



RURAL INDUSTRIES RESEARCH  
& DEVELOPMENT CORPORATION

# **Antifungal Activity of Tea Tree Oil In Vitro**

**A report for the Rural Industries Research  
and Development Corporation**

by KA Hammer, CF Carson & TV Riley

February 2001

RIRDC Publication No 01/11  
RIRDC Project No UWA-50A

© 2001 Rural Industries Research and Development Corporation.  
All rights reserved.

ISBN 0 642 58234 3  
ISSN 1440-6845

*Antifungal Activity of Tea Tree Oil In Vitro*  
Publication No. 01/11  
Project No. UWA-50A

The views expressed and the conclusions reached in this publication are those of the author and not necessarily those of persons consulted. RIRDC shall not be responsible in any way whatsoever to any person who relies in whole or in part on the contents of this report.

This publication is copyright. However, RIRDC encourages wide dissemination of its research, providing the Corporation is clearly acknowledged. For any other enquiries concerning reproduction, contact the Publications Manager on phone 02 6272 3186.

#### **Researcher Contact Details**

Associate Professor Thomas V. Riley  
Department of Microbiology  
The University of Western Australia  
Queen Elizabeth II Medical Centre  
Nedlands, Western Australia, 6009

Phone: 08 9346 3690  
Fax: 08 9346 2912  
Email: [triley@cyllene.uwa.edu.au](mailto:triley@cyllene.uwa.edu.au)  
Website: <http://www.meddent.uwa.edu.au/TeaTree/>

#### **RIRDC Contact Details**

Rural Industries Research and Development Corporation  
Level 1, AMA House  
42 Macquarie Street  
BARTON ACT 2600  
PO Box 4776  
KINGSTON ACT 2604

Phone: 02 6272 4539  
Fax: 02 6272 5877  
Email: [rirdc@rirdc.gov.au](mailto:rirdc@rirdc.gov.au)  
Website: <http://www.rirdc.gov.au>

Published in February 2001  
Printed on environmentally friendly paper by Canprint

# Foreword

Several comprehensive studies have already been conducted investigating the antibacterial activity of *Melaleuca alternifolia* (tea tree) oil. Since fungi have emerged only relatively recently as important pathogens, they have been neglected in terms of the development of antifungal agents and the more basic science of how antifungal agents work. Therefore the aim of this project is to determine the in vitro susceptibility of a range of medically important yeasts and filamentous fungi to tea tree oil.

While the market for tea tree oil has expanded significantly over the last decade, continued expansion and extension of the market will only occur if new supporting data are available. The purpose of this report is to describe research that has been conducted into the activity of tea tree oil against a range of medically relevant fungi and to discuss potential areas of further research in this area.

This report, a new addition to RIRDC's diverse range of over 600 research publications, forms part of our Tea Tree Oil R&D program, which aims to support the continued development of a profitable tea tree oil industry. This project was funded from industry revenue which is matched by funds provided by the Federal Government.

Most of our publications are available for viewing, downloading or purchasing online through our website:

- downloads at [www.rirdc.gov.au/reports/Index.htm](http://www.rirdc.gov.au/reports/Index.htm)
- purchases at [www.rirdc.gov.au/eshop](http://www.rirdc.gov.au/eshop)

**Peter Core**  
Managing Director  
Rural Industries Research and Development Corporation

# Acknowledgments

The authors thank Australian Plantations Pty. Ltd., Wyrallah, NSW for the provision of tea tree oil samples throughout the course of this study.

We are grateful for the technical, financial and institutional support of the Department of Microbiology, The University of Western Australia, and the Division of Microbiology and Infectious Diseases, The Western Australian Centre for Pathology and Medical Research (PathCentre).

Particular thanks go to the laboratory staff of the Mycology Section (PathCentre) for providing advice, expertise and fungal strains.

# Abbreviations

cfu/ml	colony forming units per millilitre
MIC	minimum inhibitory concentration
MIC <sub>90</sub>	minimum concentration at which 90% of isolates are inhibited
MFC	minimum fungicidal concentration
MFC <sub>90</sub>	minimum concentration at which 90% of isolates are killed
spp.	species (plural)

# Contents

<b>Foreword</b> .....	<b>iii</b>
<b>Acknowledgments</b> .....	<b>iv</b>
<b>Abbreviations</b> .....	<b>v</b>
<b>Executive Summary</b> .....	<b>vii</b>
<b>1. Introduction</b> .....	<b>1</b>
<b>2. Objectives</b> .....	<b>3</b>
<b>3. Methodology</b> .....	<b>4</b>
3.1 Tea tree oil .....	4
3.2 Organisms .....	4
3.2.1 Yeast isolates .....	4
3.2.2 Dermatophytes .....	5
3.2.3 Other fungal isolates .....	5
3.3 In vitro susceptibility assays .....	5
3.3.1 Inoculum preparation .....	5
3.3.2 Broth microdilution assay .....	6
3.3.3 Agar dilution assay .....	7
3.4 Time-kill assays .....	8
<b>4. Results</b> .....	<b>9</b>
4.1 Activity of tea tree oil against yeasts .....	9
4.2 Activity of tea tree oil against dermatophytes .....	9
4.2.1 Broth microdilution method .....	9
4.2.2 Agar dilution method .....	11
4.3 Activity against <i>Aspergillus niger</i> .....	15
4.4 Time-kill assays .....	15
<b>5. Discussion</b> .....	<b>18</b>
5.1 Activity against yeasts, dermatophytes and other fungi in vitro .....	18
5.2 Previous clinical trials .....	20
5.3 Further work .....	21
<b>6. Implications and Recommendations</b> .....	<b>22</b>
6.1 Implications .....	22
6.2 Recommendations .....	22
<b>7. References</b> .....	<b>23</b>

# Executive Summary

Tea tree oil has emerged as a popular and relatively well known topical treatment. Its applications range from purely cosmetic through to pharmaceutical and medical. The acceptance of tea tree oil by consumers as 'natural' and 'safe' has contributed immensely to its popularity and success. However, medical professionals are less likely to adopt tea tree oil for treatment because of the apparent lack of scientific data supporting many of the claims that have been made about the oil.

This investigation is the first step in a series of studies necessary to determine the suitability of tea tree oil for the treatment of fungal infections in humans. In this study, basic information about the concentrations of tea tree oil required to inhibit the growth of, and kill, fungi was determined. The use of methods based on internationally accepted and standardised protocols means the results are scientifically credible and are more likely to be accepted by health and medical professionals.

This study shows that a wide range of fungi are susceptible to tea tree oil. For many isolates, the amount required to inhibit the organisms was several fold less than the amount required to kill. This indicates that tea tree oil has only inhibitory effects over a range of concentrations. In terms of clinical use of the oil, this suggests that the use of too low a concentration may result in inhibition, but not killing of the infecting organism and after ceasing treatment the infection may recur. However, this is only speculation and the exact relationship of this laboratory data to effectiveness in human infections or clinical outcome remains unclear. Clinical trials evaluating the effectiveness of tea tree oil or tea tree oil products are necessary to determine this.

The susceptibility of three groups of fungi was investigated by two methods. The first group of organisms was the yeasts: members of the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Saccharomyces* and *Trichosporon* were tested. Various *Candida* species - primarily *C. albicans* - cause mainly superficial infections including oral thrush, vaginal thrush and nappy rash. Of the other yeast genera, the non-*Candida* genera rarely cause disease in humans. These relatively 'non-pathogenic' organisms were included in the study to establish the range of activity of tea tree oil against yeasts. A total of 115 yeast isolates was tested against tea tree oil by the broth microdilution method. The minimum inhibitory concentrations (MICs) obtained by this method ranged from 0.016% for *Rhodotorula* spp. to 0.5% for several *Candida* species. Minimum fungicidal concentrations (MFCs) ranged from 0.06% for isolates of *C. guilliermondii*, *Cryptococcus neoformans* and *Trichosporon* spp. to 1.0% for isolates of *C. albicans*, *C. glabrata* and *C. parapsilosis*.

The second group of organisms tested against tea tree oil was the dermatophytes. This group of filamentous fungi contains the genera *Epidermophyton*, *Microsporum* and *Trichophyton*. These organisms cause fungal infections of the skin commonly known as ringworm, tinea or athlete's foot. These infections are treated either topically or systemically with oral medication, depending on the type and severity of the infection. A total of 106 clinical dermatophyte isolates was tested against both tea tree oil and griseofulvin, a standard agent used for treating fungal infections. Isolates were tested by the broth microdilution and agar dilution methods. The MICs obtained by the broth microdilution method ranged from 0.004% to 0.016%. The MFCs obtained ranged from <0.03% to 1.0%. All organisms had MICs of griseofulvin in the range of 0.25 µg/ml to 2.0 µg/ml.

Five dermatophyte isolates were tested against tea tree oil in the range of 0.25% to 0.016% by the agar dilution method. By this method, the MIC of oil against each isolate of *M. canis*, *M. gypseum*, *T. mentagrophytes* var *interdigitale* and *T. rubrum* was 0.25% and, for *E. floccosum*, was 0.12%. The growth of isolates was reduced compared to growth on the control plate at tea tree oil concentrations between 0.03 and 0.25%. No isolate showed growth at any time on plates containing 0.25% tea tree oil. The MICs obtained by the agar dilution method were several concentrations higher than those obtained by the broth microdilution method and resembled more closely the MFC results obtained by the broth microdilution method.

Time-kill studies were performed with an isolate each of *T. mentagrophytes* var *interdigitale* and *T. rubrum*. There was a reduction in the numbers of viable organisms recovered over the course of the experiment. However, even at concentrations four to eight-fold higher than the MFC for each organism, complete killing of either isolate did not occur over the 6 hours of the experiment.

The third group of organisms was the filamentous fungi, comprising nine isolates of *Aspergillus niger*. These organisms can cause severe diseases in humans that are not amenable to topical treatment and usually require systemic antifungal therapy. Isolates were tested against tea tree oil by the broth microdilution method. The MICs ranged from 0.06 to 0.12% and the MFCs ranged from 2.0 to 8.0%.

# 1. Introduction

Currently, there is still a paucity of information published in appropriate peer-reviewed journals about the antimicrobial activity of tea tree oil in general and, in particular, its antifungal activity. Although a few reports have been published describing the activity of tea tree oil against yeasts [1-3], there is still a lack of data describing the activity of tea tree oil against filamentous fungi.

Studies investigating the antimicrobial properties of tea tree oil frequently use a range of bacteria as test organisms and do not often include any fungal isolates [4-7]. Only a few studies have been published that focus exclusively on the antifungal properties of the oil. In vitro susceptibility data for yeasts have been published by several different research groups [1-3, 8]. The concentrations of tea tree oil that have been reported to inhibit yeasts of the genus *Candida* range from 0.04% [4] to approximately 1.0% [9]. Concentrations that have been reported to inhibit *Malassezia* species range from 0.12% [10] to 0.25% [1, 8, 10]. Several authors have also published susceptibility data for medically relevant filamentous fungi [1, 11-13]. The concentrations of tea tree oil that have been reported to inhibit filamentous fungi range from 0.016% for *A. niger* [4] to 1.0% for *A. niger*, *T. mentagrophytes* and *T. rubrum* [8, 9]. Furthermore, three studies have investigated tea tree oil as a potential post-harvest mould preventative [14-16].

The inhibitory values cited above have been obtained from several different studies and represent the range of published values. Each study has used a different method for obtaining values and has generally only used one yeast or fungal isolate of each species. A major restriction on the usefulness and comparability of the available antifungal data created to date is the methods that have been used to generate the results. Studies published to date have used the broth microdilution method [2, 3] the agar dilution method [1, 2, 4, 8, 13] agar diffusion assays [11, 12] and growth inhibition assays [14]. Frequently the data have been generated by methods that cannot readily be compared. Some of these methods have been derived from methods originally intended for investigating the potential for essential oils (or their components) to be used as crop or food preservatives and thus are different from the methods that have now been developed for the in vitro susceptibility testing of medically important filamentous fungi [17, 18]. The use of the protocols and methods that have been developed for the in vitro susceptibility testing of medically important yeasts and fungi in vitro, means that the data produced are seen as credible and more palatable to the broader medical and scientific community. Despite the limitations of the data, it is apparent that tea tree oil inhibits the growth of fungi.

On this basis, several clinical trials have been conducted that evaluate the usefulness of tea tree oil as an antifungal agent. Conditions that have been investigated include the treatment of oral candidiasis [19], tinea pedis [20, 21] and onychomycosis [22, 23]. The outcomes of these trials cover a range of clinical efficacies and clearly indicate that while tea tree oil has the potential to be effective against fungal disease in vivo, there is still much that is not understood. The generation of new data relating to the in vitro susceptibility of fungi to tea tree oil should therefore be a priority. The most important organisms to test against tea tree oil are those that cause superficial infections. This includes the yeasts, in particular members of the genus *Candida*, and the dermatophytes. These data will contribute to the development and evaluation of tea tree oil products for the treatment of fungal infections and will add greatly to our understanding of the clinical efficacy of tea tree oil.

## 2. Objectives

The aim of this project, carried out as part of RIRDCs Tea Tree Oil Research and Development Program, is to increase the acceptability of tea tree oil as a naturally occurring antifungal agent, both nationally and internationally. This will be achieved in three stages:

- The development and assessment of methods for testing the susceptibility of yeasts and filamentous fungi to tea tree oil.
- The use of these methods to determine the susceptibility of recent clinical isolates of yeasts and other fungi to tea tree oil.
- Making the data available via publications in international medical/scientific refereed journals.

## 3. Methodology

### 3.1 Tea tree oil

*Melaleuca alternifolia* (tea tree) oil was kindly donated by Australian Plantations Pty Ltd., Wyrallah, NSW. Batch 97/1 was used for all studies and had the composition shown in Table 3.1, as determined by gas-chromatography mass spectrometry performed by the Wollongbar Agricultural Institute, Wollongbar, NSW.

**Table 3.1:** Composition of *M. alternifolia* oil batch 97/1

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

### 3.2 Organisms

#### 3.2.1 Yeast isolates

A collection of 115 yeast isolates was obtained. Isolates were obtained from the Department of Microbiology at The University of Western Australia, Royal Perth Hospital, The Western Australian Centre for Pathology and Medical Research and some were isolated and identified in our laboratory. Isolates were identified based on colony and microscopic morphology, in addition to using the ID 32C yeast identification kit (bioMerieux sa, 69280 Marcy-l'Etoile, France). Reference isolates were *Candida albicans* ATCC 10231, ATCC 90028, ATCC 90029, *Candida glabrata* ATCC 90030, *Candida parapsilosis* 90018 and *Saccharomyces cerevisiae* ATCC 10716. The remaining isolates were obtained from different kinds of specimens including clinical specimens and normal human skin. All isolates were maintained on Sabouraud's Dextrose Agar (SDA) stored at 4°C.

### 3.2.2 Dermatophytes

A total of 106 dermatophytes was obtained from the Mycology Section of the Western Australian Centre for Pathology and Medical Research. They were as follows: *Epidermophyton floccosum* (n=15), *Microsporum canis* (16), *Microsporum gypseum* (6), *Trichophyton mentagrophytes* var *mentagrophytes* (14), *Trichophyton mentagrophytes* var *interdigitale* (21), *Trichophyton rubrum* (19) and *Trichophyton tonsurans* (15). Cultures were maintained on Potato Dextrose Agar (PDA) slopes stored at room temperature.

### 3.2.3 Other fungal isolates

Nine isolates of *Aspergillus niger* were obtained from the Mycology Section of the Western Australian Centre for Pathology and Medical Research. Isolates were maintained and stored as for the dermatophytes.

## 3.3 In vitro susceptibility assays

### 3.3.1 Inoculum preparation

Yeast isolates were subcultured onto SDA plates and incubated at 35°C for 24h. Suspensions of each organism were made in 0.85% saline and suspensions were then adjusted to 1.0 McFarland (approx 70% transmission) using a Vitek colorimeter. This was found to correspond to approximately  $10^7$  cfu/ml, as confirmed by viable counts. After inoculation of tests, final inocula concentrations were  $1.0 \times 10^3$  -  $3.5 \times 10^3$ , confirmed by viable counts which were performed by serially diluting the sample 10-fold in sterile distilled water (SDW) and spot inoculating 10 $\mu$ l aliquots onto SDA. After incubation, colonies were counted and the inoculum concentration was determined.

Dermatophyte inocula were prepared based on methods described by the National Committee for Clinical Laboratory Standards [17] taking into account the optimal growth conditions for dermatophytes as described by Norris *et al.* [24]. Briefly, isolates were subcultured onto PDA slopes and incubated at 30°C for 7d [24]. Slopes were then flooded with 0.85% saline and the growth was gently probed. The resulting suspension was removed into a sterile glass Bijoux bottle. The suspension was mixed thoroughly with the use of a vortex mixer and the larger particles allowed to settle. Suspensions were adjusted (see Table 3.2) as described above for yeasts. Suspensions were diluted as necessary to correspond to final inocula concentrations of approximately  $2.5 \times 10^3$  -  $2.5 \times 10^4$  cfu/ml. Viable counts were performed initially to confirm inocula concentrations but were not performed every time.

Inocula for *A. niger* were prepared as described above, except that PDA slopes were incubated at 35°C and suspensions were adjusted to correspond to a final inocula concentration of  $0.4 \times 10^4$  -  $5.0 \times 10^4$  cfu/ml.

**Table 3.2:** Adjustment of inocula suspension for filamentous fungi

Organism	% transmission	cfu/ml
<i>A. niger</i>	70%	$8.5 \times 10^5$ - $1.6 \times 10^6$
<i>E. floccosum</i>	55-65%	$2.5 \times 10^4$ - $1.0 \times 10^5$
<i>M. canis</i>	60-70%	$2.5 \times 10^4$ - $2.75 \times 10^4$
<i>M. gypseum</i>	50-60%	$2.0 \times 10^5$ - $4.75 \times 10^5$
<i>T. mentagrophytes</i> var <i>interdigitale</i>	50-60%	$3.0 \times 10^6$ - $7.5 \times 10^6$
<i>T. mentagrophytes</i> var <i>mentagrophytes</i>	50%	$1.25 \times 10^6$ - $5.5 \times 10^6$
<i>T. rubrum</i>	60-70%	$1.7 \times 10^6$ - $2.5 \times 10^6$
<i>T. tonsurans</i>	50-60%	$3.5 \times 10^5$ - $2.5 \times 10^6$

### 3.3.2 Broth microdilution assay

The broth microdilution method was based on reference methods M27-P and M38-P recommended by the National Committee for Clinical Laboratory Standards [17, 25] for yeasts and conidium-forming filamentous fungi, respectively. Microdilution trays contained a series of doubling dilutions of the test agent in 100µl volumes of the growth medium RPMI 1640 (Gibco BRL) with L-glutamine, without sodium bicarbonate, buffered to pH 7.0 with morpholinopropane-sulfonic acid (Sigma Chemical Co.). Tea tree oil was tested in the range of 8% to 0.002% (v/v) and Tween 80 (Sigma) was included at a final concentration of 0.001% (v/v) to enhance oil solubility. An initial griseofulvin (Sigma) stock was prepared at 6.4mg/ml in dimethylsulfoxide (DMSO) and was diluted as required to result in final test concentrations of 4µg/ml to 0.06µg/ml. The highest concentration of DMSO was 3.125% (v/v). One column served as growth control, containing only 100µl media (with or without 0.001% Tween 80), and 100µl inocula.

After inoculation, tests with yeasts were incubated at 35°C for 48h. After incubation, subcultures of 10µl were taken from each well and spot inoculated onto SDA. Subcultures were incubated aerobically at 35°C and MICs and minimum fungicidal concentrations (MFCs) determined. For yeasts, the MIC was defined as the lowest concentration of oil resulting in the maintenance or reduction of the inoculum, and the MFC was determined as the lowest concentration of oil resulting in the death of 99.9% of the inoculum.

Tests with dermatophytes were as above, except they were incubated at 30°C for 96h [24]. Tests with *A. niger* were incubated at 35°C for 46-50h. After this time MICs were determined visually with the aid of a reading mirror. Growth in each well was compared to that of the control and was scored numerically as follows: 4, no reduction in growth; 3, approximately 75% of the growth control; 2, approximately 50% of the growth control; 1, approximately 25% of the growth control; 0, optically clear or no visible growth [17]. The MIC was determined as the lowest concentration of tea tree oil or griseofulvin corresponding to a 75% reduction in growth, compared to the control [18]. MFCs of tea tree oil were determined by subculturing 10µl from wells not visibly turbid and spot inoculating onto SDA plates. MFCs were not determined for griseofulvin as this agent is fungistatic only. Subcultures for dermatophytes were incubated at 30°C for at least 7d [26] and at 35°C for 48h for *A. niger*. MFCs were determined as the lowest concentration resulting in no growth in the subculture.

The MIC<sub>90</sub> was determined as the lowest concentration of oil inhibiting 90% of isolates, while the MFC<sub>90</sub> was defined as the concentration of tea tree oil fungicidal for at least 90% of the isolates tested. Isolates were tested on at least two separate occasions and were re-tested if resultant MIC or MFC values differed. Modal values were then selected.

### 3.3.3 Agar dilution assay

One isolate each of *E. floccosum*, *M. canis*, *M. gypseum*, *T. mentagrophytes* var *interdigitale* and *T. rubrum* was tested against tea tree oil by the agar dilution assay. This method was based on the 'poisonous-medium technique' of Grover and Moore [27]. A series of 20ml SDA plates was made containing the following doubling dilutions of tea tree oil: 0.25, 0.125, 0.06, 0.03, 0.016 and 0%. A final concentration of 0.5% Tween 20 (Sigma) was included in the agar to facilitate oil solubility. Agar plates were dried for 15 min prior to inoculation. Inocula were prepared as described for the broth microdilution assay. Standardised inocula were diluted as necessary to contain approximately  $5.0 \times 10^4$  -  $5.0 \times 10^5$  cfu/ml. A sterile 6mm blank paper disc was placed in the centre of each plate and inoculated with 20µl of the prepared inoculum suspension. Plates were incubated inverted in plastic bags at 30°C for 14d. The fungal colony diameter was determined at days 7, 11, and 14. The fungal colony diameter was measured by taking four individual measurements across the fungal colony and then determining the average. Percentage growth inhibition was determined using the formula of Pandey *et al.* cited by Zambonelli *et al.* [28]. The agar dilution assay was performed 2 to 3 times per isolate. MICs on each occasion were determined as the lowest concentration where there was no visible growth. The modal values of 2 to 3 replicates were selected as the final MICs.

### 3.4 Time-kill assays

An isolate each of *T. rubrum* (isolate 25) and *T. mentagrophytes* var *interdigitale* (isolate 8) were used in this assay. Inocula were prepared as above, except that both suspensions were adjusted to 60% transmittance, in phosphate buffered saline (PBS) to result in starting inocula concentrations of approximately  $3.0 \times 10^6$  cfu/ml. A series of tea tree oil treatments were prepared in 1ml volumes, at twice the desired final concentrations in PBS, with a final concentration of 0.002% Tween 80. The 0% control contained only 0.002% Tween 80 in PBS. Each treatment vial and control was inoculated with 1ml of inoculum and a sample was taken immediately from the 0% control for viable counts. Treatments and controls were incubated at 35°C with shaking. Further samples were taken from each vial at 1, 2, 3, 4, 5 and 6h for viable counting. Four 10µl drops were spot inoculated onto SAB plates for each viable count dilution. Viable count plates were incubated at 30°C and were read after 3d for *T. mentagrophytes* var *interdigitale* and 6d for *T. rubrum*. The lowest detectable count was determined as 250 cfu/ml, based on one colony in four 10µl spots.

## 4. Results

### 4.1 Activity of tea tree oil against yeasts

Tea tree oil showed activity against all yeast isolates, and these results (obtained by the broth microdilution method) are shown in Table 4.1. The MICs ranged from 0.016% for *Rhodotorula rubra* to 0.5% for several *Candida* species. The MFCs ranged from 0.06% for *C. guilliermondii*, *Cryptococcus neoformans* and *Trichosporon* spp. to 1.0% for *C. albicans*, *C. glabrata* and *C. parapsilosis*. MIC and MFC values for individual isolates were usually either equivalent or differed by only one concentration.

### 4.2 Activity of tea tree oil against dermatophytes

#### 4.2.1 Broth microdilution method

Data obtained by the broth microdilution method are shown in Table 4.2. The MICs obtained for tea tree oil ranged from 0.004% for *T. tonsurans* to 0.06% for *T. mentagrophytes* var *mentagrophytes*. The MFCs obtained ranged from <0.03% for *T. rubrum* to 1.0% for *T. mentagrophytes* var *interdigitale* and *T. mentagrophytes* var *mentagrophytes*. MIC and MFC values for individual isolates were at least one concentration different and, for some isolates were up to 6 concentrations apart. For griseofulvin, all organisms had MICs in the range of 0.25µg/ml to 2.0µg/ml. The organisms with MICs of 2µg/ml were one isolate each of *M. gypseum*, *T. mentagrophytes* var *mentagrophytes* and *T. rubrum*.

**Table 4.1:** In vitro susceptibility of yeasts to tea tree oil, as determined by the broth microdilution method

Organism	no.	Tea tree oil (% v/v)			
		MIC		MFC	
		Range	90%	Range	90%
<i>C. albicans</i>	14	0.25-0.5	0.5	0.25-1.0	1.0
<i>C. ciferrii</i>	2	0.06-0.12		0.25	
<i>C. colliculosa</i>	1	0.06		0.25	
<i>C. famata</i>	1	0.12		0.25	
<i>C. glabrata</i>	11	0.25-0.5	0.5	0.25-1.0	0.5
<i>C. guilliermondii</i>	5	0.06-0.25		0.06-0.5	
<i>C. humicola</i>	2	0.12		0.25	
<i>C. krusei</i>	4	0.25-0.5		0.5	
<i>C. lusitaniae</i>	1	0.5		0.5	
<i>C. parapsilosis</i>	11	0.25-0.5	0.5	0.5-1.0	1.0
<i>C. pelliculosa</i>	1	0.25		0.25	
<i>C. pseudotropicalis</i>	1	0.25		0.5	
<i>C. tropicalis</i>	11	0.12-0.25	0.25	0.25-0.5	0.5
<i>Cryptococcus laurentii</i>	2	0.03-0.06		0.12	
<i>Cryptococcus neoformans</i>	12	0.06-0.25	0.12	0.06-0.25	0.25
<i>R. rubra</i>	21	0.016-0.12	0.06	0.25-0.5	0.5
<i>S. cerevisiae</i>	5	0.12-0.25		0.25-0.5	
<i>Trichosporon</i> spp.	10	0.03-0.12	0.12	0.06-0.25	0.12

**Table 4.2:** In vitro susceptibility of dermatophytes to tea tree oil and griseofulvin as determined by the broth microdilution method

Organism	n	Tea tree oil (% v/v)				Griseofulvin ( $\mu\text{g/ml}$ )	
		MIC		MFC		MIC	
		Range	90%	Range	90%	Range	90%
<i>E. floccosum</i>	15	0.008-0.03	0.03	0.12-0.25	0.25	0.25-1.0	1.0
<i>M. canis</i>	16	0.004-0.03	0.03	0.06-0.25	0.25	0.25-1.0	1.0
<i>M. gypseum</i>	6	0.016-0.03	-	0.25-0.5	-	0.5-2.0	-
<i>T. mentagrophytes</i> var <i>interdigitale</i>	21	0.008-0.03	0.03	0.25-1.0	1.0	0.25-1.0	0.5
<i>T. mentagrophytes</i> var <i>mentagrophytes</i>	14	0.008-0.06	0.03	0.25-1.0	0.5	0.5-2.0	1.0
<i>T. rubrum</i>	19	0.008-0.03	0.03	<0.03-0.25	0.25	0.25-2.0	1.0
<i>T. tonsurans</i>	15	0.004-0.016	0.016	0.12-0.5	0.5	0.5-1.0	1.0

#### 4.2.2 Agar dilution method

The data obtained by the agar dilution method are shown in Table 4.3 and Figure 4.1. These data demonstrate the reduction of fungal growth on plates containing increasing amounts of tea tree oil. As compared to controls, the growth of isolates was reduced on plates containing tea tree oil, except for a few instances where growth was increased at concentrations of 0.016% and 0.03% (see Table 4.3). Growth was visible by day 5 on all control plates. No isolate showed growth on the 0.12% plate before day 7, and none of the isolates showed growth on plates containing 0.25% tea tree oil at any time. Representative pictures of fungal growth on plates containing tea tree oil are shown in Figure 4.2.

The MICs determined at day 14 were 0.12% for *E. floccosum* and 0.25% for the remaining 4 isolates. Comparison of data obtained by the agar and broth dilution methods (Table 4.4) shows that MICs obtained by the agar dilution method were several concentrations higher than those obtained by the broth microdilution method. The MIC values obtained by the agar dilution method were either the same as, or differed by one concentration, from the MFCs obtained by broth microdilution.

**Table 4.3:** Minimum inhibitory concentrations and reduction in growth (%) of dermatophytes, as determined by the agar dilution method.

Organism	Day	MIC <sup>a</sup>	Reduction in growth (%) at the following tea tree oil concentrations (% v/v)			
			0.016	0.03	0.06	0.12
<i>E. floccosum</i>	7	0.06	15.0 ± 10.0	18.0 ± 16.3	41.8 ± 9.3 <sup>b</sup>	ngv
	14	0.12	8.4 ± 9.6	10.2 ± 7.8	35.8 ± 10.2	ngv
<i>M. canis</i>	7	0.06	-4.2 ± 11.6	3.5 ± 9.4	32.2 ± 3.8 <sup>b</sup>	ngv
	14	0.25	3.6 ± 2.4	-1.5 ± 5.1	9.0 ± 7.6	79.2 ± 7.3 <sup>b</sup>
<i>M. gypseum</i>	7	0.12	10.7 ± 4.5	18.0 ± 6.8	47.3 ± 7.8	ngv
	14	0.25	-8.8 ± 3.3	5.7 ± 1.6	35.7 ± 4.2	69.4 ± 7.6
<i>T. mentagrophytes</i> <i>var interdigitale</i>	7	0.12	1.0 ± 11.0	10.5 ± 5.6	35.0 ± 10.0	ngv
	14	0.25	-0.3 ± 1.2	2.2 ± 2.1	14.8 ± 7.0	77.3 ± 2.9
<i>T. rubrum</i>	7	0.12	-3.0 ± 8.2	1.8 ± 3.4	31.9 ± 9.1 <sup>c</sup>	ngv
	14	0.25	-7.0 ± 5.8	-2.2 ± 3.2	26.9 ± 8.2	72.2 ± 2.9 <sup>1</sup>

ngv = no growth visible

<sup>a</sup> Modal MICs are shown

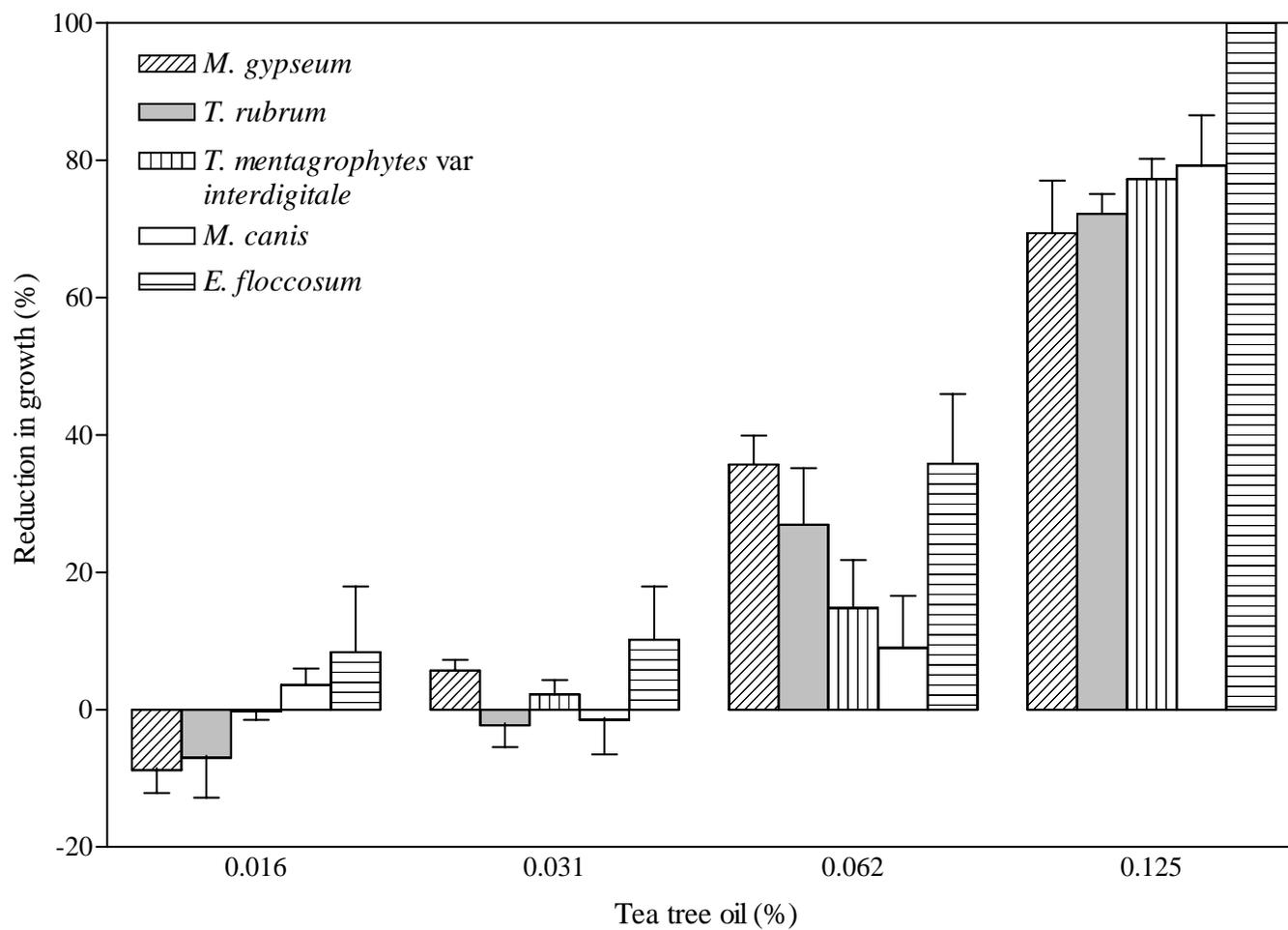
<sup>b</sup> 2 out of 3 repeats were ngv (converted to 6mm)

<sup>c</sup> 1 out of 3 repeats were ngv (converted to 6mm)

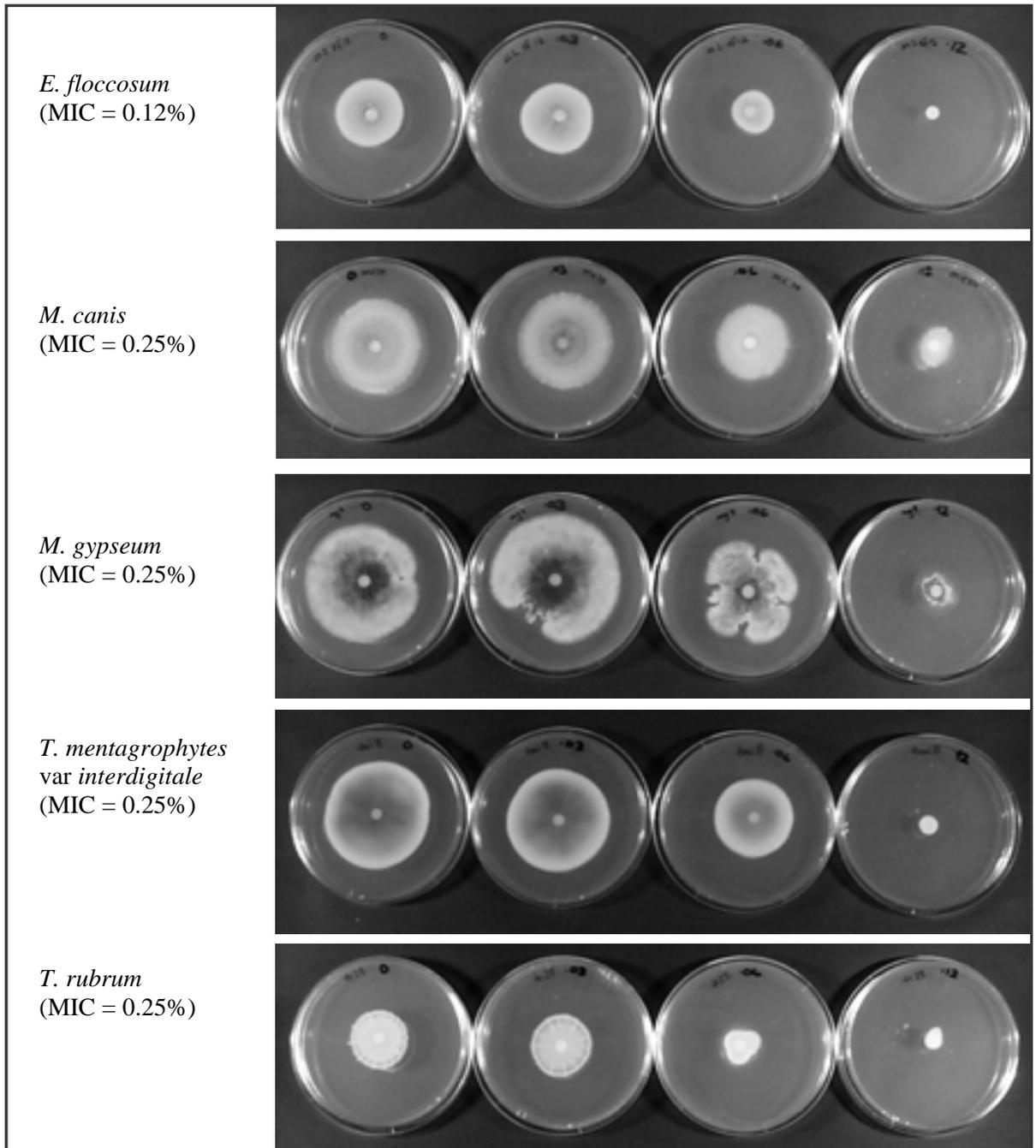
**Table 4.4:** Comparison of susceptibility data (%v/v) obtained by broth and agar dilution methods

	Broth microdilution		Agar dilution
	MIC	MFC	MIC <sup>a</sup>
<i>E. floccosum</i>	0.03	0.25	0.12
<i>M. canis</i>	0.016	0.25	0.25
<i>M. gypseum</i>	0.016	0.5	0.25
<i>T. interdigitale</i> <sup>b</sup>	0.03	0.5	0.25
<i>T. rubrum</i>	0.016	0.12	0.25

<sup>a</sup> MIC determined at day 14. <sup>b</sup> *T. mentagrophytes var interdigitale*



**Fig 4.1:** Reduction in growth (%) of dermatophytes on agar plates containing various amounts of tea tree oil (% v/v) after incubation for 14d at 30°C.



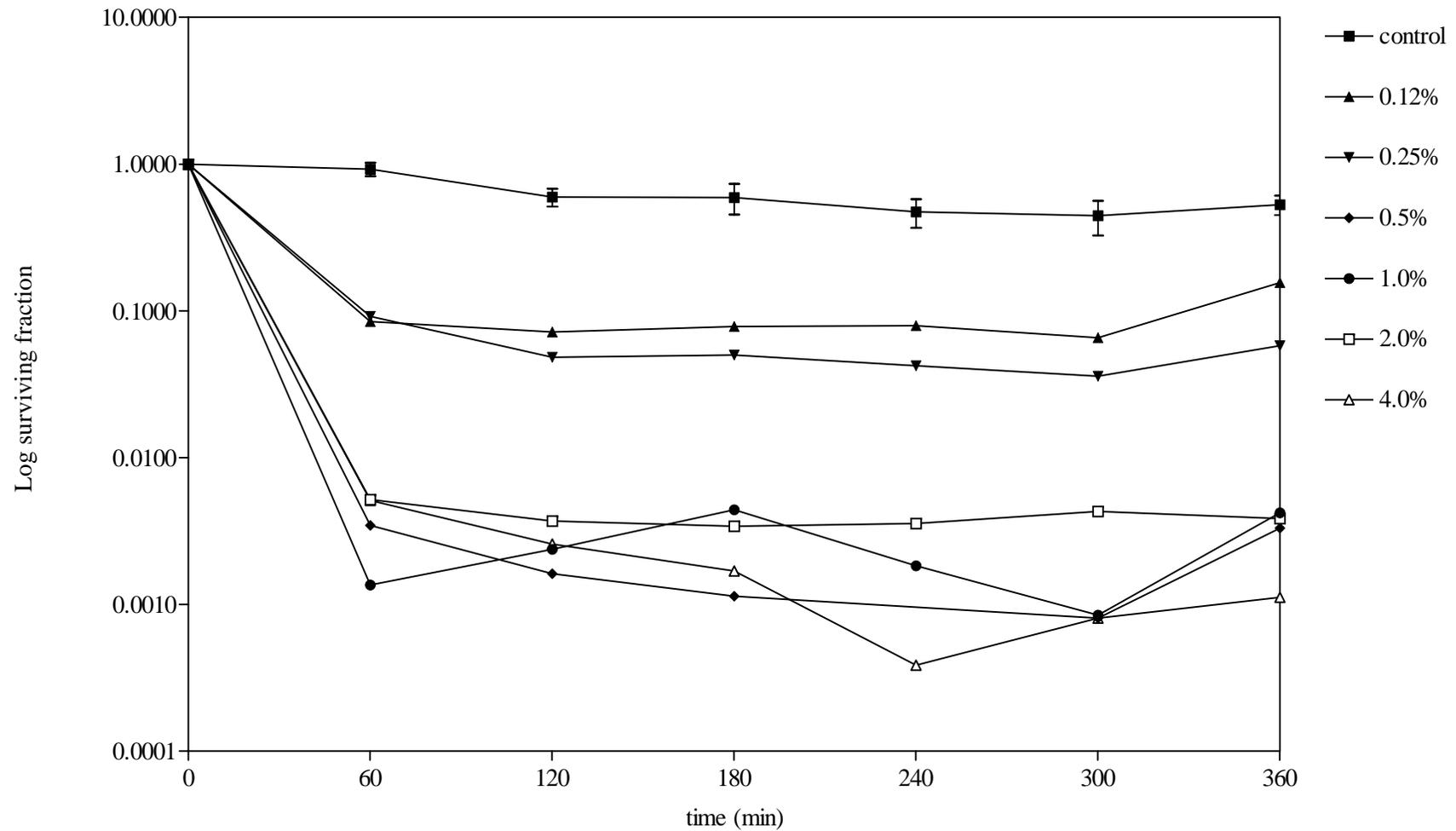
**Fig 4.2:** Growth of dermatophytes on SDA containing tea tree oil (%v/v) after incubation for 14d at 30°C (left to right; 0% control, 0.03%, 0.06%, 0.12%).

### **4.3 Activity against *Aspergillus niger***

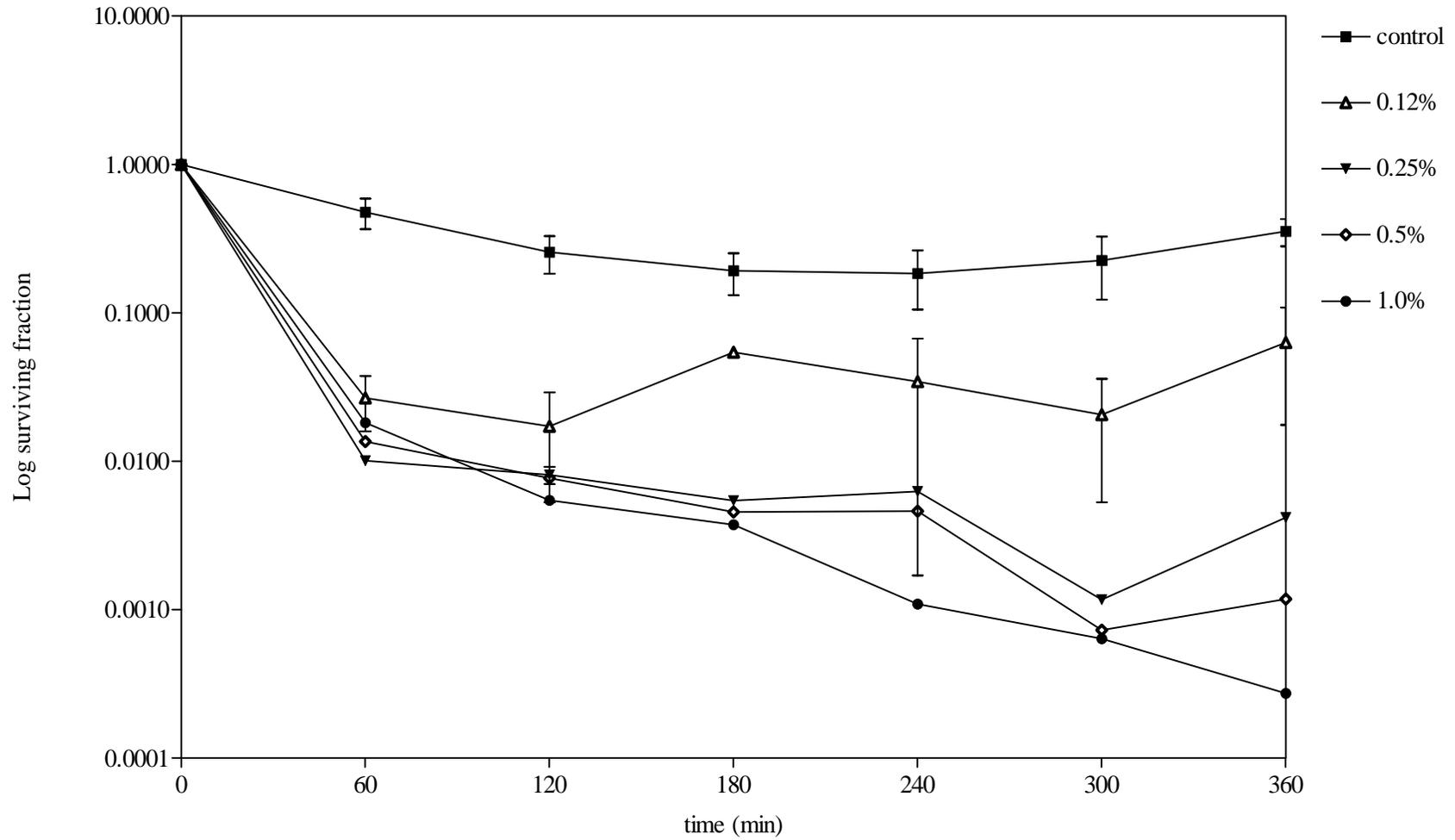
Of nine isolates of *A. niger* tested by the broth microdilution method, six had MICs of 0.12% and the remaining three isolates had MICs of 0.06%. One isolate had an MFC of 2.0%, five isolates had MFCs of 4.0%, one had an MFC of 6.0% and the remaining two had MFCs of 8.0%. MIC and MFC values differed by at least 5 concentrations for individual isolates. The inhibitory values were approximately equivalent to those obtained for the yeasts and were several concentrations higher than those obtained for dermatophytes in this study. The fungicidal values obtained were several fold higher than those obtained for the yeasts and dermatophytes.

### **4.4 Time-kill assays**

The results of the time-kill assays are shown in Figures 4.3 and 4.4, for *T. rubrum* and *T. mentagrophytes* var *interdigitale*, respectively. The concentrations of tea tree oil used were several fold in excess of the MFC values obtained for the isolates by the broth microdilution assay. Neither isolate was completely killed by any concentration of tea tree oil over the 6h time period of the experiment. The most rapid decreases in viable organisms recoverable from tea tree oil treatments occurred in the first hour of the experiment, after which time the numbers of organisms recovered remained approximately constant.



**Fig 4.3:** Time-kill curves for *T. mentagrophytes* var *interdigitale* with different concentrations of tea tree oil. Data points are either from single experiments or are mean  $\pm$  standard error (0% control).



**Fig 4.4:** Time-kill curves for *T. rubrum* with different concentrations of tea tree oil. Data points are either from single experiments or are mean  $\pm$  standard error (0% control and 0.12%).

## 5. Discussion

### 5.1 Activity against yeasts, dermatophytes and other fungi in vitro

The results obtained in this study demonstrate that tea tree oil has activity against a wide range of yeasts and dermatophytes and that the oil has both inhibitory and fungicidal activity. In addition, in this study data were generated for many species for which no data currently exist.

The inhibitory and fungicidal values obtained for yeasts in this study are very similar to those published previously [1, 2, 29]. MICs recently reported for *C. albicans* (obtained by the agar dilution method) were 0.63% [29], 0.44% [1], 0.2% [8]. A previous study published by our research group reported that both the MIC<sub>90</sub> and MFC<sub>90</sub> values, obtained by broth microdilution, were 0.25% for *C. albicans* [2], compared to the values of 0.5% and 1.0% obtained in this study. Comparison of the data obtained in these two studies showed that the MIC<sub>90</sub> and MFC<sub>90</sub> values were approximately one value higher by the NCCLS method used in the current study, compared to those obtained previously [2]. The main methodological differences were the inoculum size and test medium, which may have contributed to the slight differences in results obtained by each method.

The susceptibility data obtained for dermatophytes against tea tree oil by the broth microdilution method showed that all isolates were inhibited in the range of 0.004% to 0.06% tea tree oil and were killed in the range of <0.03% to 1.0%. The agar dilution method gave MICs that were much higher at 0.12-0.25%. It is difficult to compare either set of results to previous studies because so very few studies have investigated the activity of tea tree oil against dermatophytic fungi and also because of differences in methodology. Previously published studies have commonly used the disc diffusion method, which gives an indication of the activity of tea tree oil against dermatophytes but does not give specific, quantifiable data. Ånséhn [11] tested 27 dermatophyte isolates including *T. rubrum*, *T. mentagrophytes*, *E. floccosum*, *M. audouinii* and *M. canis* against 10µl tea tree oil in a disc diffusion method. He found all isolates showed zones of inhibition of greater than 3.5cm. Concha *et al.* [12] found zones of inhibition for 29 out of 30 dermatophyte strains (*T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *E. floccosum* and *M. gypseum*), when using 20µl of oil, also in an agar diffusion method. The isolate not inhibited was a strain of *E. floccosum*. Rushton *et al.* [13] examined one isolate each of *T. rubrum*, *T. mentagrophytes*, *E. floccosum* and *M. audouinii* by an agar dilution method. Isolates were inoculated onto plates containing 0.1% and 1.0% tea tree oil as well as a control. They observed that after 8 weeks incubation all isolates showed growth on the 0.1% plate but none showed growth on the 1.0% plate. Nenoff *et al.* [1] 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). These values are similar to those obtained by the agar dilution method in this study. Although the agar dilution method is not used as a conventional method for testing the susceptibility of dermatophytes to antifungal agents, it has commonly been used for investigating the activity of essential oils against fungi [30-32]. Our study showed that the MICs determined by the agar method were several concentrations higher than those obtained by the broth dilution method. This may be due to differences in the degree of contact between the oil and organism between the two methods. Presumably the organisms come into greater direct contact with the oil in the broth medium compared to agar. Other differences that may account for the different results include the two different growth media used (RPMI 1640 for the broth assay and SAB for the agar assay) and the length of incubation of tests (four days compared to 14 days). Also, solid agar may be a more favourable growth medium for the dermatophytes, as compared to broth. Niewerth *et al.* [33] compared MICs for five antimycotics obtained by agar and broth methods and also found that MICs were consistently higher by the agar method. It is interesting that they found a similar trend, although they did not speculate as to why this effect occurred.

The results of the time-kill experiments, albeit preliminary, showed that neither dermatophyte isolate was killed completely within the six hours of the experiment, at concentrations that were several times in excess of their respective MFC values. This demonstrates that at those concentrations, the killing effect is neither instantaneous nor rapid. Other time-kill studies with yeasts or fungi have generally conducted these experiments over at least a 24h period [34, 35] so a longer incubation period may be necessary to see significant decreases in viability. A slight decrease in recovered organisms was seen between time zero and one hour, at particular tea tree oil concentrations. A possible explanation for this is that the fungal morphologies more susceptible to the lethal effects of tea tree oil were killed within this first hour of the experiment and the less susceptible forms remained viable. Since the inocula for the experiment was likely to contain both hyphae and conidia this suggests that one of these morphologies is more susceptible than the other to tea tree oil.

*A. niger* was less susceptible to tea tree oil than the dermatophytes. The MICs obtained in this study were comparable to those obtained by the agar dilution method by other authors, such as 0.2% [36] and 0.3-0.4% [8]. No MFC values have been reported previously. It was interesting to note that the MFC values were several concentrations higher than the MIC results, a trend that was also seen for the dermatophytes. Another interesting result was that although *A. niger* was inhibited by 0.06-0.12% tea tree oil, some isolates remained viable at concentrations up to 8%. This result is in keeping with other data showing that *A. niger* is comparatively less susceptible to antimicrobial agents such as phenol, benzyl alcohol and benzalkonium chloride than non-sporulating bacteria and yeasts [37].

More isolates, both of *A. niger* and other filamentous fungi, need to be tested to obtain a more complete picture of the susceptibility of filamentous fungi to tea tree oil.

Several generalisations can be made from these data about the susceptibility of filamentous fungi to tea tree oil. The first is that the filamentous fungi appear to be relatively difficult to kill in vitro. Even though the broth microdilution data showed that the dermatophytes have MIC and MFC values that are very similar to bacteria and yeasts, the time-kill data indicate that fungicidal activity is neither rapid nor immediate. Also, the difference between inhibitory and fungicidal value for filamentous fungi demonstrates that to a certain degree, tea tree oil displays fungistatic rather than fungicidal activity. It is tempting to assume that fungal spores are resistant to antimicrobial agents in the same manner as bacterial spores. However, there is no evidence to suggest that this is a valid assumption. In fact, Russell [37] commented that fungi are often more susceptible than bacterial spores but are less susceptible than non-sporulating bacteria.

## 5.2 Previous clinical trials

Several clinical trials have been conducted using tea tree oil or tea tree oil products in the treatment of fungal infections. A study by Jandourek *et al.* [19] investigated the efficacy of a tea tree oil mouthwash for treating fluconazole-refractory oral candidiasis in HIV patients. They found that of 12 evaluable patients at the end of the four week treatment period, two were cured mycologically and an additional six had improved symptoms. Two trials have investigated the treatment of onychomycosis with tea tree oil. The trial by Buck *et al.* [22] found that 60% of trial participants had full or partial resolution at the end of six months of therapy. In addition, 18% of participants were culture negative at the end of therapy. The trial by Syed *et al.* [23] compared 5% tea tree oil alone to 5% tea tree oil combined with 2% butenafine in treating toenail onychomycosis. They found that none of the tea tree only group had mycological cure, compared to 80% cure in the tea tree oil/butenafine group. In comparison to the trial by Buck *et al.*, it is not surprising that the participants in the trial by Syed *et al.* did not achieve mycological cure, given that they used a much lower amount of tea tree oil (5% tea tree oil in an unstated vehicle, compared to neat oil) and had a much shorter treatment period (eight weeks compared to six months). A trial by Tong *et al.* [21] compared 10% tea tree oil in sorbolene to 1% tolnaftate and placebo for the treatment of tinea pedis. They found mycological cure rates of 30%, 85% and 21% for the tea tree oil, tolnaftate and placebo groups, respectively. Overall, 73%, 97% and 53% from the tea tree oil, tolnaftate and placebo groups, respectively, experienced some clinical improvement.

It is evident from the results of these clinical trials that there has been only moderate success in treating fungal infections with tea tree oil. This has also been noted in a recent review of published

tea tree oil clinical trials [38]. Many variables may account for the lack of in vivo efficacy. Failure of treatment may be due to 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 such as the agent having fungistatic and not fungicidal activity. The vehicle in which tea tree oil is formulated is known to affect the availability of the oil and the use of inappropriate vehicles may reduce the overall efficacy. There are factors to do with the fungal infection itself that contribute to the treatability of an infection. These include the fact that the fungi are located within dead keratinised tissue and they are physically separated from topical agents. Also, biological variation amongst the actual organisms themselves has meant that uniform treatment approaches have been difficult to formulate [39]. Particular dermatophyte infections are notoriously difficult to treat topically, and several authors comment that tinea capitis, tinea unguium and onychomycosis either have no effective topical therapies or the available topical therapies are ineffective [39, 40].

On the basis of the results of the above trials, and with some knowledge of the pathogenesis of fungal infections, it is likely that treating fungal infections with tea tree oil will require a relatively high concentration of oil and a reasonably long duration of treatment. It is vitally important that careful consideration be given to the product vehicle as this can affect treatment efficacy.

### **5.3 Further work**

This study has provided a comprehensive body of knowledge describing the in vitro susceptibility of fungi to tea tree oil. Further work is required to determine the in vitro susceptibility to tea tree oil of additional fungal species such as *Aspergillus*, *Alternaria*, *Penicillium*, *Fusarium* and *Cladosporium*. The importance of this is that these organisms are found in indoor air, and the presence of some of these species may be associated with sick building syndrome and respiratory symptoms in children [41, 42]. The testing of these species will also determine the range of filamentous fungi that are susceptible to tea tree oil. MICs and MFCs should be determined by a broth microdilution method. The completion of the time-kill studies will add to our understanding of the activity of tea tree oil against fungi. Once these mostly descriptive studies are complete, investigations into the mechanism of action of tea tree oil are warranted. An understanding of the mechanisms causing the inhibition and killing of fungi will provide a basis for the formulation and design of products for specific fungal conditions, and the use of tea tree oil in vivo.

# 6. Implications and Recommendations

## 6.1 Implications

The results of this study demonstrate unequivocally that tea tree oil has antifungal activity. Although not all of the factors contributing to the in vivo efficacy have been elucidated, the data provided here demonstrate that tea tree oil does have promise as an antifungal agent. These data benefit the industry by providing the bases on which tea tree oil can be marketed and tea tree oil products can be formulated and provided to the consumer.

## 6.2 Recommendations

It is recommended that these results be publicised at tea tree oil industry meetings or in industry publications. It is also recommended that these results be published in medical or scientific journals as a means of making the data available to clinicians, medical researchers and other health professionals, as well as tea tree oil industry personnel. Further research into the antifungal properties of tea tree oil needs to be pursued. This will allow tea tree oil products for the treatment of superficial fungal infections, such as athlete's foot, to be developed and then evaluated in clinical trials. The availability of such products that were known to be effective, would be of great commercial benefit to the tea tree oil industry.

## 7. References

1. Nenoff P, Hausteil UF, Brandt W. Antifungal activity of the essential oil of *Melaleuca alternifolia* (tea tree oil) against pathogenic fungi in vitro. *Skin Pharmacology* 1996; **9**: 388-94
2. Hammer KA, Carson CF, Riley TV. In-vitro activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against *Candida* spp. *Journal of Antimicrobial Chemotherapy* 1998; **42**: 591-5
3. Hammer KA, Carson CF, Riley TV. In vitro activities of ketoconazole, econazole, miconazole, and *Melaleuca alternifolia* (tea tree) oil against *Malassezia* species. *Antimicrobial Agents & Chemotherapy* 2000; **44**: 467-469
4. Beylier MF. Bacteriostatic activity of some Australian essential oils. *Perfumer and Flavourist* 1979; **4**: 23-25
5. Smith MD, Navilliat PL. A new protocol for antimicrobial testing of oils. *Journal of Microbiological Methods* 1997; **28**: 21-24
6. Mann CM, Markham JL. A new method for determining the minimum inhibitory concentration of essential oils. *Journal of Applied Microbiology* 1998; **84**: 538-44
7. May J, Chan CH, King A, Williams L, French GL. Time-kill studies of tea tree oils on clinical isolates. *Journal of Antimicrobial Chemotherapy* 2000; **45**: 639-643
8. Griffin SG, Markham JL. An agar dilution method for the determination of the minimum inhibitory concentration of essential oils. *Journal of Essential Oil Research* 2000; **12**: 249-255
9. Altman PM. Australian tea tree oil. *The Australian Journal of Pharmacy* 1988; **69**: 276-278
10. Hammer KA, Carson CF, Riley TV. In vitro susceptibility of *Malassezia furfur* to the essential oil of *Melaleuca alternifolia*. *Journal of Medical & Veterinary Mycology* 1997; **35**: 375-7
11. Ånséhn S. The effect of tea tree oil on human pathogenic bacteria and fungi in a laboratory study. *Swedish Journal of Biological Medicine* 1990; **2**: 5-8
12. Concha JM, Moore LS, Holloway WJ. Antifungal activity of *Melaleuca alternifolia* (tea tree) oil against various pathogenic organisms. *Journal of the American Podiatric Medical Association* 1998; **88**: 489-492
13. Rushton RT, Davis NW, C. PJ, A. DC. The effect of tea tree oil extract on the growth of fungi. *The Lower Extremity* 1997; **4**: 113-116
14. Bishop CD, Thornton IB. Evaluation of the antifungal activity of the essential oils of *Monarda citriodora* var. *citriodora* and *Melaleuca alternifolia* on post-harvest pathogens. *Journal of Essential Oil Research* 1997; **9**: 77-82
15. Bishop CD, Reagan J. Control of the storage pathogen *Botrytis cinerea* on Dutch White cabbage (*Brassica oleracea* var. *capitata*) by the essential oil of *Melaleuca alternifolia*. *Journal of Essential Oil Research* 1998; **10**: 57-60

16. Washington WS, Engleitner S, Boontjes G, Shanmuganathan N. Effect of fungicides, seaweed extracts, tea tree oil, and fungal agents on fruit rot and yield in strawberry. *Australian Journal of Experimental Agriculture*. 1999; **39**: 487-494
17. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi; Proposed Standard M38-P. Wayne, PA: NCCLS, 1998
18. Espinel-Ingroff A, Bartlett M, Bowden R, Chin NX, Cooper Jr C, Fothergill A, McGinnis MR, Menezes P, A. MS, Nelson PW, Odds FC, Pasarell L, Peter J, Pfaller MA, Rex JH, Rinaldi MG, Sharkland GS, Walsh TJ, Weitzman I. Multicentre evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. *Journal of Clinical Microbiology* 1997; **35**: 139-143
19. Jandourek A, Vaishampayan JK, Vazquez JA. Efficacy of melaleuca oral solution for the treatment of fluconazole refractory oral candidiasis in AIDS patients. *Aids* 1998; **12**: 1033-7
20. Walker M. Clinical investigation of Australian *Melaleuca alternifolia* oil for a variety of common foot problems. *Current Podiatry* 1972; **1972**: 7-15
21. Tong MM, Altman PM, Barnetson RStC. Tea tree oil in the treatment of tinea pedis. *Australasian Journal of Dermatology* 1992; **33**: 145-9
22. Buck DS, Nidorf DM, Addino JG. Comparison of two topical preparations for the treatment of onychomycosis: *Melaleuca alternifolia* (tea tree) oil and clotrimazole. *Journal of Family Practice* 1994; **38**: 601-5
23. Syed TA, Qureshi ZA, Ali SM, Ahmad S, Ahmad SA. Treatment of toenail onychomycosis with 2% butenafine and 5% *Melaleuca alternifolia* (tea tree) oil in cream. *Tropical Medicine & International Health*. 1999; **4**: 284-287
24. Norris HA, Elewski BE, Ghannoum MA. Optimal growth conditions for the determination of the antifungal susceptibility of three species of dermatophytes with the use of a microdilution method. *Journal of the American Academy of Dermatology* 1999; **40**: S9-13
25. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts; Proposed Standard M27-P. Wayne, PA: NCCLS, 1992
26. Aguilar C, Pujol I, Guarro J. In vitro antifungal susceptibilities of *Scopulariopsis* isolates. *Antimicrobial Agents and Chemotherapy* 1999; **43**: 1520-1522
27. Grover RK, Moore JD. Toximetric studies of fungicides against the brown rot organisms, *Sclerotinia fructicola* and *S. laxa*. *Phytopathology* 1962; **52**: 876-880
28. Zambonelli A, Daulerio AZ, Bianchi A, Albasini A. Effects of essential oils on phytopathogenic fungi in vitro. *Journal of Phytopathology-Phytopathologische Zeitschrift* 1996; **144**: 491-494
29. Bassett IB, Pannowitz DL, Barnetson RSC. A comparative study of tea-tree oil versus benzoylperoxide in the treatment of acne. *Medical Journal of Australia* 1990; **153**: 455-8
30. Dikshit AHA. Antifungal action of some essential oils against animal pathogens. *Fitoterapia* 1984; **55**: 171-176

31. El-Naghy MA, Maghazy SN, Fadl-Allah M, El-Gendy K. Fungistatic action of natural oils and fatty acids on dermatophytic and saprophytic fungi. *Zentralblatt für Mikrobiologie* 1992; **147**: 214-220
32. Mishra AK, Dubey NK. Evaluation of some essential oils for their toxicity against fungi causing deterioration of stored food commodities. *Applied & Environmental Microbiology* 1994; **60**: 1101-1105
33. Niewerth M, Splanemann V, Korting HC, Ring J, Abeck D. Antimicrobial susceptibility testing of dermatophytes - comparison of the agar macrodilution and broth microdilution tests. *Chemotherapy* 1998; **44**: 31-35
34. Klepser ME, Ernst EJ, Lewis RE, Ernst ME, Pfaller MA. Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. *Antimicrobial Agents and Chemotherapy* 1998; **42**: 1207-1212
35. Nakajima R, Kitamura A, Someya K, Tanaka M, Sato K. In vitro and in vivo antifungal activities of DU-6859a, a fluoroquinolone, in combination with amphotericin B and fluconazole against pathogenic fungi. *Antimicrobial Agents and Chemotherapy* 1995; **39**: 1517-1521
36. Southwell IA. The search for optimally bioactive Australian tea tree oil. *Acta Horticulture* 1993; **344**: 256-265
37. Russell AD. Antifungal activity of biocides. In: Russell AD, Hugo WB, Ayliffe GAJ, eds. *Principles and Practice of Disinfection, Preservation and Sterilization*. 3rd ed. Osney Mead, Oxford: Blackwell Science, 1999; pp 149-167
38. Ernst E, Huntley A. Tea tree oil: a systematic review of randomised clinical trials. *Forsch Komplimentärmed* 2000; **7**: 17-20
39. Weitzman I, Summerbell RC. The dermatophytes. *Clinical Microbiology Reviews* 1995; **8**: 240-259
40. Smith EB. Topical antifungal drugs in the treatment of tinea pedis, tinea cruris, and tinea corporis. *Journal of the American Academy of Dermatology* 1993; **28**: S24-S28
41. Cooley JD, Wong WC, Jumper CA, Straus DC. Correlation between the prevalence of certain fungi and sick building syndrome. *Occupational and Environmental Medicine* 1998; **55**: 579-584
42. Garrett MH, Rayment PR, A. HM, Abramson MJ, M. HB. Indoor airborne fungal spores, house dampness and associations with environmental factors and respiratory health in children. *Clinical and Experimental Allergy* 1998; **28**: 459-467



