Nielsen, 1995).

Many essential oils other than tea tree oil have been investigated for antifungal activity for a range of applications. Areas that have received the most attention are the potential use of essential oils in the treatment of dermatophytic infection or as crop preservatives to inhibit or prevent the growth of fungal post-harvest pathogens.

Some of those showing potential as topical anti-dermatophytic agents include oils of Origanum vulgare subsp. hirtum (greek oregano), Mentha spicata (spearmint), Salvia fruticosa (greek sage), Chenopodium abrosioides (wormseed), Cymbopogon citratus (lemongrass), Mentha arvensis (cornmint) clove, peppermint, Inula viscosa and Ammi visnaga (El-Naghy et al., 1992; Kishore et al., 1993; Adam et al., 1998; Maoz & Neeman, 1998). While most of these studies have not ventured beyond determining in vitro susceptibility data, two conducted further experiments to examine the efficacy of
preparations containing 1% essential oil in experimental animal models of dermatophytic infection. Both studies showed promising results (Kishore et al., 1993; Adam et al., 1998). These oils may be useful as treatments for dermatophytic infections, however, data are required describing the toxicity of each oil, and the clinical parameters such as concentrations of oil, optimal formulations, and the duration of treatment that will maximise efficacy.

Many essential oils have been investigated for activity against post-harvest pathogens and food spoilage fungi, some of which are the traditional spice oils that have been used to flavour foods over the centuries. The oils showing the most activity in some of these studies were oils of Thymbra spicata, Satureja thymbra, Origanum minutiflorum (Müller-Riebau et al., 1995) Allium ramosum, Allium sativum, Capsicum annuum (Wilson et al., 1997), Thymus vulgaris, Mentha piperita (Zambonelli et al., 1996) and Cymbopogon citratus (Wilson et al., 1997), Thymus vulgaris, Mentha piperita (Zambonelli et al., 1996) and Cymbopogon citratus (Mishra & Dubey, 1994). Studies with essential oil components showed that carvacrol, eugenol (Thompson, 1989), cinnamic aldehyde and citral (Moleyar & Narasimham, 1986) had promising activity. In addition to in vitro susceptibility data, some of these studies showed other effects on fungi. Zambonelli et al. (1996) reported that Colletotrichum lindemuthianum and Fusarium solani treated with thyme or lavender oil had collapsed hyphae and formed far fewer conidia than controls, using electron microscopy (Zambonelli et al., 1996). Similar effects were seen in A. niger hyphae treated with Cymbopogon nardus oil. In this study, hyphal walls and the hyphae themselves were thinner than control hyphae and walls, and the cytoplasm of treated fungi appeared granular, in contrast to the uniform, clear appearance of the cytoplasm of untreated fungi (de Billerbeck et al., 2001). Further alterations in fungal morphology or function include the inhibition of aflatoxin production by A. flavus after treatment with various essential oil constituents (Mahmoud, 1994) and inhibition of conidia germination in a range of seven food spoilage fungi by several different essential oil components (Caccioni & Guizzardi, 1994). These studies show a range of effects of essential oils on fungi, different from those demonstrated in the present Chapter. It may be interesting to investigate whether tea tree oil produces these same effects on fungi.

The present study aimed to investigate the antifungal activity of tea tree oil, and to determine whether tea tree oil has potential as a topical antifungal agent. Based on the
data from the current Chapter, it would appear that tea tree oil possesses sufficient in vitro anti-dermatophytic activity. However, exactly how this in vitro activity translates into in vivo effectiveness is unclear, in particular since the clinical relevance of the in vitro activity of antifungal agents is still being determined (Balkis et al., 2002). The previously published trials using tea tree oil for the treatment of dermatophyte infections have showed only moderate success (Tong et al., 1992; Buck et al., 1994; Syed et al., 1999), suggesting that more work is required to determine how to increase clinical efficacy. There is also a need for more clinical trial data, particularly in relation to tinea pedis, which can often be treated successfully topically (Weitzman & Summerbell, 1995b).

4.4 Conclusions

Tea tree oil has inhibitory and fungicidal activity in vitro. For most fungi there was a difference of several dilutions between inhibitory and fungicidal concentrations indicating that at particular concentrations tea tree oil is fungistatic only. Some of the components of tea tree oil were also fungicidal. Time-kill studies showed a relatively slow rate of kill which may be attributed to the time required for the oil components to penetrate the dense conidial wall and gain entry to the cell interior. Studies with A. niger conidia showed that germinated conidia were more susceptible to tea tree oil than non-germinated conidia which may be because the germinated conidia lack the intact outer conidial layers that normally confer resistance to environmental conditions.
5.1 Introduction
Yeasts have emerged in the last few decades as important medical pathogens. In particular, the increasing numbers of chronically immunocompromised patients has meant a dramatic increase in the frequency of opportunistic infections caused by yeasts. Although tea tree oil is limited to topical applications, there is considerable interest in the use of tea tree oil to treat infections that have not responded to standard therapies. Furthermore, the development of safe, effective, alternative antifungal therapies would mean that the standard agents could be used less, thus potentially reducing the rate at which resistance develops to these drugs.

The effects of tea tree oil on several species of Candida, in particular Candida albicans, have been investigated, and a moderate amount of MIC data are now available. However, there are a paucity of data on the effects of tea tree oil on other clinically relevant yeasts. Therefore the aims of this Chapter were to investigate the activity of tea tree oil and components against yeasts by determining MICs and MFCs, and conducting both time-kill and growth assays.

5.2 Results

5.2.1 In vitro susceptibility data

5.2.1.1 Activity of tea tree oil against yeasts
A total of 153 yeast isolates were tested against tea tree oil and MICs ranged from 0.016% for isolates of R. rubra to 0.5% for isolates of C. albicans, C. glabrata, C. krusei and C. parapsilosis (Table 5.1). MFCs ranged from 0.06% for C. guilliermondii, Cr. neoformans and Trichosporon spp., to 1% for isolates of C. albicans, C. glabrata and C. parapsilosis. There was either no difference or a difference of one dilution only, between MIC and MFC values, for 44 and 85 of the isolates, respectively. For the remaining 24 isolates (20 of which were R. rubra), MIC and MFC values differed by
Table 5.1 In vitro susceptibility of yeasts \( (n = 153) \) to tea tree oil

<table>
<thead>
<tr>
<th>Organism ((n))</th>
<th>MIC (% v/v) Range</th>
<th>MIC(_{90})</th>
<th>MFC (% v/v) Range</th>
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<tbody>
<tr>
<td><em>Candida albicans</em> (49)</td>
<td>0.12-1.0</td>
<td>0.5</td>
<td>0.25-1</td>
<td>1</td>
</tr>
<tr>
<td><em>Candida cifferrii</em> (2)</td>
<td>0.06-0.12</td>
<td></td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td><em>Candida colliculosa</em> (1)</td>
<td>0.06</td>
<td></td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td><em>Candida famata</em> (1)</td>
<td>0.12</td>
<td></td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td><em>Candida glabrata</em> (11)</td>
<td>0.25-0.5</td>
<td>0.5</td>
<td>0.25-1</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Candida guilliermondii</em> (5)</td>
<td>0.06-0.25</td>
<td></td>
<td>0.06-0.5</td>
<td></td>
</tr>
<tr>
<td><em>Candida humicola</em> (2)</td>
<td>0.12</td>
<td></td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td><em>Candida krusei</em> (5)</td>
<td>0.25-0.5</td>
<td></td>
<td>0.25-0.5</td>
<td></td>
</tr>
<tr>
<td><em>Candida lusitaniae</em> (1)</td>
<td>0.5</td>
<td></td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td><em>Candida parapsilosis</em> (11)</td>
<td>0.25-0.5</td>
<td>0.5</td>
<td>0.5-1</td>
<td>1</td>
</tr>
<tr>
<td><em>Candida pelliculosa</em> (1)</td>
<td>0.25</td>
<td></td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td><em>Candida pseudotropicalis</em> (1)</td>
<td>0.25</td>
<td></td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td><em>Candida tropicalis</em> (11)</td>
<td>0.12-0.25</td>
<td>0.25</td>
<td>0.25-0.5</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Cryptococcus laurentii</em> (2)</td>
<td>0.03-0.06</td>
<td></td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em> (12)</td>
<td>0.06-0.25</td>
<td>0.12</td>
<td>0.06-0.25</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Rhodotorula rubra</em> (21)</td>
<td>0.016-0.12</td>
<td>0.06</td>
<td>0.25-0.5</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> (5)</td>
<td>0.12-0.25</td>
<td></td>
<td>0.25-0.5</td>
<td></td>
</tr>
<tr>
<td><em>Trichosporon spp.</em> (10)</td>
<td>0.03-0.12</td>
<td>0.12</td>
<td>0.06-0.25</td>
<td>0.12</td>
</tr>
</tbody>
</table>
two dilutions. Comparison of data obtained for *Candida*, *Cryptococcus*, *Rhodotorula*, *Saccharomyces* and *Trichosporon* showed that MIC and MFC data did not appear to vary considerably between genera.

MIC<sub>90</sub>s obtained in MHB were one or two concentrations lower than those obtained in RPMI Medium 1640 (Table 5.2). MFC<sub>90</sub>s were either equivalent, one, or two dilutions lower in MHB as compared to RPMI Medium 1640.

### 5.2.1.2 Activity of the components of tea tree oil against yeasts

The in vitro susceptibility data for six tea tree oil components are shown in Table 5.3. Concentrations of p-cymene up to and including 8% did not inhibit or kill any of the yeast isolates (data not shown in Table). 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.

Data obtained for tea tree oil components against *C. albicans* 10231 by the broth macrodilution method are shown in Table 5.4, along with data obtained by the broth microdilution method for comparison. When Tween 80 was used initially at a concentration of 0.001%, results were not reproducible for terpinolene and γ-terpinene. For terpinolene, MICs and MFCs were not reproducible at both 24 and 48 h with standing. With shaking, MICs and MFCs were 0.5% and 1% at 24 h and were both 1% at 48 h. The only reproducible result for γ-terpinene was a modal MIC of 2% obtained at 24 h with shaking. Where tea tree oil was tested using 0.001% Tween 80, data obtained were reproducible, regardless of standing or shaking. All MIC and MFC results for tea tree oil were 0.25%, except the MFC at 48 h, with shaking, which was >0.25%.
Table 5.2 In vitro susceptibility data obtained by two different methods for *Candida* spp. and tea tree oil

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mueller Hinton Broth</th>
<th>RPMI Medium 1640</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC Range 90%</td>
<td>MIC Range 90%</td>
</tr>
<tr>
<td><em>C. albicans (n = 10)</em></td>
<td>0.12 - 0.25 0.12</td>
<td>0.25 - 0.5 0.5</td>
</tr>
<tr>
<td><em>C. glabrata (n = 10)</em></td>
<td>0.12 - 0.5 0.25</td>
<td>0.25 - 0.5 0.5</td>
</tr>
<tr>
<td><em>C. parapsilosis (n = 11)</em></td>
<td>0.12 - 0.25 0.25</td>
<td>0.25 - 0.5 0.5</td>
</tr>
</tbody>
</table>
Table 5.3 In vitro susceptibility of yeasts to tea tree oil and components, as determined by the broth microdilution method.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Tea tree oil</th>
<th>Terpinen-4-ol</th>
<th>α-Terpinol</th>
<th>1,8-Cineole</th>
<th>α-Terpinene</th>
<th>γ-Terpinene</th>
<th>Terpinolene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
</tr>
<tr>
<td>C. albicans ATCC 10231</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.12</td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td>C. albicans ATCC 90028</td>
<td>0.25</td>
<td>0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>0.12</td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td>C. parapsilosis ATCC 90018</td>
<td>0.25</td>
<td>0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>8</td>
</tr>
<tr>
<td>S. cerevisiae ATCC 10716</td>
<td>0.25</td>
<td>0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>0.12</td>
<td>0.12</td>
<td>1</td>
</tr>
<tr>
<td>R. rubra</td>
<td>0.06</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.12</td>
<td>0.12</td>
<td>1</td>
</tr>
<tr>
<td>Trichosporon sp.</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.25</td>
<td>0.06</td>
<td>0.12</td>
<td>2</td>
</tr>
</tbody>
</table>

nd, not done
Table 5.4 MIC and MFC data for tea tree oil and components against *C. albicans* ATCC 10231 obtained by the broth macro- and microdilution methods

<table>
<thead>
<tr>
<th>Tea tree oil or component</th>
<th>Time (h)</th>
<th>Microdilution¹</th>
<th>Macrodilution²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Standing</td>
<td>Shaking</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC</td>
<td>MFC</td>
</tr>
<tr>
<td>Tea tree oil</td>
<td>24</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>24</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>24</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>24</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>24</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>24</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>α-Terpinene</td>
<td>24</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>ρ-Cymene</td>
<td>24</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>&gt;8</td>
<td>&gt;8</td>
</tr>
</tbody>
</table>

¹ Final concentration of 0.001% Tween 80; ² Final concentration of 0.1% Tween 80
Further studies were conducted with Tween 80 at a final concentration of 0.1%. MICs and MFCs for tea tree oil were both 0.25% by this 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.

5.2.1.3 Combinations of tea tree oil and 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 Σ 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 Σ 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 Σ 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.
5.2.2 Growth curves of \textit{C. albicans} in the presence of tea tree oil

The growth of \textit{C. albicans} in the presence of tea tree oil is shown in Table 5.5. Control cells and cells grown in the presence of 0.016\% tea tree oil did not differ significantly, whereas cells grown with 0.03\% (1/16 x MIC) and 0.06\% (1/8 x MIC) showed reduced growth when compared with controls, 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\% (1/4 x 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 values for control cells were lower than those of cells grown in the presence of 0.016\% tea tree oil at several time points, but this difference was significant 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 the 3 h time point 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 ($P < 0.05$).

5.2.2.1 Growth curves of cells pre-conditioned with 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 5.1 demonstrates that differences between the two growth curves were not great. Log$_{10}$ (OD$_{540}$) values for pre-conditioned cells were significantly higher than non-conditioned cells 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 $3.32 \times 10^2$ cfu/ml.
Table 5.5 Growth parameters of *C. albicans* ATCC 10231 in the presence and absence of tea tree oil

<table>
<thead>
<tr>
<th>Tea tree oil (%)</th>
<th>Mean cell yield in mg wet wt/ml (± SEM)</th>
<th>Mean generation time* Hours (range)</th>
<th>Relative increase in log₁₀ cfu/ml (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30.63 ± 0.72</td>
<td>1.38 (1.29 - 1.48)</td>
<td>4.63 × 10¹ (1.22 × 10¹) 3.64 × 10² (9.11 × 10¹)</td>
</tr>
<tr>
<td>0.016</td>
<td>30.02 ± 1.08</td>
<td>1.39 (1.33 - 1.45)</td>
<td>2.91 × 10¹ (4.9 × 10⁶) 9.41 × 10² (6.3 × 10²)</td>
</tr>
<tr>
<td>0.031</td>
<td>27.43 ± 0.61</td>
<td>1.35 (1.26 - 1.42)</td>
<td>2.20 × 10¹ (7.8 × 10⁶) 5.30 × 10² (2.3 × 10²)</td>
</tr>
<tr>
<td>0.062</td>
<td>22.45 ± 0.28</td>
<td>2.49 (1.76 - 3.75)</td>
<td>2.89 × 10⁰ (1.37 × 10⁶) 3.45 × 10² (5.26 × 10¹)</td>
</tr>
</tbody>
</table>

*average of 3-4 experiments*
Fig 5.1 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 ($n = 4$).
whereas non-pre-conditioned cells increased by $1.76 \times 10^2$ and this difference was significant ($P = 0.012$). However, wet weight cell yields at 24 h were not significantly different ($P = 0.25$).

5.2.3 Time kill studies

Time kill data are shown in Figures 5.2 to 5.8. The viability of control cells at both 25°C and 35°C did not change significantly over time, as compared to time zero viable counts.

5.2.3.1 Tea tree oil

At 35°C, the viability of cells treated with 0.12% tea tree oil did not differ significantly from control cells until 6 h ($P = 0.035$). 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 $\geq 3 \log_{10} \text{cfu/ml}$ (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.

For assays conducted at 25°C, cells treated with 0.25% tea tree oil differed significantly from control cells from 1 h onwards ($P < 0.01$) and cells treated with 0.5% tea tree oil differed significantly from controls after 30 min treatment ($P < 0.01$). Cells were recovered from the 0.5% treatment at 1, 2 and 3 h but not at 4 h. Decreases in cell viability of $\geq 3 \log_{10} \text{cfu/ml}$ were seen after 4 h with 0.25% and after 2 h with 0.5% tea tree oil.

Comparison of viable count data at each concentration of tea tree oil from both temperatures did not show significant differences. However, cells treated with 0.5% tea tree oil were recoverable after 3 h at 25°C, whereas 1 h was the last time point where viable cells were recovered when treated at 35°C.
Fig 5.2 Tea tree oil time kill curve (35°C). *C. albicans* ATCC 10231 was incubated at 35°C with several concentrations of tea tree oil and viable counts were performed at different time points. Experiments were repeated 2-5 times and mean values (± SEM) are shown.
Fig 5.3 Tea tree oil time kill curve (25°C). *C. albicans* ATCC 10231 was incubated at 25°C with two concentrations of tea tree oil and viable counts were performed at different time points. Experiments were repeated 2-5 times and mean values (± SEM) are shown.
Fig 5.4 Terpinen-4-ol time kill curve. *C. albicans* ATCC 10231 was incubated at 35°C with two concentrations of terpinen-4-ol and viable counts were performed at different time points. Experiments were repeated 2-5 times and mean values (± SEM) are shown.
Fig 5.5 α-Terpineol time kill curve. *C. albicans* ATCC 10231 was incubated at 35°C with two concentrations of α-terpineol and viable counts were performed at different time points. Experiments were repeated 2-5 times and mean values (± SEM) are shown.
Fig 5.6 1,8-Cineole time kill curve. *C. albicans* ATCC 10231 was incubated at 35°C with several concentrations of 1,8-cineole and viable counts were performed at different time points. Experiments were repeated 2-5 times and mean values (± SEM) are shown.
Fig 5.7 Terpinolene time kill curve. *C. albicans* ATCC 10231 was incubated at 35°C with 1% terpinolene and viable counts were performed at different time points. Experiments were repeated 2-5 times and mean values (± SEM) are shown.
Fig 5.8 α-Terpinene, γ-terpinene and ρ-cymene time kill curves. C. albicans ATCC 10231 was incubated at 35°C with 1% α-Terpinene, γ-terpinene or ρ-cymene and viable counts were performed at different time points. Experiments were repeated 2-5 times and mean values (± SEM) are shown.
5.2.3.2 Components of tea tree oil

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% $\alpha$-terpineol differed significantly from control cells at 6 h ($P = 0.011$) whereas cells treated with 0.25% $\alpha$-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% $\alpha$-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 of 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% $\gamma$-terpinene, $\alpha$-terpinene or $\rho$-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.

5.3 Discussion

5.3.1 Introduction

Little comprehensive data exist pertaining to the spectrum of activity of tea tree oil and components against clinically important yeasts. Similarly, little data exist with regards to which concentrations of tea tree oil inhibit the growth of, or kill C. albicans. In view of this lack of information, the aims of this Chapter were to examine the in vitro susceptibility of a range of medically important yeasts to tea tree oil and components using several different investigative tools.
5.3.2 In vitro susceptibility data

5.3.2.1 Tea tree oil

Of the in vitro susceptibility data obtained for tea tree oil against yeasts, most pertains to *C. albicans*. MICs obtained for this species by the agar dilution method were (% v/v) 0.2 (Griffin & Markham, 2000) and 0.44 (Nenoff *et al.*, 1996) and those obtained by the broth dilution method were 0.25 (Vazquez *et al.*, 2000), 0.3 (Christoph *et al.*, 2000) and 0.5 (D'Auria *et al.*, 2001). Despite slight variations in experimental techniques, most of these previously reported MICs are similar to each other, and to the values obtained in the present study. MICs of tea tree oil obtained previously for non-albicans *Candida* species ranged from 0.22 to 0.5% for *C. glabrata*, *C. famata*, *C. intermedia*, *C. parapsilosis*, *C. tropicalis*, *C. kefyr*, *C. krusei*, *C. guilliermondii* and *C. lusitaniae* (Nenoff *et al.*, 1996; Banes-Marshall *et al.*, 2001; D'Auria *et al.*, 2001). These values are also similar to those obtained in the present study.

Few studies have determined the susceptibility of non-*Candida* yeasts to tea tree oil. Nenoff *et al.* (1996) reported an MIC of 0.22% for an isolate of *Trichosporon cutaneum* and D’Auria *et al.* (2001) found MICs of 0.5% for isolates of *Schizosaccharomyces pombe* and *Debaromyces hansenii*. No information exists for *Rhodotorula*, *Cryptococcus* or *Saccharomyces* spp.

Differences in susceptibility to tea tree oil within a single species appear to be minimal, since ranges of MICs and MFCs within each species did not exceed three concentrations. This generalisation is limited by the numbers of isolates tested, the highest number being 49 isolates of *C. albicans*. However, similarly small MIC ranges have been found in another study, where 50 isolates of *C. albicans* had an MIC range of 0.06 - 0.25% and 21 isolates of *C. glabrata* had an MIC range of 0.25 – 0.5% (Vazquez *et al.*, 2000). Likewise, little variation in susceptibility was seen between different *Candida* species in the present study, although comparisons are again limited by numbers. D’Auria *et al.* (2001) tested a number of *Candida* species against tea tree oil and MIC<sub>90</sub>s were either 0.25 or 0.5% for all species, including *C. albicans*.

MICs and MFCs obtained for each yeast isolate differed by two or fewer dilutions for 94% of isolates (144 of 153). Most *Rhodotorula* isolates had MICs and MFCs that
differed by two or more dilutions. The relatively small difference (if any) between the MIC and MFC for each isolate suggests that tea tree oil, for the most part, has fungicidal activity against yeasts.

MIC/MFC results obtained in RPMI were only slightly higher than the method using MHB. The main methodological differences between these two assays were the growth medium, inoculum size and duration of microdilution tray incubation and, despite these differences, there were no great discrepancies between the results obtained by each assay.

5.3.2.2 Tea tree oil in combination with other agents

MICs for boric acid and nystatin were similar to previously published results (Scott et al., 1995; Otero et al., 1999) but MICs for miconazole were comparatively high (Scott et al., 1995). When each of these agents was tested in combination with tea tree oil, the MICs were generally lowered by one or more concentrations, indicating some synergistic activity. The most interesting observation was the striking synergy seen with miconazole and tea tree oil, against C. albicans only. Studies with more isolates are required to confirm this outcome, and time-kill studies may better illustrate the dynamics of the synergistic effects. This preliminary result suggests that these two agents may have complementary modes of action, with the action of each enhancing the activity of the other.

Little work, either in vivo or in vitro, has been published on the efficacy of combinations of essential oils and conventional agents. One study of combinations of terpenes with antibiotics or antifungal agents did find some synergy but this study was largely qualitative (Jedlicková et al., 1992). Syed et al. assessed a combination of tea tree oil and butenafine in a clinical trial, although they were not treating a candidal infection (Syed et al., 1999). In the study, a combination of 2% butenafine and 5% tea tree oil was compared to 5% tea tree oil alone for the treatment of toenail onychomycosis caused by Trichophyton spp. Cure rates of 80% and 0% were found for the two treatments, respectively. Although their study gave no information about the advantages, if any, of butenafine with tea tree oil over butenafine alone, it indicates that the combination was effective, and that the study of tea tree oil in combinations is of scientific interest. The agents assessed in the current study were chosen because they are
commonly used topical antifungal agents and the possibility remains that tea tree may be effectively combined with these or other agents for the treatment of superficial infections. Such combination therapy may reduce the rate at which fungi develop resistance to conventional antifungal agents.

5.3.2.3 Components of tea tree oil
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. Adam et al. (1998) assessed the activity of the main components of several essential oils (not including tea tree oil) against M. furfur and T. beigelii by a disc diffusion method. They found either no zones or only small zones of inhibition of 1 - 4 mm for γ-terpinene, α-pinene, ρ-cymene and 1,8-cineole. The relatively small amount of each agent (5 μl of component per disc) may explain the small or absent zones. Adegoke et al. (2000) studied the activity of the components of Aframomum danielii extract against six food spoilage yeasts and found MICs of 4.9 - 312 μg/ml and MFCs of 9 – 1250 μg/ml for α-terpinene. MICs of α-pinene and 1,8-cineole ranged from 78 – 156 μg/ml and MFCs ranged from 312 – 625 μg/ml. Lastly, Himejima et al. (1992) found MICs for terpinolene of 50 and 100 μg/ml for S. cerevisiae and C. utilis, respectively. 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 reported similar overall trends to those found by the microdilution method in the present study, each using one strain of C. albicans (Carson & Riley, 1995c; Griffin et al., 1999b). 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 (Carson & Riley, 1995c), 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 are dissimilar to those obtained by the microdilution assay in the present Chapter and closer to those obtained by the macrodilution method.

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

5.3.2.4 Differences between the macro and microdilution methods

Considerably lower results were obtained for some components by the macrodilution method, as compared to the microdilution method. Results obtained 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 differed by several dilutions.

Several differences between these test methods may account for the dissimilar results. These include technical factors such as different assay volumes, degrees of sealing in each assay system and the vessels in which the tests are performed. Several studies have compared the activity of antifungal agents such as amphotericin B and fluconazole by both macro- and micro-dilution methods and have generally found that MICs are either equivalent or vary only by 1 – 2 dilutions (Espinel-Ingroff et al., 1995; Tornatore et al., 1997; Pelletier et al., 2000). That these studies did not see greatly different MICs or MFCs by each method may indicate that the differences seen in the current Chapter are 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 vessel in which the assay is
performed, and the relationship of this to 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 (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. Since many of the components of tea tree oil, and other essential oils, are only sparingly soluble in water or may interact with plastics used in vitro tests, assays for evaluating antimicrobial activity must be designed with this in mind.

5.3.3 Growth in the presence of tea tree oil

The presence of 0.03 and 0.06% tea tree oil caused a decrease in both the growth rate and the cell population of *C. albicans* at 24 h, although these effects were not seen with 0.016% tea tree oil. The concentrations of 0.03 and 0.06% tea tree oil are less than those shown to inhibit the growth of *C. albicans* in standard MIC assays.

Cinnamon and thyme oils have similar effects against *C. albicans* (Ferhout et al., 1999). The MICs of cinnamon and thyme oils were determined as 350 µg/ml and 300 µg/ml, respectively, however, both oils at concentrations of 100 µg/ml extended the lag phase and reduced the final cell yield of *C. albicans* as compared to controls. Thus both oils affected growth at sub-MIC concentrations (Ferhout et al., 1999), similar to the effect seen with tea tree oil in the present study. Adam et al. (1998) investigated the effects of several essential oils on the growth of *Trichosporon beigeli*, Boonchird & Flegel (1982) investigated the effects of eugenol and vanillin on the growth of *C. albicans* and *Cr. neoformans* (Boonchird & Flegel, 1982) and Ultee et al. (1998) investigated the effects of low concentrations of carvacrol on *Bacillus cereus*. All found dose-dependent decreases in growth rates and a decrease in the final cell density, as compared to controls. These effects may be occurring by inhibition of microbial respiration, since tea tree oil and other essential oils and components inhibited respiration in fungi, including *C. albicans* (Bard et al., 1988; Inouye et al., 1998; Cox et al., 2000). In particular, it has been 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 this study (Cox et al., 2000). Alternatively, inhibition of growth
may be occurring because the plasma membrane ATPase, an enzyme that consumes large amounts of cellular ATP and is mostly responsible for regulating cell homeostasis, has been activated by the presence of tea tree oil, leaving little ATP available for cell growth (Bracey et al., 1998).

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 the study by Ferhout et al. (1999), who suggest that the growth enhancement may be due to the utilisation of the essential oil compounds as growth substrates. King and Dickinson (2000) showed previously that some yeasts (although they did not assess *C. albicans*) were able to convert terpenoids into other compounds, so it may be that where the amounts of oil present are not high enough to be toxic or harmful to the cells, they are in fact able to utilise one or more of the components as substrates.

Ferhout et al. (1999) also suggest that the enhanced growth may be due to cellular stress responses caused by the presence of low concentrations of oil. Induction of stress responses would presumably confer greater fitness to the cells and render them more capable of dealing with further stresses or insults. However, in the present study, pre-conditioning cells with tea tree oil did not confer greater fitness when cells were subsequently grown in tea tree oil. Very few studies investigating pre-conditioning and subsequent in vitro susceptibility have been conducted. Ultee et al. (2000) found that *B. cereus* exposed to sub-inhibitory amounts of carvacrol had a greater survival rate upon subsequent exposure to carvacrol, as compared to cells not pre-conditioned with carvacrol. They attributed this adaptation to a decrease in membrane fluidity and an alteration of membrane composition, which helped to maintain membrane function. Also, Chambel et al. (1999) compared the growth of pre-conditioned and non pre-conditioned *S. cerevisiae* cells in media containing 20 mg/l cinnamic acid and found that the growth of non pre-conditioned cells was significantly slower than the pre-conditioned cells. They attributed the greater fitness of pre-conditioned cells to a stimulation of membrane H⁺-ATPase as a compensatory response to low levels of cinnamic acid. The fact that in the current study pre-conditioning did not show any such effect suggests that either tea tree oil does not induce stress responses or that different kinds of assays are needed to show these effects. It is also possible that the effects of
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pre-conditioning may be more apparent if studies were conducted with only a single component of tea tree oil.

5.3.4 Time kill studies

Time kill studies showed that negligible killing of \textit{C. albicans} ATCC 10231 occurred with 0.125% tea tree oil. Tea tree oil at 0.25% caused a gradual loss of viability over the 6 h time period, and 0.5 and 1.0% caused a rapid loss of viability which was more rapid at 35°C than at 25°C. These results are similar to those of a previous study that showed a total loss of viability after 30 min treatment with 0.5% tea tree oil, and after 120 min treatment with 0.25% tea tree oil (Cox et al., 2000).

Killing at 25°C was slightly slower than at 35°C, for 0.5% but not for 0.25%. This could be because at the higher temperature the components of tea tree oil are more soluble in both the test solution and the cell membranes (Sikkema et al., 1995). The partitioning of terpenes into cell membranes will be discussed in greater depth in Chapter 6.

The components terpinen-4-ol and 1,8-cineole both produced very rapid rates of killing at 0.25% for terpinen-4-ol and at 0.5 and 1.0% for 1,8-cineole. At 0.25% \( \alpha \)-terpineol there was also a relatively rapid rate of kill, although not as rapid as for 0.25% terpinen-4-ol. The rapid rates of kill seen by these components were in contrast to the relatively slow rate of kill seen for \( \gamma \)-terpinene, \( \rho \)-cymene, terpinolene and \( \alpha \)-terpinene over the 6 h time period. Similarly, Cox et al. (2001a) saw little killing of \textit{C. albicans} with \( \gamma \)-terpinene but terpinen-4-ol produced rapid killing. It is interesting that while relatively low MICs were obtained for \( \gamma \)-terpinene by the macrodilution method using glass, this component still produced only a relatively slow rate of kill. This may be because this component has poor solubility, and had a slow rate of solubilisation into the cell membranes during the 6 h time period of the time kill assay.

5.4 Conclusions

The work of this Chapter has shown that tea tree oil has inhibitory and fungicidal activity against a range of clinically important yeasts. Also, the components of tea tree oil were active against yeasts although the degree of activity seen appeared to be highly method-dependent and was most likely to be related to the solubility of these compounds. Growth inhibition and time kill studies with tea tree oil showed that \textit{C.}
*Candida* albicans was rapidly killed with 1.0 and 0.5%, slowly killed when treated with 0.25% and growth was inhibited at 0.06 and 0.03, but not 0.016% tea tree oil.
CHAPTER 6 - STUDIES OF THE MECHANISM OF ACTION OF TEA TREE OIL AND COMPONENTS AGAINST CANDIDA ALBICANS, CANDIDA GLABRATA AND SACCHAROMYCES CEREVISIAE

6.1 Introduction

The mechanism of action of tea tree oil and components against yeasts is not fully understood. Early studies with both yeasts and bacteria suggested that terpenes affect membrane structure and function; therefore studies of these types were conducted. The membrane properties of permeability and fluidity were investigated and, since the plasma membrane ATPase plays a significant part in maintaining cell homeostasis, the functioning of this enzyme was also investigated. The effects of specific alterations in cell functioning, such as the depletion of membrane ATPase and membrane depolarisation on susceptibility to tea tree oil, were investigated to determine what role these factors play in the susceptibility of organisms to tea tree oil. Other aspects of cell functioning, such as the ability of cells to form germ tubes or accumulate trehalose in the presence of tea tree oil were also investigated.

6.2 Results

6.2.1 Alterations in cell permeability

6.2.1.1 Methylene blue dye exclusion assay

Some of the results of the methylene blue dye exclusion assays are shown in Figs 6.1 - 6.4, with the remainder described below. The percentage staining of control cells over the 6 h time period did not increase significantly.

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 (Fig 6.1). 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 $\alpha$-terpineol showed similar trends whereby treatment with 0.12% of either component did not cause a significant increase in the percentage of cells staining
Fig 6.1 Tea tree oil-treated cells stained with methylene blue. Cells of \textit{C. albicans} ATCC 10231 were treated with several concentrations of tea tree oil then stained with 0.1\% (w/v) methylene blue, to which cells are normally impermeable. Mean ± SEM ($n = 3$).
Fig 6.2 Terpinen-4-ol-treated cells stained with methylene blue. Cells of *C. albicans* ATCC 10231 were treated with several concentrations of terpinen-4-ol then stained with 0.1% (w/v) methylene blue, to which cells are normally impermeable. Mean ± SEM (*n* = 2).
Fig 6.3 α-Terpineol-treated cells stained with methylene blue. Cells of *C. albicans* ATCC 10231 were treated with several concentrations of α-terpineol then stained with 0.1% (w/v) methylene blue, to which cells are normally impermeable. Mean ± SEM (*n* = 2).
**Fig 6.4** 1,8-Cineole-treated cells stained with methylene blue. Cells of *C. albicans* ATCC 10231 were treated with several concentrations of 1,8-cineole then stained with 0.1% (w/v) methylene blue, to which cells are normally impermeable. Mean ± SEM (*n* = 3).
blue over the 6 h time period, however, 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 (Fig 6.4).

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 did not differ significantly from controls. $\gamma$-Terpinene, $\rho$-cymene and $\alpha$-terpinene, all examined at 1%, did not cause significant increases in the percentages of cells staining blue over the 6 h time period. By 6 h, the percentages of cells staining blue had increased to between 9.85 and 12.95%, compared to 5.79% of control cells.

### 6.2.1.2 Leakage of 260 nm-absorbing material

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 Figs 6.5 to 6.9 or described in the text below. *C. albicans* ATCC 10231 was tested against both 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. 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 time zero to 0.09 at 6 h, and values differed significantly from time zero from 2 h.

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 (Fig 6.5). However, leakage after 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
Fig. 6.5 Leakage of 260 nm-absorbing materials from tea tree oil-treated cells. Cells of *C. albicans* ATCC 10231 were treated with several concentrations of tea tree oil and the optical density of cell free filtrates at 260 nm was determined. Mean ± SEM (*n* = 3).
Fig. 6.6 Leakage of 260 nm-absorbing materials from tea tree oil-treated cells. Cells of *C. glabrata* ATCC 15545 were treated with several concentrations of tea tree oil and the optical density of cell free filtrates at 260 nm was determined. Mean ± SEM (*n* = 3).
Fig. 6.7 Leakage of 260 nm-absorbing materials from terpinen-4-ol-treated cells. Cells of *C. albicans* ATCC 10231 were treated with several concentrations of terpinen-4-ol and the optical density of cell free filtrates at 260 nm was determined. Mean ± SEM (n = 3).
Fig. 6.8 Leakage of 260 nm-absorbing materials from 1,8-cineole-treated cells. Cells of *C. albicans* ATCC 10231 were treated with several concentrations of 1,8-cineole and the optical density of cell free filtrates at 260 nm was determined. Mean ± SEM (n = 4).
Fig. 6.9 Leakage of 260 nm-absorbing materials from terpinolene-treated cells. Cells of *C. albicans* ATCC 10231 were treated with several concentrations of terpinolene and the optical density of cell free filtrates at 260 nm was determined. Mean ± SEM (*n* = 4).
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 however, this did not differ significantly from controls (Fig 6.6). 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$_{260}$ compared to controls, whereas changes caused by treatment with 0.25% were significant at 1, 4 and 6 h (Fig 6.7). Treatment with either 0.12 or 0.25% α-terpineol did not cause any significant increases in OD$_{260}$ compared to controls.

Treatment with 0.5 and 1% 1,8-cineole caused significant increases in the OD$_{260}$ after 1 h, compared to controls (Fig 6.8). However, these two treatments 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.

Treatment with terpinolene caused significant increases in OD$_{260}$ values after 1 h with both 0.25 and 0.5%, and after 2 h with 1.0% (Fig 6.9). 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$_{260}$, with values at all time points ranging from -0.238 to 0.189 and 0.004 to 0.155 for both components, respectively, compared to -0.001 to 0.052 for controls. Values for γ-terpinene-treated cells differed significantly from controls after 2 h.

**Influence of cations on the leakage of 260 nm-absorbing materials**

For control cells, the presence of either 10 or 50 mM cations alone did not produce additional leakage, compared to the buffer control without additional cations (Fig 6.10). For treatments without cations, cells treated with 0.5 and 1.0% tea tree oil produced significantly more leakage than control cells after 6 h, and these two treatments were not significantly different from each other at this time ($P = 0.407$).

Leakage in the presence of additional cations was not significantly different from leakage produced in their absence. Comparison of all treatments containing 1% tea tree
Fig 6.10 Leakage of 260nm-absorbing material from *C. albicans* ATCC 10231 treated with tea tree oil in the presence of cations. Cells were treated for 6 h with tea tree oil in the presence and absence of 10mM or 50mM cations. Mean ± SEM (*n = 4*).
oil (with or without cations) showed no significant differences. Similarly, comparison of all treatments containing 0.5% tea tree oil did not show significant differences.

6.2.2 Medium acidification during treatment with 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* (Figs 6.11 – 6.13). 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.
Fig 6.11 Relative decrease in the pH of the external medium of suspensions of *S. cerevisiae* NCTC 10716 in the presence of tea tree oil, following the addition of glucose. *Relative pH values were derived by dividing pH values for 5 min and onwards by the pH value for that treatment at 0 min. Mean ± SEM (n = 4).
Fig 6.12 Relative decrease in the pH of the external medium of suspensions of *C. albicans* ATCC 10231 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. Mean ± SEM (n = 4).
Fig 6.13 Relative decrease in the pH of the external medium of suspensions of *C. glabrata* ATCC 15545 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. Mean ± SEM (*n* = 5).
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 to data at 60 min did not show significant differences.

When total changes in pH (determined by subtracting the pH at time zero from each subsequent pH reading) were analysed statistically results differed little from the statistical analysis of relative pH values. The only changes in significant differences were that decreases in pH in the presence of 0.1% tea tree oil differed significantly from controls at 40 min (instead of 30 min) for *S. cerevisiae*, *C. albicans* treated with 0.2% tea tree oil differed significantly from the control at 20 min (compared to 30 min), *C. glabrata* treated with 0.3% tea tree oil differed from the control at 20 min (compared to 30 min) and treatment with 0.4% differed from the control at 10 min, compared to 20 min.

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

In the following section, organisms that were not pre-treated or were pre-treated with vehicle only are referred to as ‘control’ cells.

6.2.3.1 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 6.14). 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 in itself did not cause a significant decrease in cell viability, as seen by comparing control cells and CCCP pre-treated 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
Fig. 6.14 Viability of *C. albicans* ATCC 10231 cells pre-treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) then post-treated with tea tree oil. Test cells were pre-treated with 200μM CCCP for 2 h and were then post-treated for 1 h with 0, 0.2, 0.3 or 0.4% tea tree oil, or 0.3% tea tree oil with 200μM CCCP for 1 h. Control cells were pre-treated with vehicle (methanol) only. Mean ± SEM (*n* = 3).
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.

6.2.3.2 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 6.15. 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, for both DES concentrations.

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.

6.2.3.3 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
Fig. 6.15 Viability of *C. albicans* ATCC 10231 cells pre-treated with diethylstilboestrol (DES) and post-treated with tea tree oil. Test cells were pre-treated for 2 h with 100µM or 125µM DES and were post-treated for 1 h with 0, 0.2, 0.3 or 0.4% tea tree oil, 2M NaCl or 0.3% tea tree oil with 125µM DES. Control cells were pre-treated with vehicle (methanol) only. Mean ± SEM (n = 4).
compared to control cells post-treated with no tea tree oil ($P = 0.001$ for both) (Fig 6.16). 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 ($9.2 \times 10^3$ cfu/ml) on one occasion of 5 repeats and once each from calcium pre-treated cells that were post-treated with 0.4% ($3.1 \times 10^2$ cfu/ml) and 0.5% ($3.45 \times 10^2$ cfu/ml). These post-treatment concentrations had been repeated 5 and 4 times, respectively.

6.2.4 Alterations in membrane fluidity as measured by DPH fluorescence intensity

6.2.4.1 *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 when compared to control cells, whereas increases seen for cells grown with 0.03 and 0.06% were (Fig. 6.17). 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.

6.2.4.2 *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 6.18.
Fig. 6.16 Viability of *C. albicans* ATCC 10231 cells pre-treated with calcium then post-treated with two concentrations of tea tree oil. Cells were pre-treated for 2 h with 100mM Ca$^{2+}$ then post-treated for 1 h with 0, 0.2 or 0.3% tea tree oil. Mean ± SEM (*n* = 5).
Fig. 6.17 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. Mean ± SEM (*n* = 4).
**Fig 6.18** 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 (n = 4).
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 the increase seen for γ-terpinene 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.

6.2.5 Trehalose content of cells grown in the presence of tree oil

The trehalose content of cells of *S. cerevisiae* and *C. albicans*, expressed as mg trehalose/g dry weight, is shown in Fig 6.19. Assays were performed in duplicate only, so the ranges expressed below give both values. Statistical analyses were limited. Baseline levels of trehalose were low, between 0.24 and 0.64 mg/g dry wt, for both organisms.

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, however, 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 time zero. 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.
Fig 6.19 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 (n = 2).
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 time zero to 3 h. However, trehalose levels in control cells increased 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). No statistically significant differences were seen between tea tree oil treatments and controls. 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), compared to baseline levels.

6.2.6 Germ tube formation both during and after treatment with tea tree oil

Mean percentage germ tube formation (GTF) in the presence of tea tree oil for *C. albicans* isolates ATCC 10231 and 88E is shown in Fig 6.20. The highest concentration of tea tree oil used in these assays was 0.25%, and this represents \( \frac{1}{2} \times \text{MIC} \) for both organisms (broth microdilution method), or is equivalent to the MIC for ATCC 10231 (broth macrodilution method). No GTF was seen for either isolate in the presence of 0.125% and 0.25% tea tree oil. For all other concentrations of tea tree oil, and at all other time points, mean percentage GTF was less than the controls, except isolate 88E at 1 h where this was greater than the control in the presence of 0.004%, 0.008% and 0.016% tea tree oil. However, these differences were not statistically significant.

The morphology of cells after 4 h in the presence of tea tree oil in HS is shown in Table 6.1. For both isolates, cells treated with 0.25% tea tree oil showed no change in the proportions of cells of each morphology type over time. Cells treated with 0.062% and 0.125% tea tree oil showed an increase in cells bearing multiple buds and single buds, and a decrease in single cells, as compared to the morphologies of cells at time zero. Cells treated with 0.031% showed mean percentage GTF of 44.0% and 51.3%, for 10231 and 88E respectively, and the next largest proportion of cells was those showing a multibudded morphology. Analysis of viable count results from cells treated with 0.25% tea tree oil showed no difference in numbers of viable cells at any time point, for both isolates.
Fig. 6.20 Germ tube formation by *C. albicans* in the presence of tea tree oil. *C. albicans* ATCC 10231 (A) and clinical isolate 88E (B) were incubated in horse serum with several different concentrations of tea tree oil (% v/v) and were examined microscopically at several time points for the formation of germ tubes. Mean (% germ tube formation) ± SEM (*n* = 3).
Table 6.1 Morphology (mean percentage ± SEM) of *C. albicans* ATCC 10231 and clinical isolate 88E at 0 and 4 h in the presence of tea tree oil (% v/v)

<table>
<thead>
<tr>
<th>Tea tree oil (%)</th>
<th>Morphology*</th>
<th><em>C. albicans</em> ATCC 10231</th>
<th><em>C. albicans</em> 88E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC</td>
<td>68.0 ± 5.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>SB</td>
<td>24.0 ± 5.3</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>MB</td>
<td>9.3 ± 1.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>0.0 ± 0.0</td>
<td>99.5 ± 0.5</td>
</tr>
<tr>
<td>0.016</td>
<td>SC</td>
<td>0.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SB</td>
<td>1.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MB</td>
<td>23.3 ± 7.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>77.5 ± 6.0</td>
<td></td>
</tr>
<tr>
<td>0.031</td>
<td>SC</td>
<td>1.5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SB</td>
<td>8.7 ± 5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MB</td>
<td>54.0 ± 4.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>42.5 ± 7.2</td>
<td></td>
</tr>
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<td>0.062</td>
<td>SC</td>
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<td>SB</td>
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<td>MB</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>0.7 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Tea tree oil (%)</td>
<td>Morphology*</td>
<td>C. albicans ATCC 10231</td>
<td>C. albicans 88E</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------</td>
<td>------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>4 h</td>
<td>0 h</td>
</tr>
<tr>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>4.0 ± 3.0</td>
<td></td>
<td>17.3 ± 4.0</td>
</tr>
<tr>
<td>SB</td>
<td>32.0 ± 1.1</td>
<td></td>
<td>42.0 ± 5.0</td>
</tr>
<tr>
<td>MB</td>
<td>3.3 ± 1.8</td>
<td></td>
<td>40.7 ± 5.0</td>
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<tr>
<td>SC</td>
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<td></td>
<td>80.0 ± 2.3</td>
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<td>SB</td>
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<td>17.3 ± 1.3</td>
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<tr>
<td>MB</td>
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</tr>
<tr>
<td>GT</td>
<td>0.0 ± 0.0</td>
<td></td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Key: SC Single cells; SB Single bud; MB Multiple bud; GT Germ tube.

Protuberances were not seen at 0 or 4 h. Data for 0.004% and 0.008% not shown.
The mean percentage GTF for all 10 isolates in the presence of 0.031% tea tree oil is shown in Table 6.2. For all isolates, mean percentage GTF ranged from 91.0% to 100.0% for controls and from 10.0% to 68.5% in the presence of 0.03% tea tree oil. Comparison of tea tree oil-treated cells with controls showed that GTF in the presence of tea tree oil differed significantly from controls for eight of the 10 isolates \( (P < 0.05) \).

Correlation of MICs and MFCs with mean percentage GTF in the presence of 0.031% tea tree oil gave correlation coefficients of 0.548 and 0.858, respectively.

GTF after 1 h pre-treatment with subinhibitory concentrations of tea tree oil is shown in Fig 6.21. For both isolates, mean GTF in cells pre-treated with 0.125% and 0.25% tea tree oil differed significantly from controls at 1 h \( (P < 0.05) \) however, at all later time points only cells pre-treated with 0.25% differed significantly from controls \( (P < 0.01) \). Mean percentage GTF in control cells was always greater than in cells pre-treated with tea tree oil, except for \( C. \) albicans ATCC 10231 at 1 h, where mean percentage GTF was greater in cells that had been pre-treated with 0.016% tea tree oil than in control cells (not statistically significant, \( P > 0.05 \)). Analysis of viable count results for \( C. \) albicans 10231 pre-treated with 0.25% tea tree oil over time showed no difference in viability, as determined by ANOVA, however, Dunnett's Multiple Comparison Test showed that at 5 h and 6 h viable counts were significantly lower than time zero values \( (P < 0.05) \).

6.3 Discussion

6.3.1 Introduction

Several studies investigating the mechanism of action of tea tree oil and components against yeasts are reported in this Chapter. Previous studies with tea tree oil and terpenes have demonstrated a range of effects against microorganisms and other cells, including changes in cell membrane permeability (Lambert et al., 2001), polarity and fluidity (Bard et al., 1988) and inhibition of respiration (Uribe et al., 1985; Mucciarelli et al., 2001). In the following discussion, parallels will be drawn with studies investigating several key compounds such as ethanol, polygodial, thymol and carvacrol, which display some mechanisms of antimicrobial activity similar to those of tea tree oil (Cartwright et al., 1986; Lunde & Kubo, 2000; Ultee et al., 2000).
Table 6.2 MICs and MFCs for tea tree oil (% v/v), and mean percentage germ tube formation (GTF) at 4 h in the presence of 0.031% tea tree oil for 10 C. albicans isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC</th>
<th>MFC</th>
<th>Mean percentage GTF ± SEM</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0.03% TTO</td>
</tr>
<tr>
<td>231</td>
<td>0.25</td>
<td>0.25</td>
<td>91.0 ± 5.3</td>
<td>10.0 ± 5.6</td>
</tr>
<tr>
<td>204</td>
<td>0.25</td>
<td>0.25</td>
<td>98.0 ± 1.2</td>
<td>19.5 ± 10.8</td>
</tr>
<tr>
<td>245</td>
<td>0.25</td>
<td>0.25</td>
<td>98.5 ± 0.5</td>
<td>23.0 ± 12.2</td>
</tr>
<tr>
<td>39M</td>
<td>0.5</td>
<td>0.5</td>
<td>98.5 ± 1.5</td>
<td>26.0 ± 6.7</td>
</tr>
<tr>
<td>202</td>
<td>0.25</td>
<td>0.5</td>
<td>100.0 ± 0.0</td>
<td>29.5 ± 10.4</td>
</tr>
<tr>
<td>45S</td>
<td>0.25</td>
<td>0.5</td>
<td>99.5 ± 0.5</td>
<td>30.0 ± 7.4</td>
</tr>
<tr>
<td>ATCC 10231</td>
<td>0.5</td>
<td>0.5</td>
<td>99.5 ± 0.5</td>
<td>42.5 ± 7.2</td>
</tr>
<tr>
<td>27U</td>
<td>0.25</td>
<td>0.5</td>
<td>98.5 ± 0.5</td>
<td>57.5 ± 17.6</td>
</tr>
<tr>
<td>88E</td>
<td>0.5</td>
<td>1.0</td>
<td>99.5 ± 0.5</td>
<td>57.5 ± 9.3</td>
</tr>
<tr>
<td>64T</td>
<td>0.5</td>
<td>1.0</td>
<td>96.0 ± 1.6</td>
<td>68.5 ± 12.3</td>
</tr>
</tbody>
</table>

* Students 2-tailed T-test.
Fig. 6.21 Germ tube formation by *C. albicans* after 1 h pre-treatment with tea tree oil. *C. albicans* ATCC 10231 (A) and clinical isolate 88E (B) were pre-treated with several different concentrations of tea tree oil (% v/v) for 1 h. Cells were then washed and inoculated into horse serum and examined for the formation of germ tubes. Mean (% germ tube formation) ± SEM (n = 4).
6.3.2 Alterations in cell permeability

Many compounds alter cell permeability. These include, but are not limited to, lipophilic compounds such as amphotericin B (Beggs, 1994), ketoconazole (Anséhn & 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 the present Chapter.

Changes in cell permeability can be assessed by staining with the membrane-impermeable dye methylene blue. 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 such as 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 optical density 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 by 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 had been determined by the macrodilution assay. The exception was terpinolene, which caused permeability changes at concentrations below the MIC, however, this was only seen with the assay for detecting the leakage of 260 nm-absorbing materials but not by the MB dye exclusion assay. 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, severe effects may be expected.

Differences between the chemical composition, shape and solubilities of tea tree oil components may explain the differences in activity. One distinct difference is that terpinen-4-ol, α-terpineol and 1,8-cineole are all oxygenated monoterpenes and 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 solubilities of 1827, 1491 and 907 ppm, respectively (Griffin et al., 1999b).

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 used was that permeability changes were apparent much more quickly with the methylene blue assay. Where *C. albicans* cells were treated with 0.25% tea tree oil, no significant leakage of 260 nm-absorbing materials was seen after 6 h treatment, whereas after 2 h a significant proportion of cells (30.63%) was stained with methylene blue. A similar trend was seen by Cox *et al.* (2000) who found that although tea tree oil-treated *C. albicans* cells were permeable to propidium iodide, they did not leak potassium ions. These differences 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 discrepancies 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. Cox et al. (2000) suggested in their study that the thickness and impermeability of the yeast cell wall may have prevented the potassium ions from diffusing into the external medium. 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 (\(\Delta \psi\)), which consists of both an electrical potential (\(\Delta \Psi\)) and a pH gradient (\(\Delta \rho\)), and depolarises the membranes. Studies by Sikkema et al. (1994) support this theory since they showed using model membranes that several cyclic hydrocarbons, including \(\alpha\)-pinene, benzene and tetralin dissipate both \(\Delta \rho\) and \(\Delta \psi\). 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 \(\Delta \rho\). 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 and \(S.\) cerevisiae (Cartwright et al., 1986).

Tea tree oil or components may dissipate \(\Delta \rho\) because of their lipophilicity, which means that they preferentially partition into the lipophilic portion of the PM bilayer and cause the PM to expand, as shown previously (Sikkema et al., 1994). In addition to the PM expansion, 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 may also occur. One or more of these changes may cause an increase in the passive flux of ions across the membrane, resulting in the dissipation of \(\Delta \rho\).
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). This decrease in internal pH is caused by the rapid influx of protons and 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.

6.3.3 Medium acidification during treatment with tea tree oil

The addition of glucose to starved yeast cells causes a rapid decrease in the pH of the medium in which the cells are suspended. This is because the glucose is rapidly taken up via the proton motive force generated by the rapid expulsion of intracellular protons by the PM H⁺ATPase (Manavathu et al., 1999b; Lunde & Kubo, 2000). The efficiency of the proton-pumping capabilities of the PM H⁺ATPase can therefore be monitored by changes in the pH of the external medium (Manavathu et al., 1999b). 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).

In the present Chapter, all cell suspensions showed an immediate glucose-induced decrease in pH, but by 10 min, differences between tea tree oil treatments 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 and results were similar for all three test organisms. 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.

Changes in the functioning of the PM ATPase 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 which then changes the ‘matrix’ within which the enzyme
normally functions (Kubo et al., 2001). Isolated membrane fractions have commonly
been used to confirm the PM ATPase as the target of an agent (Surarit & Shepherd,
1987), however, this still does not distinguish between effects directly on the ATPase
itself and those on surrounding molecules (Alexandre et al., 1996; Manavathu et al.,
1999b).

Alternatively, the inhibition of medium acidification may be caused by effects on
acellular 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,
as has been shown in bacteria and yeasts (Uribe et al., 1985; Cox et al., 1998), the
energy deficit caused by the inhibition of respiration 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 pumping out
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), which was postulated as
being due to either increased use or decreased production, or both. Also, the dose-
dependent inhibition of acidification 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 section 6.2.2,
dissipation of Δp of the yeast mitochondria may, in part, explain how respiration is inhibited.

Studies with other lipophilic compounds such as decanoic acid, ethanol, nonylphenol, β-pinene and in particular polygodial, have shown that these compounds also 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). Kubo *et al.* (2001) showed that polygodial inhibited medium acidification using whole cells of *S. cerevisiae*. Based on their results and the lipophilic, surfactant-like properties of polygodial, they postulated that polygodial adversely affects the PM H⁺ATPase by disrupting the normal hydrogen bonding at the lipid-protein interface that holds the PM H⁺ATPase proteins in place (Kubo *et al.*, 2001). However, when Lunde & Kubo (2000) added polygodial to PM ATPase extracted from cells, they showed that polygodial did not directly inhibit the PM ATPase, but affected medium acidification indirectly by inhibiting the mitochondrial ATPase. Inhibition of the mitochondrial ATPase meant that the large amounts of cellular ATP required to fuel the PM ATPase were not available (Lunde & Kubo, 2000). This illustrates the need for further studies to determine which mechanisms underlie the tea tree oil-induced inhibition of medium acidification in yeasts.

An interesting observation from several studies with lipophilic antifungal compounds is 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. Studies that investigated how susceptibility to tea tree oil is affected when the PM ATPase is inhibited are discussed in section 6.3.4.2.

### 6.3.4 Effects of pre-treatment on susceptibility to tea tree oil

Compounds that alter specific aspects of cell functioning may be useful for elucidating the mechanism of action of antimicrobial agents. These studies focus on several membrane functions or properties that may be important in cell homeostasis.
6.3.4.1 CCCP

Several studies have shown that the in vitro susceptibility of microorganisms to particular antimicrobial agents can be altered after pre-treatment with ionophores such as carbonylcyanide m-chlorophenylhydrazone (CCCP) (Thevissen et al., 1999). For example, the killing and permeabilisation of *E. coli* and *C. albicans* by the membrane-active antimicrobial peptides indolicidin and histadin 5, respectively, was much reduced when cells were pre-treated with CCCP (Falla et al., 1996; Koshlukova et al., 1999). From these studies, the suggestion was made that an intact membrane potential is required for the activity of these compounds (Falla et al., 1996; Thevissen et al., 1999).

CCCP is a proton ionophore that acts in yeasts by shuttling ions across plasma, vacuolar and mitochondrial membranes, resulting in their depolarisation. In mitochondria, this results in the uncoupling of respiration 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). There are also indications that the dissipation of Δp renders cells incapable of a heat shock response (Cheng & Piper, 1994). Both the importance of Δp to normal cell functioning and the indications that pre-treatment with CCCP may alter the antimicrobial susceptibility of microorganisms, prompted investigations into what effects, if any, the dissipation of Δp had on the susceptibility of *C. albicans* to tea tree oil.

Results showed that cells pre-treated with CCCP were acutely susceptible to tea tree oil, compared to non pre-treated cells. This outcome is different to those found above but 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. The destabilising effects of tea tree oil may represent an additional 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 functions, 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 and their overexpression has been implicated in drug resistance in yeasts (Kohli et al., 2002). The terpene eugenol has been reported to stimulate the expression of the genes encoding the ABC transporters in Mycosphaerella graminicola (Zwiers & De Waard, 2000) although little other information is available describing whether terpenes in particular are exported by yeast cells. Regardless, depolarisation of the PM would remove 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.

In conclusion, the pre-treatment of C. albicans cells with the ionophore CCCP rendered cells acutely susceptible to tea tree oil, which in turn demonstrated that $\Delta p$-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 $\Delta p$, either $\Delta \Psi$ or $\Delta p\text{H}$, may prove useful.

### 6.3.4.2 DES

Diethylstilbestrol (DES) is an inhibitor of the PM ATPase in yeasts and other organisms (Dawson et al., 1986). The role of the PM ATPase, as discussed in section 6.3.3, is to maintain cell homeostasis by regulating intracellular pH and the PM electrochemical gradient ($\Delta p$). In addition to the PM ATPase, yeast cells contain mitochondrial and vacuolar ATPases (van der Rest et al., 1995) and each ATPase has been shown to have inhibitors specific to that enzyme. Orthovanadate, DES, and $N, N'$-dicyclohexylcarbodiimide (DCCD) have been shown to specifically inhibit the PM
ATPase (Henschke & Rose, 1991) and DES has been shown to have only slight inhibitory effects on mitochondrial and vacuolar ATPases (Henschke & Rose, 1991; van der Rest et al., 1995).

In the present Chapter, 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 and that an activated PM ATPase is one of the responses used by yeasts to counter the proton-translocating effects of different antifungal agents (see section 6.3.3). 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 an ionic imbalance that cannot be compensated for and results in a loss of homeostasis that 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.

6.3.5 Influence of cations on the activity of tea tree oil

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 because cations may inhibit interactions between antimicrobial agents and cell membranes (Ben-Josef et al., 1999), or the 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 susceptibility to compounds such as ethanol and polygodial has been significantly altered in the presence of cations.

In the present Chapter, the presence of cations was not shown to significantly alter tea tree oil-induced leakage or cell death. Both remained essentially unchanged in the
presence of additional Ca\(^{2+}\) or Mg\(^{2+}\) 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.

### 6.3.6 Alterations in membrane fluidity

Membrane fluidity (MF) is a measure of the lateral motion of molecules within a lipid bilayer and can be measured with the use of fluorescent probes (Lentz, 1988; Slavik, 1994). The probe used in this Chapter, 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, as does the DPH derivative 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene \(p\)-toluenesulfonate (TMA-DPH) (Lentz, 1988). The difference between these two probes is that TMA-DPH has a hydrophilic headgroup that acts as an anchor, securing the probe near the headgroups of the phospholipid molecules (Lentz, 1988). Since DPH has no such 'anchor', the depth at which DPH is positioned within the bilayer is still a matter of debate (Lentz, 1988; Slavik, 1994; Laroche et al., 2001) and it has been observed that the DPH molecule will occasionally position itself between the monolayers of the membrane, where the ends of the fatty acyl chains meet (Lentz, 1988). Membrane fluidity is most often measured by determining fluorescence polarisation or anisotropy, which is inversely related to MF, such that a decrease in anisotropy corresponds to an increase in MF.

#### 6.3.6.1 Cells grown for 24 h with tea tree oil

The MF of cells grown with low concentrations of tea tree oil was increased, and increases were dose-dependent with greater changes occurring with higher concentrations 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 \(C.\) \emph{albicans} have been shown to have increased MF as compared to susceptible control strains (Kohli et al., 2002). In all of these studies, MF changes 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 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 membranes, which represents an adaptive response (Sikkema et al., 1995).

In contrast to the findings of the present study, cells of B. cereus that were adapted to grow in low concentrations of the terpene carvacrol had lower MF than non-adapted cells (Ultee et al., 2000). These modifications were due to changes in fatty acid and head group composition, with an increase in the proportion of shorter chain length fatty acids. Studies with miconazole also showed that miconazole-grown cells had less fluid membranes than control cells, although in this instance these membranes also lacked ergosterol which would greatly influence fluidity (Ansari & Prasad, 1993b). Changes in membrane composition in response to lipophilic hydrocarbons has been shown to correlate with the solubility of the compound in water whereby treatment with more polar compounds such as ethanol and acetone resulted in an increase the ratio of saturated to unsaturated fatty acids and treatment with more apolar compounds such as benzene decrease this ratio. From this generalisation, it could be inferred that the MF changes seen by Ultee et al. (2000) can be ascribed to carvacrol belonging to the 'more apolar' compound category. However, where carvacrol fits in this polarity 'scale' is unclear and may be subject to interpretation. Other important factors to consider are possible differences in the kinds of compositional changes that occur in bacteria and yeasts, 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 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.

The increase in the MF of tea tree oil-grown cells is probably reflecting changes in the composition of the PM lipids, which in turn alters the physical characteristics of the membrane. However, since the DPH fluorescence measurements in the current study represent whole cell measurements and not just PM measurements, it cannot be discounted that fluidity changes may be occurring in other internal cell membranes. Analysis of the composition of the PM lipids of cells grown with tea tree oil would be required to determine if modifications in lipid composition have occurred. In addition to alterations in fluidity, it must also be considered that changes in the physical properties of membranes are likely to affect other membrane properties such as membrane embedded proteins and their functions (Slavik, 1994; Weber & de Bont, 1996).

### 6.3.6.2 Immediate changes in membrane fluidity

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 treatment. The largest change in fluidity at 30 min was also caused by 1,8-cineole, followed by treatments with tea tree oil, terpinolene, γ-terpinene (although these results were not significant) and terpinen-4-ol. The component α-terpineol caused a slight decrease in fluidity, as compared to controls. It is important to consider that changes in MF are likely to be concentration, time and organism dependent although these variables were not investigated to any great extent in the current Chapter.

Previous studies have also found that short-term treatments with terpenes caused increases in MF. Studies with yeasts have shown that both geraniol, at concentrations up to 10 mM and β-pinene at concentrations of 100 - 1000 μM, caused decreases in fluorescence polarisation values for *C. albicans* (Bard *et al.*, 1988) and *S. cerevisiae*, respectively (Uribe *et al.*, 1985). In a study with *B. cereus*, the addition of carvacrol to cells resulted in an immediate decrease in phase transition temperature of membrane
lipids, which also corresponds to an increase in MF (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 (Sikkema et al., 1994; Engelke et al., 1996). In the study by Uribe et al. (1985), the decrease in anisotropy after treatment with β-pinene was further characterised by repeating their studies using both isolated PMs and mitochondria and results showed the greatest change in MF was 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). The 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 (terpinen-4-ol) and 4.36 ($\gamma$-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.

A decrease in fluidity, albeit slight, was seen for terpinolene at 10 min and $\alpha$-terpineol at both 10 and 30 min. While it may be postulated that the MF of cells treated with $\alpha$-terpineol may have increased at subsequent time points, some cyclic hydrocarbons, such as menthone and $\beta$ ionone, have been shown to cause decreases in fluidity (Bouchard et al., 1996). Engelke et al. (1996) showed that the hydrocarbons $n$-hexane and cyclohexane decreased MF in artificial membranes whereas benzene and toluene increase MF, and they proposed that this was because the former compounds contained only single bonds and therefore did not cause great dislocation of the fatty acyl chains (Engelke et al., 1996). Molecules of $\alpha$-terpineol are unsaturated and more similar in structure to benzene than $n$-hexane so this theory does not seem to apply in the present work. Another suggestion is that membrane ordering may be increased because some compounds may interact with both monolayers (Sikkema et al., 1995).

Another interesting observation from the current study was that $\alpha$-terpineol and terpinen-4-ol showed quite different changes in MF after 30 min treatment. This was unexpected since $\alpha$-terpineol and terpinen-4-ol only differ structurally in the position of their hydroxyl groups, and they have virtually identical log $K_{ow}$ values. However, a similar discrepancy in activity between these two compounds was noted by Griffin et al. (1999) who ascribe this to differences in the molecular properties of each terpene, such as H-bonding capacity.
These experiments have shown that the fluidity of the cell membranes of *C. albicans* is altered by treatment with tea tree oil and components, however, several important methodological factors need to be considered. Firstly, fluidity in the current Chapter was estimated by fluorescence intensity measurements whereas the majority of membrane fluidity studies measure fluorescence polarisation or anisotropy. This is because lipid bilayers are considered to be highly anisotropic, which describes the rates and axes of motion of the phospholipid molecules with the bilayer, and the vast differences in the physical properties encountered in different areas within the bilayer (Yeagle, 1993; Weber & de Bont, 1996). Since the probe is inserted between, and parallel to, the fatty acyl chains, the motion of the probe must also be described as anisotropic. Anisotropy measurements are also less susceptible to slight variations in assay conditions such as pH changes, as compared to fluorescence intensity measurements (Haugland, 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.* (1994) observed no changes in the MF of liposomes treated with cyclic hydrocarbons when TMA-DPH was used as a probe, whereas changes were seen with 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.
6.3.7 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). Of great interest is the observation that 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). It is also interesting that trehalose accumulation occurs in a manner very similar to the expression of heat shock proteins (hsp), both occurring rapidly after an increase in temperature and both declining when cells are returned to normal physiological temperatures (Attfield, 1987). Trehalose accumulation 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.

In the present Chapter, 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 different 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). However, studies in this Chapter examining trehalose levels need to be repeated before firm conclusions can be drawn.

Most studies on trehalose accumulation have investigated responses to environmental conditions such as heat and osmotic shock and very few antifungal agents have been investigated. 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 (Attfield, 1987). An alternative explanation is that the yeast cells may be unable to accumulate trehalose because tea tree oil is acting as a general inhibitor of growth and metabolism, disallowing energy-requiring processes such as trehalose production. In support of this theory, it has already been shown in Chapter 5 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). Furthermore, there are data to suggest that de novo RNA synthesis is required for the accumulation of trehalose during heat shock (Attfield, 1987; Blazquez *et al.*, 1994) and this process may not be possible in energy-deficient cells. A study with amphotericin B has shown that after the addition of the agent to *C. albicans* cells, there was a gradual decrease in glucose consumption and corresponding decreases in trehalose, ethanol and glycerol production (Rabaste *et al.*, 1996). Since amphotericin B and tea tree oil are similar in that both induce alterations in cell permeability and inhibit the growth of yeast cells (Surarit & Shepherd, 1987; Bracey *et al.*, 1998), it is possible that these agents may be preventing trehalose accumulation by similar means.

6.3.8 Effects of tea tree oil on germ tube formation

*Candida albicans* is unique amongst *Candida* yeasts in its ability to form germ tubes, which are the first stage in the transition from blastospores to hyphae (Odds, 1988). Germ tube formation (GTF) has been suggested to be a virulence factor (Sobel *et al.*, 1984), based in part on the observation that germ tubes have an increased capacity to adhere to and penetrate epithelial cells, as compared to blastospores (Odds, 1988). Also, the ability of *C. albicans* to form germ tubes and hyphae may play an important role in the pathogenesis of vaginal and oral candidiasis since hyphae (usually combined with blastoconidia and/or pseudohyphae) are almost always present in smears from patients with either condition (Odds, 1988; Kobayashi & Cutler, 1998).

The formation of germ tubes by *C. albicans* was affected by the presence of, or pre-treatment with, tea tree oil, and some of these results were similar to those found by another author (D'Auria *et al.*, 2001). In the presence of 0.25% tea tree oil, germ tubes were not seen, and there were no changes in cell morphologies from 0 to 4 h. This suggests that no growth was occurring, which is supported by results of viable counts which were also unchanged from 0 to 4 h. Similarly, no GTF occurred in the presence
of 0.125% tea tree oil, however, there was a general trend of blastospores changing from single or singly budding morphologies to multiply budding morphologies over the 4 h test period. These changes in morphology suggest that these cells were actively growing, although still unable to form germ tubes, implying that there is specific inhibition of morphogenesis occurring, rather than a total inhibition of growth. An explanation for the predominance of budded forms may be that the presence of tea tree oil represents an 'environmental stress' and that under these conditions growth by budding is favoured (Odds, 1988). Most publications describing inhibition of GTF do not mention the morphologies of those cells not bearing germ tubes, therefore comparisons are limited. However, Odds et al. (1985) noted that in the presence of azoles the development of hyphae was severely restricted and that growth occurred in the form of clumped budding yeast cells.

Pre-treatment experiments showed that inhibition of GTF was reversible, since tea tree oil-treated cells were able to form germ tubes when washed and resuspended in fresh HS. Compared to the GTF of control cells, cells pre-treated with tea tree oil appeared to have a concentration-dependent 'lag phase' in germination, as seen by the differences in GTF at 1 h. However, after 2 h, GTF was approaching that of control cells, except for those cells pre-treated with 0.25% tea tree oil. Viable counts from cells pre-treated with 0.25% tea tree oil indicated that there was a decrease in viability over the course of the experiment. The lag phase in germination and the profound inhibition of GTF after pre-treatment with 0.25% tea tree oil suggests that there are restorative or repair mechanisms taking place that must occur before GTF can take place. Ellepola and Samaranayake (1998a) found a similar effect for some antifungal agents when they treated cells for 1 h, removed the antifungal agent and then induced germ tubes for 1 h in serum. They theorised that treatment with antifungal agents induced a post-antifungal effect which has been described as the suppression of fungal growth that persists after limited exposure to antifungal agents, and this effect has been shown nystatin, amphotericin B, ketoconazole and 5-fluorocytosine against C. albicans and C. tropicalis (Anil et al., 2001).

The MIC/MFC results, and the results of GTF for 10 isolates in the presence of 0.031% tea tree oil, showed some variability between isolates, as has been seen by other authors in germ tube inhibition studies (Johnson et al., 1983; Ellepola & Samaranayake, 1998a).
The correlation seen between MFC and degree of GTF suggests that the results obtained by these two quite different assays are related, with the result of one assay giving an indication of the likely result in the other assay, as seen previously (Ha & White, 1999). However, another study showed no relationship (Ellepola & Samaranayake, 1998a).

The terpenes eugenol and vanillin also both inhibited GTF by *C. albicans* (Boonchird & Flegel, 1982). These authors speculated that since eugenol inhibits mitochondrial respiration and energy production (Cotmore *et al.*, 1979), and GTF requires energy, the lack of energy production prevented the morphological transition from blastospore to hyphae (Boonchird & Flegel, 1982). However, inhibition of respiration is more likely to cause a non-specific inhibition of growth than the specific inhibition of GTF (Odds, 1988). The possibility remains that tea tree oil or terpenes affect the functioning of membrane-associated enzymes specifically required for GTF such as the chitin, mannan and 1,3-β-D-glucan synthases, especially since tea tree oil has been shown in this study to have several effects against the PM of *C. albicans*.

In conclusion, tea tree oil reversibly inhibits GTF in *C. albicans*. Inhibition may be due to a generalised inhibition of growth at the higher concentrations of oil, however, at lower concentrations, GTF was specifically inhibited while growth continued by budding. These findings may be due to effects on cell membranes and associated functions, including the inhibition of respiration.

**6.3.9 General discussion**

From this and other studies it is 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.

Studies showed that tea tree oil, at concentrations of 0.25% and above, caused membrane permeability and fluidity changes, and cell death. At concentrations of 0.125% and below, tea tree oil did not affect permeability or viability, but cells grown with concentrations between approximately 0.03 - 0.12% had significantly reduced growth rates, increased membrane fluidity and at these concentrations the formation of germ tubes was inhibited.
Studies with individual components of tea tree oil showed that for some, the concentrations producing detrimental effects were similar for different assays. For terpinen-4-ol, no viability or permeability changes were apparent at 0.12% but both viability and permeability were affected at 0.25%, and membrane fluidity changes were apparent. With 1,8-cineole, no changes in viability or membrane permeability were seen at 0.25%, but membrane fluidity was significantly increased. Viability and permeability were affected at 0.5% 1,8-cineole. For α-terpineol, treatment with 0.25% caused viability and permeability changes as determined by methylene blue uptake, but did not cause the leakage of 260 nm-absorbing material and at this concentration membrane fluidity was decreased.

The components that did not conform to this pattern were terpinolene, α-terpinene and γ-terpinene where some conflicting results were obtained. Each component at 1% caused moderate decreases in viability over a 6 h time scale, but no changes in permeability were detected by methylene blue staining. All three components caused increases in membrane fluidity at 0.25% although for γ-terpinene this increase was not statistically significant. The leakage of significant amounts of 260 nm-absorbing materials was also seen after cells were treated with 0.25, 0.5 and 1.0% terpinolene. These inconsistencies may be due to the extreme water-insolubility of these components which may have confounded results, however more studies need to be performed to investigate these phenomena fully.

From studies performed in this Chapter, and results seen previously by others, the following hypothetical picture may be constructed of the sequence of events occurring in the short term when cells 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.

6.4 Conclusions

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.
CHAPTER 7 – GENERAL DISCUSSION

The superficial infections caused by yeasts and dermatophytes are some of the most common diseases in humans. Infections caused by dermatophyte are usually limited to keratinised tissues such as the outer skin layers, hair and nails, whereas yeasts are more likely to infect the mucous membranes. Some of these fungal infections can be difficult to treat and may require systemic treatment. However, for many of these infections, their superficial nature means they are amenable to topical treatment.

Tea tree oil has been known for its antimicrobial and anti-inflammatory activity for many years. Until recently, these views have largely been supported by anecdotal evidence only, however, research is now showing that they do have a scientific basis (Cox et al., 1998; Hart et al., 2000; Brand et al., 2002; Carson et al., 2002). In particular, the antibacterial properties of tea tree oil and components have been the subject of several comprehensive studies which describe in detail aspects of the mechanisms of antibacterial action of tea tree oil and components. However, little similar work has been conducted for fungi. Given the importance of fungi as human pathogens, and the potential usefulness of tea tree oil as a topical antifungal agent, data on the antifungal activity of tea tree oil are urgently required. Therefore, the aim of this study was to investigate the activity of tea tree oil and components against fungi relevant to human health, using several different approaches. A primary objective was to determine the in vitro susceptibility of a wide range of yeasts, dermatophytes and other filamentous fungi to tea tree oil. A secondary objective was to determine the antifungal activity of the components of tea tree oil against a smaller collection of representative fungi. The final objective was to investigate the mechanisms of action of both whole tea tree oil and components against clinically important yeasts, in particular C. albicans.

Yeasts belonging to the genera Candida, Saccharomyces, Trichosporon, Rhodotorula and Malassezia were susceptible to tea tree oil and activity was largely fungicidal. Little variation in susceptibility was evident between species of a particular genus or between different genera. Although not all organisms belonging to these genera are considered important human pathogens, the testing of this variety of organisms established that tea
tree oil has activity against a wide range of yeasts. Investigations of potential synergy between tea tree oil and several other topically applied antifungal agents showed that tea tree oil had little synergy with nystatin or boric acid but showed significant synergy with miconazole. This result suggests that these two agents may have complementary modes of action, which may be useful clinically.

Of the yeast genera mentioned above, the collection of Malassezia species was obtained from both clinically normal skin and clinical specimens. Isolates were then identified to the species level using biochemical tests and growth characteristics. For most isolates, this was achieved relatively easily although a small number did not show characteristics that were entirely consistent with any individual species. These isolates were identified as *M. sympodialis*, which was the species they most closely resembled. However, the possibility remains that these isolates may represent new sub-species or species and this notion is supported by the recent publication describing the new species *M. dermatis* (Sugita *et al.* 2002). In vitro susceptibility testing showed little differences in tea tree oil MICs between *Malassezia* species, although low numbers of isolates for some species limited comparisons. In contrast, tea tree oil data for *M. furfur* and *M. sympodialis* obtained by the broth dilution method showed that although MICs were approximately equivalent, MFCs were higher for *M. furfur* than for *M. sympodialis*. *Malassezia* spp. varied in their susceptibility to the ketoconazole, econazole and miconazole (determined by agar dilution) with *M. furfur* being the least susceptible species but with the remaining species being relatively similar in susceptibility. The clinical relevance of this is as yet unknown as information on the specific disease involvement of the different *Malassezia* species is still being determined.

When the components of tea tree oil were tested against representative isolates from several yeast genera by the broth microdilution method, lowest MICs and MFCs were seen for terpinen-4-ol and α-terpineol, followed by 1,8 cineole. No appreciable activity was seen for γ-terpinene, α-terpinene and p-cymene by the microdilution method, however, MICs and MFCs were considerably lower when the broth macrodilution method was used. Differences in assay volume did not account for this difference, since MICs and MFCs for other compounds such as tea tree oil or terpinen-4-ol were either equivalent or only one dilution lower by macrodilution. Rather, these differing results may be related to the unique chemical attributes of the polystyrene microtitre trays or
glass McCartney bottles that the broth micro- and macro-dilution assays were performed in, respectively, and the interaction of the hydrophobic components of tea tree oil with each of these.

The activity of tea tree oil against the dermatophytes varied little between genera, with both MICs and MFCs mostly falling within ranges of 3 – 4 dilutions. A similar trend was seen for fungi of the genera *Alternaria, Aspergillus, Cladosporium, Fusarium* and *Penicillium* although these fungi were generally less susceptible than the dermatophytes with higher MIC and MFC ranges. In particular, isolates of *A. niger* were unique in that they were the least susceptible to tea tree oil as evidenced by MFCs as high as 8%. These high MFCs were attributed to the characteristics of the conidial wall that render it relatively resistant to challenges. For most fungi, there was a difference of several dilutions between inhibitory and fungicidal concentrations indicating that at particular concentrations tea tree oil is fungistatic only. When the components of tea tree oil were tested against these fungi using the broth microdilution method, the dermatophytes were generally more susceptible than the other filamentous fungi, similar to the trend seen with whole oil. Terpinen-4-ol and α-terpineol showed MICs and MFCs similar to, or less than, those of whole oil, and 1,8-cineole and terpinolene showed the next lowest MICs and MFCs. The components γ-terpinene, α-terpinene and ρ-cymene had very little in vitro activity, although different results may be evident by the broth macrodilution method.

When the susceptibility of these fungi to tea tree oil was further investigated by time kill studies, a relatively slow rate of kill was seen which may be attributed to the time required for the oil components to penetrate the dense conidial wall and gain entry to the cell interior. Studies with *A. niger* conidia showed that germinated conidia were significantly more susceptible to tea tree oil than non-germinated conidia, emphasising the importance of the intact conidial wall for protection against tea tree oil, and possibly other antifungal agents.

The studies discussed above addressed two of the fundamental aims of this thesis, which were to determine the susceptibility of a wide range of fungi to tea tree oil and components. These studies laid the groundwork for the remaining aim of this project,
which was to investigate the mechanism of action of tea tree oil and components. Experiments concentrated on analysing membrane properties and functions since the membrane has been suggested as a primary site of action of essential oils and terpenes. Studies were conducted with the test organism *C. albicans* although some studies were conducted with *C. glabrata* and *S. cerevisiae* also. Usually more than one concentration of oil or components was used in each of the experiments and as a generalisation, effects occurred in a dose-dependent manner, with greater effects occurring with increasing quantities of oil or component.

When the effects of tea tree oil on the viability and permeability of *C. albicans* was investigated over time, negligible changes occurred at 0.12%, moderate changes were seen at 0.25% and effects were relatively rapid at 0.5 and 1.0%. When these studies were conducted with terpinen-4-ol and α-terpineol, it was evident that these two components cause very rapid killing and loss of selective permeability to methylene blue, suggesting that these components contribute significantly to the total activity of tea tree oil. The components 1,8 cineole, terpinolene, γ-terpinene, α-terpinene and ρ-cymene also caused some loss of viability or changes in permeability, although these effects were generally not as rapid or to as great an extent as those seen for terpinen-4-ol and α-terpineol. These studies clearly indicated that tea tree oil and components affect both viability and permeability. Permeability changes may be caused by the direct interaction of tea tree oil components and the plasma membrane, which alters both its physical properties and functioning. In particular, it is proposed that these effects are seen because one or more of the components of tea tree oil act as ionophores and cause the non-specific movement of ions across the plasma membrane resulting in depolarisation.

Evidence that tea tree oil and components cause such alterations came from studies showing that the membrane fluidity of *C. albicans* was significantly increased after treatment for 30 min with 0.25% tea tree oil, terpinen-4-ol, 1,8 cineole, terpinolene and α-terpinene. Of these results, it was interesting to note that the greatest and most rapid change in fluidity was caused by 1,8-cineole, a component not necessarily thought of as contributing significantly to the antimicrobial activity of tea tree oil. It is thought that these increases in membrane fluidity are caused by the insertion of terpene molecules between the fatty acyl chains of the phospholipid molecules in the lipid bilayer. This
physical disruption decreases the interactions between the fatty acyl chains, which results in increased fluidity. The effects of increasing membrane fluidity on other membrane-associated functions such as enzymes or transport systems was of great interest and led to studies of the plasma membrane ATPase.

Tea tree oil was shown to affect the functioning of the plasma membrane ATPase, as estimated by medium acidification in *C. albicans*, *S. cerevisiae* and *C. glabrata*. This enzyme is critical for maintaining osmotic balance and the impairment of this enzyme may be one of the factors that contribute to either the loss of viability or selective permeability. The mechanisms by which tea tree oil inhibits medium acidification are not yet known, but it is suggested that the components of tea tree oil may be having direct inhibiting effects on the PM ATPase or they may be inhibiting respiration, which provides the energy required to fuel the PM ATPase. The importance of the PM ATPase was further emphasised in studies showing that in cells where the PM ATPase was selectively inhibited by pre-incubation with DES, susceptibility to tea tree oil was greatly increased compared to control cells. From these studies it was concluded that the PM ATPase plays a significant part in countering the ionophoric properties of tea tree oil and components and attempting to regain osmotic stability.

Using another approach to investigate the effects of tea tree oil on yeast cells, it was found that several alterations were seen when *C. albicans* was grown with sub-inhibitory quantities of tea tree oil. Firstly, the growth rate and cell biomass at 24 h had decreased incrementally with increasing quantities of oil, and this was postulated to be caused by a generalised inhibition of respiration or excessive consumption of cellular ATP by activation of the plasma membrane ATPase, which may then leave little ATP available for normal cell growth and division. In addition, the membrane fluidity of *C. albicans* cells grown with low quantities of tea tree oil for 24 h was increased compared to control cells. This increase in fluidity is suggestive of an adaptive change in the composition of the PM lipids to compensate for the effects of tea tree oil on the cells. Since *C. albicans* was shown to produce adaptive changes in response to the presence of tea tree oil, the possibility that *C. albicans* or *S. cerevisiae* also showed stress responses, in particular the accumulation of trehalose, was considered. However, it was shown that neither yeast species accumulated large amounts of trehalose after 3 h incubation with low levels of tea tree oil, as compared to the levels of trehalose accumulated by each
organism after heat shock. Although the lack of trehalose accumulation in response to tea tree oil may be because these circumstances do not trigger the accumulation of trehalose in these yeasts, it is also plausible that the presence of tea tree oil is preventing normal yeast growth and metabolism, which includes the production of trehalose.

A similar mechanism of generalised growth inhibition was postulated as one of the mechanisms preventing the formation of germ tubes in the presence of both inhibitory and sub-inhibitory quantities of tea tree oil. However, at some of the concentrations inhibiting the formation of germ tubes, growth was not totally inhibited, as shown by the observation that yeast cells continued to divide, albeit by budding only. In this instance, the possibility remains that germ tube formation was inhibited because the components of tea tree oil were inhibiting enzymes specifically required in the formation of germ tubes, such as those involved in the manufacture of cell walls. The specific inhibition of germ tubes is of particular relevance for the treatment of vaginal candidiasis, since the formation of germ tubes appears to be an important part of the infection and disease processes. In addition, the penetration of germ tubes into the vaginal epithelia initiates an inflammatory process, which may be partly responsible for the itch and discomfort associated with vaginal candidiasis (Odds, 1994). Since tea tree oil inhibits and prevents germ tube formation, and has both antimicrobial and anti-inflammatory activity, it has obvious benefits for the treatment of vaginal candidiasis.

Many of mechanisms of action described above are consistent with results seen for other lipophilic compounds such as cyclic and non-cyclic terpenes (Sikkema et al., 1994; Engelke et al., 1996), and ethanol (Alexandre et al., 1994), and suggest that tea tree oil and components act by increasing membrane fluidity which results in the depolarisation of membranes and the non-specific passive movement of ions across the plasma membrane. The exact sequence of these and other effects remains unknown, as does the contribution that each of the components makes to these deleterious effects. In particular, the contribution of each component to the effects of whole oil may be related to the percentage composition of each component. Furthermore, the possibility that two or more components act synergistically is also an area that requires investigation.

While investigations of the range and mechanisms of activity of tea tree oil are useful and interesting on their own, the greatest utilisation of these studies is in their
application to understanding in vivo efficacy. The activity of tea tree oil against both
\textit{Candida} yeasts and dermatophytes suggests that the oil may be a useful treatment
option for infections caused by these organisms. For vaginal yeast infections, products
such as tea tree oil gels, creams or pessaries may be suitable and similar products are
currently available both in Australia and overseas. For dermatophyte infections, topical
preparations such as gels, creams or lotions may be appropriate. Any such products
would require rigorous clinical investigation to establish their in vivo efficacy. Of the
clinical antifungal data published to date, one study investigated tinea pedis (Tong \textit{et al.}, 1992),
two investigated onychomycosis (Buck \textit{et al.}, 1994; Syed \textit{et al.}, 1999) and
one presented a case series of patients with oral candidiasis (Jandourek \textit{et al.}, 1998). It
was evident from the results of these clinical trials that there has been only moderate
success in treating fungal infections with tea tree oil, as has been noted in a review of
published tea tree oil clinical trials (Ernst \& Huntley, 2000). Many variables may
account for the lack of in vivo efficacy, such as insufficient duration of therapy or
concentration of agent, the inability of the agent to penetrate to the site of active
infection or insufficient activity of the antifungal agent. The vehicle in which tea tree oil
is formulated is also known to affect the availability of the oil and the use of
inappropriate vehicles may reduce the overall efficacy. The shift from thorough in vitro
investigations to in vivo studies is well overdue and is now further supported by in vitro
data from this study. The possibility remains that if clinical success cannot be achieved
with tea tree oil alone, it may prove useful in combination with other agents. Also, non-
medical uses for tea tree oil that capitalise on the antifungal properties of the oil remain.
Anecdotal evidence suggest that the dispersion of tea tree oil in air, via air conditioning
systems, results in the reduction of both fungal loads in indoor air and fungal growth on
indoor walls. Antifungal data from the present Thesis support these applications.

The key achievements of this thesis can be summarised as follows;

i) in vitro susceptibility testing showed that tea tree oil has fungicidal activity
against wide range of medically important fungi

ii) the components of tea tree oil were shown to have differing antifungal activity,
however it was also shown that the conditions of the test method greatly
influenced results
iii) when tea tree oil was used at concentrations greater than or equal to fungicidal amounts in time kill studies, *C. albicans* was killed rapidly whereas kill rates were considerably slower for *Trichophyton* and *Aspergillus* spp.

iv) germinated *A. niger* conidia were significantly more susceptible to tea tree oil than non-germinated conidia

v) treatment of *C. albicans* with tea tree oil or components caused alterations in permeability in a dose-dependent manner

vi) the actions of the plasma membrane ATPase was shown to be one of the mechanisms by which yeast cells defend themselves against tea tree oil but in separate studies it was shown that this enzymes functioning was adversely affected in the presence of tea tree oil

vii) tea tree oil inhibited the formation of germ tubes by *C. albicans* at and below inhibitory concentrations, but germ tube formation was restored when tea tree oil was removed, after an initial recovery period

viii) when *C. albicans* and *S. cerevisiae* cells were grown with sub-inhibitory tea tree oil for 3 h they did not accumulate trehalose

ix) when *C. albicans* was grown for 24 h with sub-inhibitory tea tree oil, cells showed increased membrane fluidity, representing an adaptive response

x) short-term treatment of *C. albicans* with tea tree oil and components caused immediate increases in membrane fluidity, with 1,8-cineole producing the greatest changes after both 10 and 30 min treatment

To date, the antifungal properties of tea tree oil and its components have been only poorly characterised. Work presented in this thesis shows that tea tree oil and its components have a range of antifungal effects against both medically important and environmental fungi. In addition, many of the results obtained from studies investigating the mechanism of action of tea tree oil are the first description of such effects against yeasts caused specifically by tea tree oil and components. This thesis represents a substantial contribution to the body of knowledge pertaining to the antifungal activity of tea tree oil. Furthermore, the in vitro data support a move towards clinical applications of tea tree oil. Investigations of the in vivo efficacy of tea tree oil for the treatment of oral and vaginal candidiasis, dandruff, seborrhoeic dermatitis, pityriasis versicolor, onychomycosis and tinea are now warranted. The availability of
effective and safe alternative treatments for these common conditions could have a significant impact on human health.
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