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Antimicrobial Activity of Tea Tree Oil

A report for the Rural Industries Research and Development Corporation

by C F Carson & T V Riley

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Foreword

Tea tree (*Melaleuca alternifolia*) oil has become an increasingly popular product in recent years and production has continued to expand in an effort to meet the growing demand.

Despite mounting interest in the oil for therapeutic purposes, the vast majority of information available about it was, until now, anecdotal in nature. There is enormous potential to expand the tea tree oil industry if claims about its medicinal properties are verified.

Scientific evidence regarding the antimicrobial activity of tea tree oil may assist in the accessing of international markets and the registration of the oil with national regulatory bodies. The aim of this project was to produce that scientific evidence and, to that end, it has been extremely successful.

This publication describes work which confirms and characterises the antimicrobial activity of the oil. It looks at the methods used to evaluate the oil as well as the spectrum of activity and mechanism of action.

The project is part of RIRDC's Tea Tree Oil Program which aims to support the continued development of a profitable tea tree oil industry. The results will undoubtedly enhance the profile of this unique Australian commodity.

Peter Core
Managing Director
Rural Industries Research and Development Corporation

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Executive Summary

Despite the increasing interest in tea tree oil for therapeutic purposes, the vast majority of reports of its efficacy in treating a variety of infections are anecdotal and there is a paucity of information published in appropriate peer-reviewed journals This situation constitutes a significant dilemma for the tea tree oil industry. Early submissions to the Food and Drug Administration in the United States of America for tea tree oil to be registered as an over-the-counter topical antimicrobial have not been successful. One reason for this is that published in vitro efficacy data were lacking

In 1990, the results of a clinical trial examining the efficacy of tea tree oil in the treatment of acne were published (Bassett *et al.*, 1990) and created considerable interest. The trial demonstrated quite clearly that similar trials in this and other areas should be attempted. First, however, adequate data regarding the susceptibility of various pathogens to tea tree oil are required. Indeed, this suggestion has been made by the FDA. Therefore, one of the tea tree industry's first priorities has been to accumulate substantial susceptibility data on isolates from infections potentially treatable with tea tree oil.

The second area of research requires a focus on the mechanism of action of tea tree oil as no information on this area is available. Since the mechanism of action has implications for the selectivity and safety of antimicrobial agents, this issue was becoming increasingly important. In addition, investigations into the antimicrobial activity of the individual components were required. Not only was this information necessary for our understanding of how the oil works, but it may also have implications for distillation methods and the manipulation of oil composition.

The objectives of this project therefore were to increase the acceptability of tea tree oil as a naturally-occurring antimicrobial agent, both nationally and internationally, by:

- producing scientifically valid data relating to the antimicrobial activity of tea tree oil against bacteria.
- determining which components of tea tree oil are responsible largely for antimicrobial activity of the oil.

- elucidating the mechanism of action of tea tree oil.
- publishing results of this work in peer-review medical journals.

As a first step methods were developed and validated and finally, a broth micro-dilution method was used to examine the susceptibility of *Escherichia coli* (n=110) and *Staphylococcus aureus* (n=105) to tea tree oil. The detergent Tween 80 was used successfully to enhance the solubility of tea tree oil in the test medium and the minimum inhibitory concentration (MIC) inhibiting 90% of strains for *E. coli* was 0.25%, while for *S. aureus* it was 0.50%.

The antimicrobial activity of eight components of tea tree oil was evaluated using disc diffusion and broth microdilution methods. After assessing media with and without solubilizing agents, the disc diffusion method was used to determine the susceptibility of a range of micro-organisms to 1,8-cineole, 1-terpinen-4-ol, ρ-cymene, linalool, α-terpinene, γ-terpinene, α-terpineol and terpinolene. While the disc diffusion method lacked reproducibility, it was considered useful as a procedure for screening for antimicrobial activity. Terpinen-4-ol was active against all the test organisms while ρ-cymene demonstrated no antimicrobial activity. Linalool and α-terpineol were active against all organisms with the exception of *Pseudomonas aeruginosa*. Minimum inhibitory and minimum cidal concentrations of each component against *Candida albicans*, *E. coli* and *S. aureus* were determined using a broth microdilution method. Modifications to this method previously developed overcame solubility and turbidity problems associated with the oil components and allowed the antimicrobial activity of each of the components to be quantified reproducibly.

Methicillin-resistant strains of *S. aureus* (MRSA) are a cause of important hospital-acquired infections. They are usually carried in the nose and may be amenable to topical treatment with tea tree oil. The susceptibility of a further 66 isolates of *S. aureus* to tea tree oil was determined using disc diffusion and modified broth microdilution methods. The addition of Tween 80 detergent to the test system adequately enhanced the solubility of tea tree oil in the aqueous test medium. Dissolution in ethanol further assisted solubilisation of the tea tree oil in the test broth. Of the isolates tested, 64 were MRSA and 33 were resistant to mupirocin the most

commonly prescribed topical antibiotic. All the isolates tested were susceptible to tea tree oil using both methods. The MIC and minimum bactericidal concentration (MBC) for 60 Australian isolates were 0.25% and 0.50%, respectively. Co-workers in Britain using similar methods, determined the MIC and MBC for the remaining 6 isolates as 0.312% and 0.625%, respectively. These in vitro results suggest tea tree oil may be useful in the treatment of MRSA carriage.

Streptococci may also cause skin infections that could possibly be treated with a tea tree oil product and therefore a number of strains was tested. For 19 *Streptococcus pyogenes* isolates, the MIC_{90} was 0.12%, while the MBC_{90} was 0.25%. The most susceptible streptococci were *S. dysgalactiae* and one of the *S. pyogenes* isolates with an MIC and MBC of 0.03%. *S. equi*, *S. equisimilis* and the Lancefield's group G streptococcus had an MIC and MBC of 0.12%. The MIC for *S. zooepidemicus* was 0.06% while the MBC was 0.12%.

The Food and Drug Administration requires that the in vitro antimicrobial spectrum of compounds intended for use as a health-care antiseptic be determined. In particular they are interested in the susceptibility of normal flora to intended active compounds. We therefore tested a range of normal and commensal isolates of the type found on skin *Serratia marcescens* had the lowest MIC_{90} of 0.25%. The highest MIC_{90} was 3% for *Ps. aeruginosa*. The lowest MBC_{90} was 0.25% for *S marcescens* while the highest was 8% for *S. capitis*. *S aureus* and most Gram-negative bacteria tested were more susceptible to tea tree oil than the coagulase negative staphylococci and micrococci. These results suggest that tea tree oil may be useful in removing transient skin flora while suppressing but maintaining resident flora.

The in vitro activity tea tree oil against 105 isolates of anaerobic and microaerophilic vaginal bacteria was determined. MICs and MBCs of oil were determined by agar and broth dilution methods. By agar dilution, 90% of isolates of *Bacteroides*, *Prevotella*, *Fusobacterium* and *Peptostreptococcus* were inhibited by concentrations of ≤0.5% (v/v) tea tree oil. In contrast, 2.0% tea tree oil was required to inhibit 90% of the lactobacilli tested. This difference in susceptibility suggests that tea tree oil, when formulated into appropriate products, may be useful in the treatment of bacterial vaginal infections.

Treatment of *E. coli* suspensions with tea tree oil or components resulted in significant reductions in optical density. These results suggested that the membrane was a site of action in *E. coli*. None of the treatments affected the optical density of *S. aureus* suspensions suggesting that action of the oils on the cell membrane is not the prime mechanism of action in *S. aureus* cells.

The second manner in which the putative membrane action of tea tree oil and its components was examined was by observing the leakage of nucleic acids from treated cells. While only *S. aureus* suspensions were examined in this manner, significant results were obtained. Treatment with MICs of α-terpineol, 1, 8-cineole, terpinen-4-ol and linalool all resulted in the appearance of 260nm-absorbing material outside the cells. This suggests that genetic material is being lost from the cell through a damaged membrane.

Electron microscopy of terpinen-4-ol treated cells provided further information. *C. albicans* cells were not lysed by terpinen-4-ol treatment and by electron microscopy, appeared unaltered. In contrast, electron microscopy provided useful information about terpinen-4-ol-induced damage to *E. coli*. These cells had been sensitive to lysis by oil treatment and the appearance of empty "ghost" cells by electron microscopy confirmed this effect. The appearance of mesosome-like structures in terpinen-4-ol treated *S. aureus* also suggests damage to the cell membrane or wall.

The original premise was that tea tree oil and/or its components act on the cell membrane or wall. These results indicate that this is a site of action. While further evidence is required to corroborate these observations, the possibility that other sites of action may exist, must be considered.

This project has firmly established that tea tree oil has significant antimicrobial activity. These results have been published in mainstream medical and scientific journals and generated considerable interest in Australia and, in particular, Europe and the United States. The industry now has a firm basis for the next step in the promotion of tea tree oil as a *bona fide* topical antibiotic. The next step in the process is to establish that tea

tree oil products work in the clinical setting. To do this randomised clinical trials will need to be conducted at appropriate testing centres. This is not an inexpensive exercise, however, the potential benefits to the industry should justify the outlay.

1.0 Introduction

1.1 Background to the project

Renewed interest in tea tree oil as a naturally-occurring antimicrobial agent has fostered substantial expansion of the tea tree oil industry. As additional growers are attracted to the industry, production has increased. In the absence of a corresponding increase in demand, it is possible that a surplus of oil on the world market will occur. There is enormous potential to expand the tea tree oil industry if the United States and European markets are more accessible. To enable this, approval from regulatory authorities, such as the Food and Drug Administration in the United States, is required.

Despite the increasing interest in tea tree oil for therapeutic purposes, the vast majority of reports of its efficacy in treating a variety of infections are anecdotal and there is a paucity of information published in appropriate peer-reviewed journals. There are industry reports containing some data but these are often treated as confidential and are not generally available. In addition, they have not been subject to peer review. This situation constitutes a significant dilemma for the tea tree oil industry. Early submissions to the FDA in the US for tea tree oil to be registered as an over-the-counter topical antimicrobial have not been successful. One reason for this is that published in vitro efficacy data were lacking. This lack of information is compounded by the fact that some of the methods required by the FDA for testing purposes, are inappropriate for tea tree oil. Approval is unlikely to be obtained unless tea tree oil is investigated using scientific methods currently acceptable by regulatory authorities.

In addition, for pharmaceutical and medical communities to accept tea tree oil as a *bona fide* antimicrobial agent, the results of investigations need to be published in international refereed journals acceptable to these groups. Only when this occurs will tea tree oil move out of the realms of "quackery" and "alternative medicine." In 1990 the results of a clinical trial examining the efficacy of tea tree oil in the treatment of acne were published (Bassett *et al*., 1990) and created considerable interest. It demonstrated quite clearly that similar trials in this and other areas should be attempted. First, however, adequate data regarding the susceptibility of various pathogens to tea tree oil are required. Indeed, this was suggested by the FDA after one of the tea tree oil submissions. Therefore, one of the tea tree industry's first priorities has been to

accumulate substantial susceptibility data on isolates from infections potentially treatable with tea tree oil. While data on all infectious agents, including bacteria, fungi and viruses were required, this project concentrated on bacteria. Following industry requests some additional work with fungi was also included.

The second area of research focussed on the mechanism of action of tea tree oil. No previous investigations reporting on this were available. Since the mechanism of action has implications for the selectivity and safety of antimicrobial agents, this issue was becoming increasingly important.

In addition, investigations into the antimicrobial activity of the individual components were required. Not only was this information necessary for our understanding of how the oil works, but it may also have implications for distillation methods and the manipulation of oil composition.

1.2 Objectives

At present tea tree oil is sold mainly on the basis of its alleged antimicrobial properties. If the markets for this commodity are to be increased, then scientific evidence of this activity must be produced. It is imperative that this evidence be of a calibre suitable for international regulatory authorities.

At the outset of this project, very little contemporary data of an adequate standard were available. The objectives of this project were to increase the acceptability of tea tree oil as a naturally occurring antimicrobial agent, both nationally and internationally, by:

- producing scientifically valid data relating to the antimicrobial activity of tea tree oil against bacteria.
- determining which components of tea tree oil are responsible largely for antimicrobial activity of the oil.
- elucidating the mechanism of action of tea tree oil.
- publishing results of this work in peer-review medical journals.

2.0 Materials and methods

2.1 Bacteria

It was important to test large numbers of isolates of various species and to include a selection of clinical and reference isolates. Tests with recent clinical isolates provide contemporary data about the susceptibility of organisms to tea tree oil while the inclusion of reference isolates allow other workers to repeat the work and corroborate the results.

The bacteria (n = 682) examined were as follows: *Acinetobacter baumannii* (60), *Bacteroides* spp. (12), *Corynebacterium* spp. (10), *Escherichia coli* (113), *Fusobacterium* spp. (2), *Klebsiella* spp. (63), *Lactobacillus* spp. (26), *Micrococcus luteus* (4), *M. varians* (2), *Micrococcus* spp. (5), miscellaneous anaerobic Gram positive cocci (11), *Peptostreptococcus anaerobius* (12), *Prevotella* spp. (24), *Pseudomonas aeruginosa* (53), *Serratia marcescens* (30), *Staphylococcus aureus* (105), methicillin-resistant *Staphylococcus aureus* (66), *S. capitis* (10), *S. epidermidis* (15), *S. haemolyticus* (10), *S. hominis* (10), *S. saprophyticus* (4), *S. warneri* (9), *S. xylosus* (2), *Streptococcus dysgalactiae* (1), *S. equi* (1), *S. equisimilis* (1), *S. pyogenes* (19), *S. zooepidemicus* (1), Lancefield's Group G streptococcus (1).

2.2 Susceptibility tests

2.2.1 Broth microdilution tests

The methodology for testing the susceptibility of microorganisms to tea tree oil has been the subject of some controversy. The problems have been largely due to the immiscibility of tea tree oil with various water-based testing media. However, this can be over come by the use of detergents such as Tween 80 (Beylier, 1979; Walsh and Longstaff, 1987) in broth procedures. This creates another problem in that uninoculated media are quite turbid thereby precluding the visual reading of endpoints.

A number of approaches were employed to overcome this problem. In preliminary experiments a growth indicator, triphenyl tetrazolium chloride (TTC), was added to the broths. TTC changes from colourless to red in the presence of bacterial growth. This provided clearly defined and easily readable endpoints with some organisms.

Another point considered during the selection of methods for susceptibility tests was the overall acceptability of the chosen method. In our diagnostic laboratory most

susceptibility testing of bacteria follows the procedures recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 1991). While these guidelines do not provide a solution to the problems of oil miscibility and turbidity, they do allow control over all the other aspects of the testing procedure. More important, they are recognised in the United States and Europe. Their use would also facilitate the publication of the results in peer-reviewed medical journals, one of the aims of the project.

One of the aims of the project was to obtain quantitative data, rather than qualitative data. Information about the concentrations of oil that inhibit and kill bacteria was sought. These concentrations were defined as minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). These values were also sought because they could give valuable insight as to the mechanism of action of tea tree oil.

With conventional antimicrobial agents, the correlation between MIC/MBC data and disc diffusion data is well understood and conclusions about the susceptibility of organisms may be made from disc diffusion tests. Since disc diffusion methods are much simpler to perform than MIC/MBC tests, considerable effort was put into determining if this relationship was similar for tests with tea tree oil.

To quantitate the antibacterial activity of *M. alternifolia* oil, MICs and MBCs were determined using a broth microdilution method. Initially tests were performed in heart infusion broth. Later, the medium was changed to Mueller-Hinton broth to comply with NCCLS guidelines. In some cases, nutritional requirements of the test organisms necessitated the use of alternative growth media. Where NCCLS guidelines were available, these were followed. Tween 80 detergent was added to enhance the solubility of the tea tree oil in the broth system. At first, the concentration of Tween 80 detergent was 0.5% (v/v) but later work demonstrated that non-ionic detergents such as Tween 80 compromised the activity of the oil and the concentration was reduced to 0.001% (v/v).

In some tests, triphenyl tetrazolium chloride (TTC) (Aldrich Chemical Company Inc., Milwaukee, Wis., USA) was also added to the culture medium as a growth indicator to overcome the problem of turbidity due to the solubilised oil. The final concentration of TTC after inoculation was 0.005% (w/v).

For organisms that grow aerobically, serial doubling dilutions of *M. alternifolia* oil were prepared in a 96-well tray (Falcon, Becton-Dickinson & Co., Lincoln Park, New Jersey, USA) over the range 0.03-4.0% (v/v). Overnight broth cultures of test organisms were prepared by inoculating Mueller-Hinton broth with 1-2 colonies from a blood agar plate and incubating for 18h at 35°C with shaking. The concentration of organisms was adjusted using a nephelometer (model # 41100-71, Biomerieux Vitek, Hach Company, Loveland, USA) so that, upon inoculation, each well contained approximately 5.0×10^5 cfu/ml. Positive and negative growth controls were included in every test. The concentration of each inoculum was confirmed using viable counts on blood agar plates (Oxoid). Trays were incubated aerobically at 35°C for 24h, and the MICs and MBCs determined.

In tests with TTC, the reduction of this compound from the colourless solution to red precipitate served as an indicator of bacterial growth although this did not always correlate exactly with either the MIC or MBC. To confirm MICs and to establish MBCs, 10µL of broth was removed from each well and spot inoculated onto blood agar. After aerobic incubation at 35°C overnight, the number of surviving organisms was determined. The MIC was the lowest concentration that resulted in the maintenance of reduction of the inoculum, while the MBC was the point where 99.9% of the inoculum was killed. Since sub-culture was required to determine the MBC, the TTC was omitted from subsequent tests and the MIC determined from the sub-culture results.

For anaerobic organisms, the method was similar except that inocula were usually prepared from cultures of the organism grown on blood agar for 48h. One to two colonies were emulsified in sterile distilled water, adjusted using the nephelometer and used to inoculate the tea tree oil dilutions. Trays were incubated in an anaerobic chamber (Don Whitley Scientific Ltd.) for 48h before being sub-cultured to determine the MIC and MBC.

Once the MIC and MBC of *S. aureus* NCTC 6571 and *E. coli* (NCTC 10418) had been determined, one of these organisms was included in all susceptibility tests as a control. If the resulting MIC/MBC for the control organism differed by more than one dilution

in either direction from the known MIC/MBC, the results were rejected and the test repeated.

Broth microdilution methods were also used to examine eight of the individual components of tea tree oil. The eight individual components tested were: 1,8-cineole (Sigma Chemical Co., St. Louis, MO, USA), 1-terpinen-4-ol (Aldrich Chemical Co. Inc., Milwaukee, WI, USA), ρ-cymene (Aldrich), linalool (Sigma), α-terpinene (Sigma), γ-terpinene (Sigma), α-terpineol (Aldrich) and terpinolene (Keith Harris and Co. Ltd., Thornleigh, NSW, Australia). These components were tested because they were readily available and, apart from linalool, are major individual components of tea tree oil. The test organisms were *C. albicans* (ATCC 10231), *E. coli* (NCTC 10418) and *S. aureus* (NCTC 6571). MICs and minimum cidal concentrations (MCCs) were determined.

2.2.2 Disc diffusion tests

In order to determine the correlation between broth microdilution and disc diffusion tests, the individual components of *M. alternifolia* oil, were tested using both methods. Broth microdilution tests were conducted as described above and the disc diffusion method is described here.

In preliminary experiments, the effect of solubilising agents on the diffusion of components through the agar was examined. Five sets of media were prepared. MH agar was used as the standard test medium. MH agar supplemented with Tween 80 detergent (Atlas Chemical Industries Inc.) (0.5% or 1.0% v/v) or dimethyl sulphoxide (DMSO) (1.0% or 2.0% v/v) was also prepared. All agar plates were prepared in 90mm Petri dishes with 22ml of agar giving a final depth of 4mm. *Escherichia coli* and *Staph. aureus* were tested on this range of media. Overnight broth cultures were prepared, adjusted in sterile distilled water to yield approximately 1 x 10^8 cfu/ml and used to lawn inoculate the range of solid media. Paper discs (diameter 12.7mm) were placed on the inoculated agar surfaces and impregnated with 30µl of undiluted oil component. The components tested were 1,8-cineole, ρ-cymene, linalool, α-terpinene, γ-terpinene, terpinen-4-ol, α-terpineol and terpinolene. Each individual oil component was tested on a separate plate. A duplicate set of plates was prepared. Once the plates were

inoculated and the *M. alternifolia* oil component placed on the paper disc on the agar surface, one set was left at 4°C for 2h to allow pre-diffusion of the components through the medium prior to incubation. The second set was incubated immediately. All plates were incubated aerobically at 35°C for 24h. After incubation, zones of inhibition were determined. Each test was performed in triplicate and the results analysed for statistical significance (see below).

Following these experiments, MH was selected as the most appropriate medium and the remaining organisms were tested. The pre-diffusion step was omitted. Overnight broth cultures of the organisms, except *Lact. acidophilus,* were prepared; *Bact. fragilis* and *Cl. perfringens* were grown in pre-reduced anaerobic brain heart infusion broth (Unipath Ltd.), *Mor. catarrhalis* in MH broth (Unipath Ltd.) and the remainder in HIB. Suspensions of *Lact. acidophilus* were prepared in saline (0.85%) from blood agar (Unipath Ltd.) plate cultures. All cultures/suspensions were adjusted as before and inoculated onto MH agar except for *Bact. fragilis* and *Mor. catarrhalis* which were inoculated onto MH agar supplemented with horse blood (5%) and *Lact. acidophilus* which was inoculated onto blood agar. After placement of the component-impregnated disc, plates were incubated immediately for 24h. Each test was performed in triplicate and the mean size of the zone of inhibition determined. The disc diffusion method was also used to estimate the susceptibility of 60 isolates of MRSA to *M. alternifolia* oil.

2.3 Mechanism of action

Most antimicrobial agents function by inhibiting the synthesis of macromolecules, such as protein or DNA, or by affecting the cell wall. The mechanism of action of *M. alternifolia* oil and some of its major components was investigated.

2.3.1 Optical density of microbial suspensions treated with *Melaleuca alternifolia* oil or components

The capacity of *M. alternifolia* oil and several of the individual components to cause cell lysis was examined spectrophotometrically. Fresh BA cultures of *E. coli, S. aureus* or *C. albicans* were prepared and used to inoculate MHB which was incubated at 35°C for 18h with shaking. For *E. coli,* 500ml of MHB was inoculated while for *C. albicans* and *S. aureus,* 200ml volumes were inoculated. Organisms were separated from the

growth medium by centrifugation (20min 10K rpm JA-10 rotor), washed twice with PBS and resuspended finally in PBS/Tw. All suspensions were adjusted so that the OD_{620} of a 1 in 100 dilution was 0.1-0.3. Viable counts were performed on the stock suspensions to determine the number of organisms.

Volumes of 4.6ml of test organism were prepared in 20ml McCartney bottles. The optical density of each suspension was confirmed by removing 0.1ml, diluting 1 in 100 in PBS/Tw and measuring the optical density (λ=620nm) (OD_{620}). Stock dilutions of *M. alternifolia* oil or component, at 10 times the desired final concentration, were prepared and 0.5ml was added to each 4.5ml test suspension. Each organism was tested against *M. alternifolia* oil, terpinen-4-ol, 1,8-cineole and α-terpineol at 1 x and 2 x the MIC. Untreated controls were included in each experiment. For tests with *E. coli*, each agent was tested with and without ethylene dinitrilo tetraacetic acid (EDTA) at a final concentration of 0.02%. All tests were conducted at room temperature (22°C).

At 0, 30, 60, 90 and 120min, the suspensions were mixed thoroughly using a vortex mixer and 0.1ml was removed. This was diluted 1 in 100 in PBS/Tw and the OD_{620} measured. The 1 in 100 dilution effectively reduced *M. alternifolia* oil or component concentration to a level where its contribution to absorbance was negligible. Corresponding dilutions of test agents were used as blanks in each case. Results were expressed as a ratio of the OD_{620} at 120min versus the OD_{620} at 0min (%). The effect over time of each treatment on the test organisms was graphed.

2.3.2 Leakage of 260nm-absorbing material from microbial suspensions treated with *Melaleuca alternifolia* oil or components

The capacity of several of the individual components of *M. alternifolia* oil to cause leakage of 260nm-absorbing material from microbial cells was examined spectrophotometrically. Attempts were made to evaluate the effect of *M. alternifolia* oil on leakage. However, *M. alternifolia* oil absorbed too strongly at 260nm and dilution of the samples could not be increased to remove this effect.

Fresh BA cultures of *E. coli*, *S. aureus* or *C. albicans* were prepared and used to inoculate 50ml of MHB which was incubated at 35°C for 18h with shaking. Growth

medium was removed by centrifugation (20min 10K rpm JA-10 rotor), the organisms washed twice with PBS buffer and resuspended finally in PBS/Tw. Suspensions of *E. coli* and *S. aureus* were adjusted to a final concentration of ~10^9 cfu/ml which corresponded to an OD_{620} of a 1 in 10 dilution of 0.33 and 0.18 respectively. Suspensions of *C. albicans* were adjusted to ~10^8 cfu/ml which corresponded to an OD_{620} of a 1 in 100 dilution of 0.14.

Volumes of 13.6ml were placed in McCartney bottles. The OD of each separate suspension was confirmed by removing 0.1ml, diluting in PBS/Tw and measuring the OD_{620}. Stock solutions of the test agents were prepared at 10 x the desired final concentrations. 1,8-Cineole, linalool, terpinen-4-ol and α-terpineol were tested at 0.1 x MIC, 0.5 x MIC and MIC. In some cases, 2 x MIC was tested. Volumes of 1.5ml of the test solutions were added to the microbial suspensions and mixed thoroughly using a vortex mixer. At 0, 5, 10, 15, 30, 60, 120 and 240 and 300min, 1ml samples were removed using a syringe and needle. Samples were filtered immediately through a 0.22μm filter to remove organisms and the filtrate collected. The optical density of each filtrate was measured at 260nm. Filtrates of PBS/Tw supplemented with the appropriate concentration of test agent were used as blanks.

2.3.3 Electron microscopy of terpinen-4-ol treated cells

Stationary phase cultures of *C. albicans*, *E. coli* and *S. aureus* were prepared by O/N incubation of 30ml HIB broths. Each culture was divided into 3 separate 10ml plastic V-bottom test tubes and centrifuged. The supernatant was discarded and the pellets resuspended in HIB/Tw.

Each organism was treated with terpinen-4-ol at a concentration just in excess of the MIC. *C. albicans* and *S. aureus* were treated at 0.3% and *E. coli* at 0.15%. The treatment times for *S. aureus* and *E. coli* were 4 and 10min respectively, while *C. albicans* was treated for 1 and 2h. Control tubes of each culture were allowed to stand in HIB/Tw for the maximum treatment time.

After centrifugation (Hettich Universal) at 1500*g* for 5min, the pellet was fixed in 2.5% glutaraldehyde (TAAB Laboratories, Reading, England) in 0.1M cacodylate buffer

(TAAB). Each tube was mixed gently by inversion for approximately 2min and allowed to fix O/N at room temperature. The suspensions were washed twice with 0.1M cacodylate buffer and placed in microfuge tubes. The supernatant was discarded, replaced with 10% bovine serum albumin (BSA) and the pellet resuspended. After centrifugation, the 10% BSA was removed, without disturbing the pellet surface, and replaced with 2.5% glutaraldehyde in cacodylate buffer. The preparations were allowed to stand for at least 6h or until the pellets were fixed firmly.

Processing, embedding, sectioning and staining procedures were performed by the Department of Pathology, SCGH. Semi-thin (approx. 0.5-1.0mm thick) and ultra-thin sections (50-60nm) were cut on an LKB III (LKB, Sweden) ultramicrotome using glass knives. Semi-thin sections were placed on glass slides and stained with 0.2% toluidine blue in 5% borax on a hot plate at 60°C. After mounting in DePeX they were viewed on a light microscope (Carl Zeiss Research).

Ultra-thin sections were picked up on thin bar 200 mesh copper grids (SPI Supplies, West Chester, USA). A Polaron P650A vacuum evaporator (Polaron, USA) was used to coat with 20nm of carbon. Thin sections were stained with modified lead citrate (Kay, 1965) for 5min, washed thoroughly in filtered double distilled water for 20 seconds. The stained copper grids were examined with a Philips 410 transmission electron microscope (Philips Scientific, Eindhoven, Netherlands) at an accelerating voltage of 80kV and exposures taken on Kodak Electron Microscope Film (N^{o} 4489). All prints were prepared using a Devere 504 enlarger (Devere, England).

2.3 Statistical analysis

The two-tailed student's *t*-test was used to determine the statistical significance of differences observed between zones of inhibition with and without pre-diffusion. To determine if there were any significant differences between the media used, zones of inhibition recorded from the plates which were either incubated immediately or allowed to pre-diffuse were compared using a one-way analysis of variance (ANOVA). Where a statistically significant difference occurred ($P \leq 0.05$), zones of inhibition were further compared using the student's *t*-test.

3.0 Microdilution method for *E. coli* and *Staph. aureus*

3.1 Introduction

Standard methods to evaluate the antimicrobial activity of tea tree oil are urgently required as are susceptibility data for large numbers of isolates. The purpose of this part of the project was to develop a broth micro-dilution method to determine the susceptibility of multiple isolates of *E.coli* and *S.aureus* to tea tree oil *in vitro*.

3.2 Results

The MICs of tea tree oil for the organisms tested are shown in Table 3.1. The MIC and MBC of tea tree oil for *E. coli* ATCC 10536 were both 0.12% after repeated testing. This was also the MIC and MBC for 23 of the clinical isolates while for the remaining 87 isolates the MIC and MBC were 0.25%. The MIC and MBC of tea tree oil for *S. aureus* NCTC 6571 were 0.25% and 0.50%, respectively, after repeated testing. Of the 105 clinical isolates of *S. aureus*, 3 had a MIC of 0.12%, 78 had a MIC of 0.25% while the remaining 24 had a MIC of 0.50%. Cumulative MBCs of tea tree oil are shown in Table 3.2.

Table 3.1. Susceptibility of *E. coli* and *S. aureus* to tea tree oil

	MIC (% v/v)		
Organism (No. tested)	50%	90%	Range
E.coli (110)	0.25	0.25	0.12-0.25
S. aureus (105)	0.25	0.50	0.12-0.5

Table 3.2. Cumulative % of MBCs of tea tree oil for *E. coli* and *S. aureus*

	Cumulative MBC (% v/v)				
Organism (No. tested)	0.12	0.25	0.50	1.0	2.0

E.coli (110)	20.1	100	100	100	100
S. aureus (105)	0	7.6	62.9	80	100

3.3 Discussion

A convenient and accurate method to determine the susceptibility of micro-organisms to tea tree oil is required urgently as failure to substantiate manufacturers' claims and anecdotal evidence of its efficacy with reliable scientific work will result ultimately in the decline of the tea tree oil industry. In addition, attempts to have tea tree oil recognised by national regulatory authorities will not succeed until suitable scientific evidence has been generated. In many cases, although susceptibility data have been published, insufficient information on methodology was provided (Altman, 1988; Bassett *et al.,* 1990). In addition, the range of organisms tested often did not include a specified reference strain to allow comparison of results between investigators or only one isolate of each species was tested (Low *et al.*, 1974; Walsh and Longstaff, 1987; Williams *et al.,* 1990).

Overall, *E. coli* was more susceptible to tea tree oil than *S. aureus* with all isolates having an MIC and MBC of ≤0.25%. In contrast, 77% of the *S. aureus* tested had a MIC of ≤0.25% while the MBCs spanned the range 0.25-2.0%. These results correlate with those obtained in disc-diffusion tests where *E. coli* was more susceptible to tea tree oil than *S. aureus* (Carson and Riley, 1994a).

The development of methods to determine the susceptibility of specific organisms to tea tree oil has been difficult, due partly to the inherent problems associated with testing an oil; tea tree oil is lipophilic. The insolubility of tea tree oil in standard test media has represented a serious impediment in the evaluation of its antimicrobial activity. Attempts to overcome this problem have included the use of surfactants, such as the non-ionic detergent Tween 80 (Atkinson and Brice, 1955; Low *et al.*, 1974; Beylier, 1979; Walsh and Longstaff, 1987; Carson and Riley, 1994b).

Although the incorporation of Tween 80 detergent into the test broth did not result in the formation of a completely homogeneous solution, it did enhance the solubility of tea tree oil in broth to the point where consistent results could be obtained thereby allowing

the antimicrobial activity to be evaluated. Tween 80 does not possess antibacterial activity of its own (Beylier, 1979) and the presence of Tween 80 in the HIB at a concentration of 0.5%, did not inhibit the growth of *E. coli* or *S. aureus*. While the incorporation of an extraneous compound into the test method is undesirable, it appears to represent a reasonable compromise between method integrity and expediency.

Although Tween 80 enhanced the solubility of the tea tree oil, the turbidity of the resulting solution hampered visual determination of the MICs. While this problem was overcome in part by the inclusion of TTC as a growth indicator, this remedy would not be universally applicable as many micro-organisms fail to reduce TTC. TTC colour development corresponded closely to inoculum survival or growth in tests with *E. coli*. However, in tests with *S. aureus* a distinct endpoint was less evident with viable inoculum persisting 1-2 wells further than colour development. Since subculture is required to establish the MBCs, TTC can be omitted and the presumptive visual MIC can be confirmed in this step.

We have previously used similar modifications successfully with a broth macro-dilution system to determine the MBC of tea tree oil for 32 isolates of *Propionibacterium acnes* (Carson and Riley, 1994b). The application of these modifications to a broth micro-dilution system should allow the rapid determination of MICs and MBCs of tea tree oil for a larger number of isolates.

4.0 Activity of tea tree oil components

4.1 Introduction

The antimicrobial activity of the terpinen-4-ol component of tea tree oil, with a Rideal-Walker (RW) coefficient of 13 (Penfold and Grant, 1925), is not disputed. This observation, along with anecdotal evidence about the enhanced antimicrobial activity of tea tree oils with high terpinen-4-ol and low cineole content, has resulted in terpinen-4-ol being regarded as the main antimicrobial component (Penfold and Morrison, 1937; Lassak and McCarthy, 1983). Other major components include ρ-cymene, linalool, α- and γ-terpinene, α-terpineol and terpinolene and, together with cineole and terpinen-4-ol, these components constitute approximately 80-90% of tea tree oil (Brophy *et al.*

1989; Williams and Home, 1989). The contribution of individual components to the overall antimicrobial activity is unknown.

The purpose of this section of the work was to examine the antimicrobial activity of eight individual components of tea tree oil using modified disc diffusion and broth microdilution methods to overcome the problems of oil dispersion and turbidity.

4.2 Results

For both *E. coli* and *S. aureus,* zones of inhibition on media containing Tween 80 were consistently smaller than on either MH or MH containing DMSO. These differences usually occurred in tests with α-terpineol, linalool or terpinen-4-ol. Zones of inhibition on both MH and DMSO containing media were similar with only three statistically significant differences in zone sizes occurring. As a result, MH agar was used for the remaining tests.

Terpinen-4-ol was the only component that was significantly affected by the pre-diffusion step when tested on MH agar. For *E. coli* and *S. aureus*, the mean zone size for pre-diffused plates was significantly larger than that for plates incubated immediately. No other combinations of components and test media were affected.

Representative results of the disc diffusion method are shown in Table 4.1. Using this method, each of the individual components demonstrated some antimicrobial activity. The growth of *Ps. aeruginosa* was inhibited by terpinen-4-ol only. *Bacteroides fragilis*, *C. albicans* and *Cl. perfringens* were inhibited by all of the components tested. While terpinen-4-ol inhibited all 12 of the test organisms, linalool and α-terpineol failed to inhibit *Ps. aeruginosa* only. The least inhibitory component was ρ-cymene which inhibited four of the test organisms. While there was little variation between triplicate zone diameter measurements, reproducability was a problem with mean zones of inhibition varying by several millimetres on occasions.

Table 4.1. Disc diffusion susceptibility of various micro-organisms to eight components of tea tree oil

Organism	Component							
	1,8-cineole	Linalool	terpinen-4-ol	α-terpineol	ρ-cymene	α-terpinene	γ-terpinene	terpinolene
B. *subtilis* (NCTC 3610)	0*	33	28	23	0	19.3	0	18.3
Bact. fragilis (NCTC 9343)	19	42	36.7	27.7	15	21.3	15	17.7
C. albicans (ATCC 10231)	16	40.3	41	33.7	15	17	15	18
Cl. *perfringens* (NCTC 8359)	18.7	6.3	38.7	29.3	20	32.3	22	28.7
Ent. faecalis (NCTC 8213)	0	23	19.7	17	0	15	14	19.7
E. coli (NCTC 10418)	25	42.5	37	33	0	16	0	23
L. acidophilus (NCTC 2949)	0	44	25	19.3	0	0	0	14.7
Mor. catarrhalis (NCTC 3622)	22	>90	32	28	0	20.3	16	20.7
M. smegmatis (NCTC 333)	14.3	27.7	35.7	41	0	19.7	0	36
Ps. aeruginosa (NCTC 10622)	0	0	14.6	0	0	0	0	0
S. *marcescens* (NCTC 1377)	15.3	22	32	20	0	0	0	15.7
Staph. *aureus* (NCTC 6571)	0	27.7	23	20.7	0	15	0	17.7

*mean zone size (mm)

In contrast, the broth microdilution method gave highly reproducible results. The MICs and MCCs determined by broth microdilution are shown in Table 4.2. Linalool, terpinen-4-ol and α-terpineol exhibited the greatest antimicrobial activity, with MICs and MCCs ≤0.25%. The lowest dilution of ρ-cymene (8.0%) failed to inhibit the growth of any of the test organisms, while 8% γ-terpinene did not inhibit *C. albicans* or *E. coli*. Eight percent α-terpinene inhibited the growth of *C. albicans*, but was not fungicidal. MIC and MCC results for *C. albicans* were, with the exception of α-terpinene, the same for each of the components. Similar results were obtained for *E. coli*, although MICs and MCCs were consistently lower. Similar MICs and MCCs of linalool, terpinen-4-ol and α-terpineol were also observed against *S. aureus*. In contrast, the MICs of 1,8-

cineole, α-terpinene, γ-terpinene and terpinolene for *S. aureus* were one dilution higher than the MCCs.

Table 4.2. Minimum inhibitory concentrations (MICs) and minimum cidal concentrations (MCCs) (expressed as % v/v) of individual components of *M. alternifolia* oil against *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus*.

Component	Organism					
	C. albicans		*E. coli*		*S. aureus*	
	MIC	MFC	MIC	MBC	MIC	MBC
1,8-cineole	1	1	0.25	0.25	0.50	1
linalool	0.25	0.25	0.06	0.06	0.25	0.25
terpinen-4-ol	0.25	0.25	0.06	0.06	0.25	0.25
α-terpineol	0.25	0.25	0.06	0.06	0.25	0.25
ρ-cymene	>8.0[#]	>8.0	>8.0	>8.0	>8.0	>8.0
α-terpinene	8.0	>8.0	2.0	2.0	4.0	8.0
γ-terpinene	>8.0	>8.0	>8.0	>8.0	4.0	8.0
terpinolene	8.0	8.0	4.0	4.0	2.0	4.0

[#]8% was the maximum concentration tested

4.3 Discussion

There is a paucity of information about the individual components of tea tree oil and their antimicrobial activity. Several properties of tea tree oil and its components would appear to render them unsuitable for examination in conventional susceptibility testing systems. A comparison of five media types demonstrated that, despite the limited solubility of the test components, MH agar could be used for the disc diffusion assay and the addition of solubilizing agents was not required. Pre-diffusion was discontinued

since terpinen-4-ol/MH was the only component/medium combination that showed increased zones of inhibition following pre-diffusion. The reduction in zone size seen when Tween 80 detergent was added to the MH agar may be due to the Tween 80 allowing better distribution of components through the agar, resulting in a lower overall concentration. Alternatively, the Tween 80 may have enhanced the growth of the test organisms as it is a source of oleic acid (Carson and Riley, 1994b). Antagonism between Tween 80 and the oil components is also possible. Despite its initial promise, however, the disc diffusion method lacked reproducibility.

The inclusion of Tween 80 detergent in the broth dilution method was more important and facilitated a relatively stable and even dispersion of the oil components in the test medium. While the incorporation of such extraneous compounds into susceptibility testing media is undesirable, the need to adequately solubilize the hydrophobic tea tree oil components, in order to accurately quantify their antimicrobial activity, was greater. Although the detergent possesses negligible antimicrobial activity of its own, the possibility that it may act synergistically with the oil components or enhance the growth of test organisms cannot be disregarded.

The addition of TTC to the HIB provided a simple method of detecting the growth of *E. coli* and overcoming the problem of oil turbidity. Growth and colour development correlated well and MICs could be determined accurately based on the presence or absence of a colour change. However, with *S. aureus*, growth often occurred in the absence of a colour change and so this method was not as useful. While a similar technique for *C. albicans* would be useful, the tendency for this organism to form pellets on the well floors allowed adequate growth detection. Since subculture was required in each test to determine the MCC, the provisional MIC may be confirmed in this step and the TTC omitted.

The disc diffusion method was useful for the preliminary examination of the antimicrobial properties of tea tree oil and oil components. All components demonstrated some antimicrobial activity confirming the suggestion that no one single component is responsible for the activity of tea tree oil. The lack of reproducibility of the disc diffusion method is however, a distinct disadvantage and it should only be used

as a screening test. Antimicrobial activity was better qualified by MIC and MCC determinations using a broth dilution technique. Despite previous reports attributing the antimicrobial activity of tea tree oil almost entirely to terpinen-4-ol, two other components, linalool and α-terpineol, demonstrated antimicrobial activity equivalent to that of terpinen-4-ol. Although the MIC and MCC results varied between test organisms, for linalool, terpinen-4-ol and α-terpineol, each MIC was equivalent to the MCC indicating that these components were biocidal. A cidal effect was also exerted by 1,8-cineole against *C. albicans* and *E. coli* while the MIC/MCC results against *S. aureus* suggest a bacteriostatic action. Linalool has previously been reported as having antibacterial (Onawunmi *et al*. 1984; Ross *et al*. 1980), antifungal (Maruzzella *et al*. 1961; Reuveni *et al*. 1984) and anthelmintic (McKern and Parnell, 1964) activity with a RW coefficient of 13 (Penfold and Grant, 1925; Lassak and McCarthy, 1983). In tea tree oil, linalool levels vary from a trace to 0.2% (Swords and Hunter, 1978; Brophy *et al*. 1989; Williams and Home 1989). The antimicrobial activity demonstrated by α-terpineol was not unexpected as it has a RW coefficient of 16 (Penfold and Grant, 1925; Lassak and McCarthy, 1983). Anthelmintic activity of this component has been reported before (McKern and Parnell ,1964). Levels of α-terpineol vary from 2.4 to 5.3% in tea tree oil (Swords and Hunter, 1978; Brophy *et al*. 1989; Williams and Home, 1989).

The antimicrobial activity demonstrated by cineole contrasted with previous reports that it had negligible antimicrobial activity (Low *et al*. 1974; Lassak and McCarthy, 1983; Cruz *et al*. 1989; Hayes *et al*. 1993). Since it appears to contribute to the antimicrobial activity of the oil, the low level stipulated by the Australian Standard (<15%) may be inappropriate. However, this level is also a function of cineole's reputation as a skin irritant (Lassak and McCarthy, 1983). This reputation is based largely on anecdotal evidence, although contact dermatitis to tea tree oil has been reported (Apted, 1991; de Groot and Weyland, 1992) with cineole being confirmed as the allergen in the latter case. Adverse skin reactions have also followed ingestion of the oil (Elliott, 1993).

The growth of *Ps. aeruginosa* was inhibited by terpinen-4-ol only. In previous work using the same disc diffusion method, whole oil failed to inhibit the growth of *Ps. aeruginosa* (Carson and Riley, 1994a) suggesting there may be antagonism between

components in the oil. This may also account for the conflicting reports regarding the susceptibility of *Ps. aeruginosa* to tea tree oil (Altman, 1988) given the variation in terpinen-4-ol content allowed by the Australian standard. The complexity of the oil, with approximately 100 components, increases the likelihood that synergistic or antagonistic interactions are occurring between components.

Although only *C. albicans*, *E. coli* and *S. aureus* were tested by both MIC/MCC and disc diffusion methods, and although the disc diffusion method lacked reproducibility, there appeared to be reasonable correlation obtained between MICs and zones of inhibition for most components. The only exception was 1,8-cineole which produced no zone of inhibition against *S. aureus* despite having a relatively low MIC of 0.5%. The size of the zone of inhibition produced by a compound in a disc diffusion test is determined by the inherent antimicrobial activity of the compound, its solubility in and diffusion through the medium and the various characteristics of the organism. For mainstream antimicrobial agents the relationships between these parameters have been well studied, however, for tea tree oil components further investigations are required.

This study has identified several components which appear to contribute significantly to the antimicrobial activity of tea tree oil, although the possibility that other oil components possess antimicrobial activity remains. Disc diffusion methods are suitable for screening purposes only and quantitative data should be sought using a broth dilution technique. In addition, the role of synergistic or antagonistic interactions needs to be evaluated. Finally, little is known about the mechanism of action of tea tree oil or the potential for the emergence of resistance. If tea tree oil continues to be marketed as an antimicrobial agent, investigation of these and other issues is essential.

5.0 Activity against methicillin-resistant *Staphylococcus aureus*

5.1 Introduction

The carriage and subsequent dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) by hospital staff and patients is a recognised risk for nosocomial infection. Attempts to eliminate MRSA carriage often include the application of topical

preparations such as the antistaphylococcal agent mupirocin which has been useful in eliminating MRSA carriage previously (Kauffman *et al*., 1993; Naguib *et al*., 1993). The emergence of mupirocin-resistant MRSA (Rahman *et al*., 1987; Janssen *et al*., 1993; Riley *et al*., 1994) represents a serious threat to our capacity to manage MRSA carriage and suitable alternatives to mupirocin are required (Maple *et al*., 1992).

Walsh and Longstaff (1987) reported the minimum inhibitory concentration (MIC) of tea tree oil for *S. aureus* was 0.08%, while Altman (1988) found that MICs for a range of common pathogens, including *S. aureus,* were between 0.5 and 1.0%. Little additional susceptibility data for tea tree oil are available, due partly to methodological problems experienced in testing the oil. Tea tree oil has limited solubility in aqueous media and this may prejudice its performance in some susceptibility tests. The purpose of our study was to examine the susceptibility of MRSA, both mupirocin-susceptible and mupirocin-resistant, to tea tree oil, by disc diffusion and broth dilution methods.

5.2 Results

All 60 MRSA tested against tea tree oil by the disc diffusion method were susceptible, with a mean zone size of 27.0mm (range=21.3-33.3mm; σ=2.9mm). The MICs and MBCs for the 60 isolates, as determined by the broth microdilution method, were all 0.25% and 0.50%, respectively. There was no difference in tea tree oil susceptibility between mupirocin-susceptible and mupirocin-resistant MRSA. Values obtained at the LHI were similar, with the MIC and MBC values determined being 0.312% and 0.625%, respectively. Again, there was no difference between mupirocin-susceptible and mupirocin-resistant strains.

5.3 Discussion

The incidence of high-level mupirocin resistance in MRSA has been reported as 0% (Maple *et al.*, 1992), 1.3% (Naguib *et al.*, 1993) and 1.5% (Kauffman *et al*., 1993). These rates contrast sharply with the rate of high-level mupirocin resistance (15.0%) exhibited in a recent study of MRSA isolated in WA (Riley *et al*., 1994). In that study, mupirocin resistance occurred mainly in WA MRSA corroborating the suggestion made by Udo *et al*., (1994) that mupirocin resistance has emerged rapidly within the WA MRSA population. Given that high-level mupirocin-resistance, in MRSA in general

(Janssen *et al*., 1993) and in WA MRSA specifically (Udo *et al.*, 1994), appears to be transmissable, mupirocin may soon be of little value in the control and treatment of MRSA. The universal susceptibility to tea tree oil of all MRSA and MSSA isolates tested, including those which were mupirocin-resistant, represents a significant result which may find application in the control of MRSA.

Despite the insolubility of tea tree oil in aqueous media, disc diffusion testing proved to be a rapid and convenient method of assessing the susceptibility of MRSA to tea tree oil. All isolates produced zones of inhibition within a range of 21.3-33.3mm and these zone diameters corresponded to low MIC and MBC results. Based on the MIC and MBC results, none of the isolates could be considered resistant. Unfortunately, in the absence of a range of MIC/MBC results it was not possible to determine the extent of correlation between zone diameter and MIC/MBC. However, the MICs and MBCs were within previously reported ranges (Walsh and Longstaff, 1987; Altman, 1988) and are well below the concentrations of tea tree oil found in most currently available product formulations (2-5%). What remains to be established is the efficacy of these products in clinical settings. Although the occurrence of dermal irritation upon exposure to *M. alternifolia* oil has been reported, it appears to be rare (Apted, 1991) and requires further investigation.

6.0 Activity against streptococci

6.1 Introduction

Chapter 5 describes the antibacterial activity of tea tree oil against *S. aureus*, both methicillin-susceptible and resistant (Carson *et al*., 1995a; Carson *et al.*, 1995b). *S. aureus*, along with *Streptococcus pyogenes*, are the main aetiological agents of the common childhood infection impetigo (Shriner *et al.*, 1995). In light of suggestions that the oil may be useful in the topical treatment of impetigo, data regarding the susceptibility of *Streptococcus* spp. were sought.

6.2 Results

For the 19 *S. pyogenes* isolates, the MIC_{90} was 0.12%, while the MBC_{90} was 0.25%. The most susceptible organisms were *S. dysgalactiae* and one of the *S. pyogenes*

isolates with an MIC and MBC of 0.03%. *S.* e*qui, S. equisimilis* and the Lancefield's group G streptococcus had an MIC and MBC of 0.12%. The MIC for *S. zooepidemicus* was 0.06% while the MBC was 0.12%.

6.3 Discussion

Several reports on the in vitro susceptibility of bacteria and fungi to tea tree oil have been published in recent years (Altman, 1988; Carson *et al*., 1995a, b). However, none has given data on the susceptibility of *S. pyogenes* to tea tree oil except for Altman's reported MICs of about 1% (v/v). The MIC_{90} of 0.12% demonstrated in our study suggests that tea tree oil may be effective against streptococci when used topically as a wound disinfectant.

Although systemic treatment is usually indicated for impetigo, topical treatment may also be of value (Barnett and Frieden, 1992; Shriner *et al*., 1995). Mupirocin has been used successfully for this problem and offers the advantage that it is effective against both *S. aureus* and *S. pyogenes* (Barnett and Frieden, 1992). However, the development of mupirocin-resistant *S. aureus* poses a problem (Riley *et al.,* 1994). Tea tree oil has demonstrated antibacterial activity against *S. aureus*, MRSA and mupirocin-resistant *S. aureus* (Carson and Riley, 1995a,b) and development of resistance to the oil has not been observed. Given that tea tree oil exhibits antibacterial activity against both of the organisms implicated in impetigo, it may prove useful in topical treatment. Careful product formulation and clinical trials are now required to evaluate the potential of this natural product.

7.0 Activity against transient and commensal flora

7.1 Introduction

The Food and Drug Administration of the USA requires that the in vitro antimicrobial spectrum of compounds intended for use as a health-care antiseptic be determined. In particular they are interested in the susceptibility of normal flora to intended active compounds. The purpose of this part of the project was to determine the susceptibility

of a range of transient and commensal skin flora to tea tree oil using a broth microdilution method.

7.2 Results

MIC and MBC results are given in Tables 7.1 and 7.2 respectively. *S marcescens* had the lowest MIC_{90} of 0.25%. The highest MIC_{90} was 3% for *P aeruginosa*. Isolates of *A baumannii*, *Corynebacterium* spp., *Micrococcus* spp., *M luteus* and *S capitis* had the lowest MIC of 0.06% while the highest MIC was 5% for one isolate of *P aeruginosa*. *S marcescens* had the lowest MBC_{90} of 0.25% while the highest was 8% for *S capitis*. The lowest MBC was 0.12% for isolates of *A baumannii*, *Corynebacterium* spp., *Micrococcus* spp. and *S epidermidis*. Isolates of *M luteus*, *Micrococcus* spp., *S capitis* and *S warneri* all had MBCs equal to or greater than 6%. The highest MBC was 10% for one *S capitis* isolate. In the present study, with Gram-negative organisms, the MIC often corresponded to the MBC and this may be indicative of a bactericidal effect. In contrast, Gram-positive organisms often exhibited trailing endpoints with a large difference between the MIC and MBC, suggesting tea tree oil may exert a bacteriostatic effect on these organisms.

7.3 Discussion

There was wide variation in the susceptibility of the staphylococci to tea tree oil with MBCs for the coagulase negative staphylococci (CNS) being higher than those for *S aureus*. Lower concentrations of tea tree oil were required for cidal activity against the Gram-negative organisms, *A baumannii, K pneumoniae* and *S marcescens*. In previous studies we obtained similar MIC_{90} and MBC_{90} results for *E coli* (Carson *et al*., 1995) and demonstrated that methicillin-resistant *S aureus* has an MIC_{90} of 0.25% and MBC_{90} of 0.5% (Carson *et al*., 1995). The results suggest that *S aureus* and most of the Gram-negative bacteria tested, are more susceptible to tea tree oil than CNS and micrococci. This may afford tea tree oil a use in removing transient skin flora, while suppressing but maintaining resident flora as a protective measure against colonisation by multiresistant pathogenic bacteria.

Table 7.1. MICs of tea tree oil for various bacteria obtained by the broth microdilution method.

Organism (no. of isolates)	MIC (%v/v)		
	Range	50%	90%
A baumannii (23)	0.06-1	0.25	1
Corynebacterium spp.(10)	0.06-2	0.5	2
K pneumoniae (14)	0.12-0.5	0.25	0.25
M luteus (4)	0.06-0.5	nd[a]	nd
M varians (2)	0.5-1	nd	nd
Micrococcus spp. (5)	0.06-0.5	nd	nd
P aeruginosa (10)	2-5	2	3
S marcescens (11)	0.25-0.5	0.25	0.25
S aureus (69)	0.12-0.5	0.25	0.5
S capitis (10)	0.06-1	0.5	1
S epidermidis (15)	0.12-1	0.5	1
S haemolyticus (10)	0.5-1	0.5	0.5
S hominis (10)	0.12-0.5	0.5	0.5
S saprophyticus (4)	0.25-1	nd	nd
S warneri (9)	0.5-3	nd	nd
S xylosus (2)	0.25-0.5	nd	nd

[a]not determined

Table 7.2. MBCs of tea tree oil for various bacteria by the broth microdilution method.

Organism (no. of isolates)	MBC (%v/v)		
	Range	50%	90%
A baumannii (23)	0.12-1	0.25	1

Corynebacterium spp.(10)	0.12-3	2	2
K pneumoniae (14)	0.12-0.5	0.25	0.25
M luteus (4)	0.25-6	nd[a]	nd
M varians (2)	1-3	nd	nd
Micrococcus spp. (5)	0.12-6	nd	nd
P aeruginosa (10)	2-5	2	3
S marcescens (11)	0.25-1	0.25	0.25
S aureus (69)	0.25-2	1	2
S capitis (10)	1-10	6	8
S epidermidis (15)	0.12-4	2	4
S haemolyticus (10)	1-4	1	2
S hominis (10)	1-4	2	4
S saprophyticus (4)	2-3	nd	nd
S warneri (9)	2-8	nd	nd
S xylosus (2)	1-3	nd	nd

[a]not determined

The MIC and MBC values obtained in our study may not be comparable to those of other studies due to methodological differences. Previously published MICs for *S aureus*, determined by agar or broth dilution methods, included 0.5% (Beylier, 1979) and 0.08% (Walsh and Longstaff, 1987). MBCs were not determined. Altman (1988) reported MICs in the range 0.5-1.0% for a range of bacteria including *E coli*, *S aureus* and *P aeruginosa*. Despite possible methodological differences, these results are all similar to those obtained in this study with the exception of the MIC of 0.08% for *S aureus* (Walsh and Longstaff, 1987) and the MIC of 1% for *P aeruginosa* (Altman, 1988). Variation between study results also highlights the value of including reference strains in any chosen method, both as an internal control and for the purposes of external comparison.

The hands of staff are one of the main modes of transmission of hospital infection, and handwashing remains the principle method for reducing cross-infection in hospital wards. Unfortunately, frequent handwashing may result in dry, cracked skin and this is

a major impediment to compliance. Consequently, the refinement of existing handwashing products and the development of novel products continues. The natural product renaissance of the last decade has seen a plethora of claims regarding the usefulness of many essential oils, including tea tree oil, in numerous products. Given the antimicrobial activity of tea tree oil seen in this study, particularly against transient bacterial pathogens, potential exists for tea tree oil to be used in hygienic hand disinfection. In addition, the lipophilic nature of tea tree oil means that it lends itself readily to incorporation in surfactant preparations. Tea tree oil is already incorporated into a number of moisturising products and this property may constitute another benefit of this natural product. The potential of this lipophilic compound to penetrate the outer layers of skin may also enhance its antimicrobial activity against transient and commensal flora by means of a residual effect.

Tea tree oil has demonstrated potentially useful antimicrobial activity in vitro and its potential as an active ingredient in handwash preparations warrants further examination. In addition to its antibacterial activity, the antifungal and antiviral activity of tea tree oil also requires investigation. When the full spectrum and extent of the antimicrobial activity of tea tree oil is known, in vivo work will be able to proceed on a sound basis.

8.0 Activity against anaerobic bacteria

8.1 Introduction

Bacterial vaginosis (BV) is a polymicrobial vaginal infection affecting women of reproductive age (Cook *et al*., 1992). The numbers of normally predominant lactobacilli are reduced, with a corresponding increase in the numbers of anaerobic and microaerophilic vaginal bacteria. These bacteria include *Gardnerella vaginalis, Mobiluncus* spp., *Prevotella* spp., *Porphyromonas* spp., *Bacteroides* spp., and anaerobic cocci such as *Peptostreptococcus*. BV is commonly treated with oral metronidazole, which usually results in the restoration of normal vaginal flora and alleviation of symptoms. However, BV can recur within 3 months in up to 40% of women who responded to the initial therapy (Cook *et al*., 1992).

Blackwell (1991) described a patient with typical signs and symptoms of BV who treated herself with tea tree oil vaginal pessaries. After treatment, the patient was

symptom free and the vaginal flora was predominantly Gram-positive bacilli. It was suggested that the application of tea tree oil for therapy of BV be further assessed and that in vitro data on the susceptibility of organisms associated with BV be sought. The aim of this study was to evaluate the activity of tea tree oil against lactobacilli and a range of organisms associated with BV.

8.2 Results

8.2.1 Broth dilution assay

All six *Bacteroides* isolates tested had MICs of 0.06%. Five isolates had MBCs of 0.06% while the remaining isolate had a MBC of 0.12%. Two *Prevotella* isolates had MICs and MBCs of 0.03%. Of five *Peptostreptococcus anaerobius* isolates, one and four isolates had MICs of 0.03% and 0.06%, respectively. MBCs were 0.03% for one isolate, 0.06% for three isolates and 0.12% for the remaining isolate. MICs for the 26 lactobacilli ranged from 0.12% to 2.0%. The MIC_{50} and MIC_{90} were 1.0% and 2.0%, respectively. The range of MBCs for lactobacilli was 0.25% to 2.0% and the MBC_{90} was 2.0%. Both MICs and MBCs were 0.12% for *S. aureus* and *E. coli*.

8.2.2 Agar dilution assay

Table 8.1 gives MIC data for when more than 10 organisms were tested. In addition, five *G. vaginalis* had MICs of 0.06% tea tree oil and, of four *Mobiluncus* spp., three had MICs of 0.03% while the other was 0.06%. MICs for *S. aureus* and *E. coli* were both 0.5%, on both agar dilution media.

Table 8.1. Tea tree oil MICs for organisms associated with bacterial vaginosis obtained using the agar dilution method

Organism	no.	Tea tree oil (% v/v)		
		MIC_{range}	MIC_{50}	MIC_{90}
Bacteroides spp.	12	0.03-0.5	0.25	0.5
Prevotella spp.	24	0.03-0.25	0.12	0.25
Fusobacterium spp.	10	0.06-0.25	0.12	0.25

P. anaerobius	12	0.06-0.25	0.25	0.25
Other anaerobic cocci	11	0.03-0.25	0.06	0.12

8.3 Discussion

Conventional treatment for BV includes systemic or topical metronidazole and topical clindamycin cream (Joesoef and Schmid, 1995). Topical treatments may be preferable because they produce fewer systemic side-effects, such as gastrointestinal upset and an unpleasant taste (Joesoef and Schmid, 1995). Tea tree oil has previously been advocated for topical use in the treatment of vaginal infections, including BV, trichomonal vaginitis and vaginal candidiasis (Humphrey, 1930; Pena, 1962; Belaiche, 1985). Intra-vaginal tea tree oil products are available in many countries and may be purchased by women wanting to treat vaginal symptoms, including BV. Self treatment with alternative medicines appears to be common amongst women with chronic vaginal symptoms (Nyirjesy *et al*., 1997), illustrating the need for in vitro and in vivo data pertaining to the efficacy of alternative products, in particular, those containing tea tree oil.

MICs and MBCs of tea tree oil have been reported for anaerobic bacteria including fusobacteria, *Bacteroides*, *Prevotella* and *Peptostreptococcus*, by agar and broth dilution methods (Walsh and Longstaff, 1987; Shapiro *et al*., 1994). Despite differences in methodology, our MIC data are similar to previous studies (Carson and Riley, 1993; Shapiro *et al*., 1994). In addition, all groups of organisms showed a range of susceptibilities to tea tree oil. However, all *Lactobacillus* spp. tested were appreciably more resistant to tea tree oil than those organisms known to be associated with BV, with at least a two-fold difference in MIC_{90} results.

The clinical success reported by Blackwell (1991) may be due, in part, to this differential susceptibility to tea tree oil between BV- associated organisms and commensal lactobacilli. We demonstrated similar variation in susceptibility to tea tree oil previously between commensal skin flora, such as coagulase negative staphylococci, and transiently colonising potential pathogens, such as *Staphylococcus aureus* (Hammer *et al*., 1996). These differences may allow products to be formulated that will

selectively kill or inhibit certain organisms while having a minimal effect on others. The susceptibility data obtained in this work suggest that tea tree oil may be a useful treatment for BV and further investigation in the form of a controlled clinical trial is warranted.

9.0 Mechanism of action of tea tree oil

9.1 Introduction

A second area of research focussed on the mechanism of action of tea tree oil. No previous investigations reporting on this were available. Since the mechanism of action has implications for the selectivity and safety of antimicrobial agents, this issue was becoming increasingly important.

9.2 Results

9.2.1 Optical density of microbial suspensions treated with *Melaleuca alternifolia* oil or individual components

Treatment of *C. albicans* suspensions with MIC and 2xMIC of *M. alternifolia* oil did not reduce the optical density. Similarly, after two hours treatment with the MIC of terpinen-4-ol, the mean optical density of four separate replicates was 93% that of the original suspensions. Treatment with 2xMIC terpinen-4-ol resulted in modest reductions in the optical densities with a mean final density of 86% of the original densities. For α-terpineol, the optical densities of untreated and MIC treated *C. albicans* suspensions were not diminished.

Treatment of *E. coli* suspensions with MIC and 2xMIC of *M. alternifolia* oil, 1,8-cineole, terpinen-4-ol and α-terpineol reduced the OD_{620}. The only exception was terpinen-4-ol treatment at the MIC. Treatment with *M. alternifolia* oil at 2xMIC reduced the OD_{620} to approximately 30% of the original, irrespective of EDTA content. 1,8-Cineole treatment at MIC and 2xMIC also reduced the OD_{620} to this level but only in the presence of EDTA. In the absence of EDTA, the OD_{620} was reduced, but not to the same extent (53% and 42%, respectively). Terpinen-4-ol treatment at 2xMIC reduced the OD_{620} in the presence and absence of EDTA. MIC treatment with α-terpineol reduced the OD_{620} and EDTA addition did not enhance this effect. In contrast,

2xMIC treatment in the presence of EDTA resulted in a larger reduction in OD_{620} than in the absence.

The ODs_{620} of *S. aureus* suspensions were unaffected by MIC or 2xMIC treatment with *M. alternifolia* oil, 1,8-cineole, α-terpineol or terpinen-4-ol.

9.2.2 Leakage of 260nm-absorbing material from microbial suspensions treated with *Melaleuca alternifolia* oil or components

Treatment of *S. aureus* suspensions with 1,8-cineole at 0.1xMIC resulted in higher absorbances compared to the control suspensions. Furthermore, 0.5xMIC, MIC and 2xMIC treatments all resulted in the appearance of approximately twice the level of 260nm-absorbing material in the filtrates compared to the control. Terpinen-4-ol treatment at 0.5xMIC and MIC, resulted in approximately 3-fold and 4-fold absorbances at 5h compared to control filtrates. Filtrates from *S. aureus* suspensions treated with 0.5xMIC and MIC of -terpineol,

9.2.3 Electron microscopy

When examined by electron microscopy, no discernible differences in cell morphology were seen between *C. albicans* cells treated with terpinen-4-ol for up to 2h and untreated control cells. In contrast, examination of terpinen-4-ol treated *S. aureus* cells revealed some gross differences in morphology. Multi-lamellar, mesosome-like structures not seen in untreated *S. aureus* cells were seen in treated cells. Furthermore, these structures were more marked and appeared more frequently in cells treated for 10 min compared to those treated for 4 min. In addition, the contents of some cells appeared depleted and amorphous. There was no discernible differences between untreated *E. coli* cells and cells treated for 4 min. However, cells treated for 10 min contained several gross changes. Almost none contained the dark granules seen in control cells or cells treated for 4 min. Many of these cells appeared empty and vacuous with only the exterior cell membrane/wall structure remaining. In those cells that still had contents, they appeared monochromatic and amorphous. In some cases the contents had contracted away from the terminal ends of the cells.

9.3 Discussion

The lipophilic walls and membranes of micro-organisms constitute a prime target for the similarly-natured tea tree oil and its individual components. Consequently, the outer microbial membranes were the first site to be investigated in work to identify the mechanism of action of tea tree oil.

Some antimicrobial agents are capable of causing such gross membrane damage as to provoke whole cell lysis. In microbial suspensions treated with the agent of interest, this lysis is reflected in reductions in optical density. Treatment of *C. albicans* suspensions with tea tree oil or -terpineol did not significantly reduce the optical density over 2 hours while the higher concentration of terpinen-4-ol had a modest affect.

Treatment of *E. coli* suspensions with tea tree oil or components resulted in significant reductions in optical density. In some cases, the presence of the chelating agent EDTA further enhanced these reductions. EDTA chelates, or binds up, the cations present in the cell membrane causing the membrane to be destabilised. Hence its presence would enhance the action of a membrane-active substance. Collectively, these results suggested that the membrane was a site of action in *E. coli*.

None of the treatments affected the optical density of *S. aureus*_suspensions and this would suggest that action of the oils on the cell membrane so as to cause gross cell lysis, is not the prime mechanism of action in *S. aureus* cells.

The second manner in which the putative membrane action of tea tree oil and its components was examined was by observing the leakage of nucleic acids from treated cells. Nucleic acids are the building blocks of the genetic material, DNA and RNA, contained in cells. In healthy cells with an intact cell membrane, these macromolecules do not leak out of the cell in significant quantities. However, if the integrity of the membrane has been compromised then these molecules may leak from the cell into the surrounding fluid. These molecules may be detected by measuring the optical density at 260nm. While only *S. aureus* suspensions were examined in this manner, significant results were obtained. Treatment with MICs of α-terpineol, 1, 8-cineole, terpinen-4-ol and linalool all resulted in the appearance of 260nm-absorbing material outside the

cells. This suggests that genetic material is being lost from the cell through a damaged membrane. While the first series of experiments demonstrated that cell membranes were not being lysed by these treatment concentrations, this later work suggested that the membranes were being compromised.

Electron microscopy of terpinen-4-ol treated cells provided further information. *C. albicans* cells were not lysed by terpinen-4-ol treatment and by electron microscopy, appeared unaltered. In contrast, electron microscopy provided useful information about terpinen-4-ol-induced damage to *E. coli*. These cells had been sensitive to lysis by oil treatment and the appearance of empty "ghost" cells by electron microscopy confirmed this effect. The cytoplasm coagulation seen in *E. coli* cells is also suggestive of more subtle membrane damage which is sufficient to allow oils to penetrate but insufficient to cause whole cell lysis. Since cells observed by electron microscopy were treated with the MIC of terpinen-4-ol, these microscopic results correlate well with the failure of MIC of terpinen-4-ol to cause lysis. The appearance of mesosome-like structures in terpinen-4-ol treated *S. aureus* also suggests damage to the cell membrane or wall. As with *E. coli*, the otherwise intact nature of the *S. aureus* cells correlates well with the failure of terpinen-4-ol to cause lysis.

The original premise was that tea tree oil and/or its components act on the cell membrane or wall. These results indicate that this is a site of action. While further evidence is required to corroborate these observations, the possibility that other sites of action may exist, must be considered. Furthermore, it is possible that other components, not examined thus far, may render significant contributions to the antimicrobial activity of this oil.

10.0 Miscellaneous

10.1 Toxicity

In 1994, a paper was published in the *Journal of Toxicology – Clinical Toxicology* relating to a case of tea tree oil poisoning (Jacobs and Hornfeldt, 1994). This afforded us an opportunity to comment on this issue and our response was published soon after (Carson and Riley, 1995) and is reproduced below.

"Jacobs and Hornfeldt (1994) make several valid points in their recent article concerning *Melaleuca alternifolia* or tea tree oil poisoning. Given the widespread availability of an extensive range of tea tree oil products, the paucity of accurate toxicity data represents a serious problem. This essential oil which has useful antimicrobial activity is a complex mixture of approximately 100 components. Little is known of the toxicity of the individual components or the whole oil. At present the levels of two components are regulated by an Australian standard (AS 2782-1985) which stipulates that terpinen-4-ol, the putative antimicrobial component, must comprise at least 30% of the oil, while 1,8-cineole, reputedly a skin irritant, must not exceed 15%. While the antimicrobial activity of terpinen-4-ol has been confirmed, the role of 1,8-cineole is less clear (Carson and Riley, 1993). The lipophilic nature of tea tree oil and its ability to penetrate skin may potentiate its toxicity.

Apart from this report of oral toxicity in a 23-month-old boy, there have been three other cases of oral toxicity reported in the literature. In the first, dermatitis associated with the topical application of tea tree oil was exacerbated following ingestion of oil (de Groot and Weyland, 1992). In the second, half a teaspoon of tea tree oil was ingested by a 60-year-old man resulting in a "dramatic rash" and a general feeling of unwellness (Elliott, 1993). The patient had taken tea tree oil on a number of prior occasions with no noticeable ill-effects. In the third case, a patient was comatose for 12h and semi-conscious for a further 36h following ingestion of half a tea cup of neat tea tree oil (Seawright, 1993). No data regarding the oral toxicity of tea tree oil in humans are available although the acute oral toxicity in rats is 1.9-2.6 mL/kg (Altman, 1990). Tea tree oil toxicity has also been reported in dogs and cats (Villar *et al*., 1994) with the main symptoms including depression, weakness, incoordination and muscle tremors.

Contact dermatitis from tea tree oil is more frequently reported although accurate estimates of its incidence are still required (Apted, 1991; de Groot and Weyland, 1992; de Groot and Weyland, 1993; Moss, 1994; Selvaag *et al*., 1994; Van der Valk *et al*., 1994). In most reports, patch tests with the major components of the oil were not performed. In the two cases where patch tests with 1,8-cineole were performed in an attempt to identify the allergen, the results were conflicting (de Groot and Weyland, 1992; Selvaag *et al*., 1994).

The limited toxicity data and few well-controlled studies that are currently available require corroboration urgently. Since clinical presentation of toxicity associated with tea tree oil products may become more common following the current interest in natural products, it is imperative that our clinical knowledge of this essential oil and its related products progresses accordingly."

10.2 Podiatry

In 1997, a paper was published in the *Journal of British Podiatric Medicine* on the use of tea tree oil as adjunctive therapy. This also allowed us to comment on some applications of tea tree oil.

"Rodger (1997) makes some interesting points about the use of tea tree oil as adjunct therapy in chronic mucocutaneous candidiasis. Tea tree oil is widely available in the United Kingdom, in products such as creams, toothpastes, soaps and antiseptic lotions. The oil is produced by steam distillation of the leaves of the Australian native plant *Melaleuca alternifolia* and is composed of approximately 100 individual components (Carson and Riley, 1993).

Tea tree oil is known to inhibit a wide range of fungi in vitro including *Aspergillus niger*, *A. flavus*, *Epidermophyton floccosum*, *Microsporum audonii*, *M. canis*, *Trichophyton mentagrophytes* and *T. rubrum* (Walker, 1972), however, few clinical trials have examined the therapeutic efficacy of tea tree oil in cutaneous fungal infections (Tong *et al*., 1992; Buck *et al*., 1994). Also, the commensal yeast *Malassezia furfur* (*Pityrosporum ovale*) is also inhibited *in vitro* suggesting a possible role for tea tree oil in the treatment of pityriasis versicolor (Hammer *et al*., 1996). Tea tree oil and several of its major components have also been shown to inhibit the growth of *Candida albicans* (Carson and Riley, 1993; Carson and Riley, 1995) and it is this activity which may have contributed to the successful outcome reported by Rodger (1997).

Apart from inhibiting the growth of *C. albicans*, tea tree oil is known to inhibit a wide range of organisms which may otherwise result in secondary infections such as *Staphylococcus aureus* and *Streptococcus* spp (Walker, 1972; Carson and Riley, 1993).

It has also been shown to inhibit many transient skin organisms at concentrations lower than those which inhibit commensal skin flora (Hammer *et al*., 1996). It is possible that this differential inhibition may reduce disturbance of the patient's commensal skin flora and, in turn, enhance the protection offered by these commensal flora against colonisation by other transient flora. Further work is required to examine this possibility.

Although there is no scientific evidence as yet, anecdotal evidence suggests that tea tree oil has mild analgesic properties and this is further supported by Rodger's observations. In addition, the lipophilic nature of tea tree oil and the ability of some of its components to penetrate skin (Obaata *et al*., 1991) may potentiate the antimicrobial activity and result in a residual antimicrobial effect of the oil in the skin.

The in vitro antibacterial activity of tea tree oil is becoming well-established and investigations into the antifungal activity are beginning. In contrast, too few clinical trials have demonstrated the efficacy of tea tree oil in vivo and much further work on this potentially useful natural product is required."

11.0 Implications, recommendations and intellectual property

11.1 Implications

It is difficult to measure the impact of our research on the tea tree oil industry in Australia. Although not directly assessable, several parameters give an indication of the impact of our work.

First, the significant number of interviews and articles provided for the popular press, both in Australia and overseas, has undoubtedly increased public awareness of tea tree oil and tea tree oil products. Publicity regarding the scientific proof that tea tree oil "works" directly enhances the profile and marketability of the oil.

Second, the publication of much of this project in peer-reviewed medical journals has had a significant and profound impact on the industry in Australia. This is evident by a number of effects. Publication has stimulated interest in the antimicrobial properties of tea tree oil in international medical and scientific fora. One practical way in which this has been manifested is the development of several collaborative projects between the Project Team and other research groups including the Laboratory for Hospital Infection at the Central Public Health Laboratory and a group at St. Mary's Hospital at Imperial College both in London. Numerous requests (~200) from other researchers for reprints of our publications, also indicate that interest in the applications of tea tree oil has been stimulated. Additional investigation into, and verification of, the potential uses of tea tree oil will further strengthen the industry.

Another way in which publications indicate the impact of this project, is the citation of these publications by other authors. During the course of the project, several publications by other researchers regarding the antimicrobial activity of tea tree oil have appeared and the results of this project have been cited (Jacobs & Hornfeldt, 1994; Raman *et al.*, 1995; Nenoff *et al*., 1996; Boon & Johnstone, 1997; Buchbauer, 1997; Faoagali *et al*., 1997; Hili *et al*., 1997; Nelson, 1997; Southwell *et al*., 1997). Furthermore, the work conducted by these independent investigators corroborates, to a large extent, the results of this project.

Publications have also allowed the Project Team to clarify or elaborate on tea tree oil work from other research groups. This has been done on at least two occasions (Carson and Riley, 1995; Carson *et al*., 1997) and, apart from sustaining scientific interest in tea tree oil, permits misconceptions or inaccuracies to be addressed.

Members of the Project Team have been asked to review research conducted by other workers prior to acceptance for publication in medical and scientific journals and this highlights the level of expertise attained by the group. More important, in an industry

that will soon come under increasing international competition, it helps consolidate the impression of Australia as the leader in the tea tree oil industry.

Third, the impact of our work can be measured by the degree of interest shown in the project results by companies in the tea tree oil industry. A significant number of reprints of our publications have been purchased by several tea tree oil companies, presumably for distribution to clients.

In addition, results of our studies are cited frequently on tea tree oil company world wide web sites, indicating that producers and distributors consider the project results beneficial in the marketing of tea tree oil and tea tree oil products.

Finally, as part of the on-going attempts to register tea tree oil as an active antimicrobial ingredient with the FDA in the United States, copies of our publications have been added to the previous submissions. Our data have also been presented in submissions to other national regulatory bodies, including those in Sweden and Germany.

11.2 Recommendations

The antimicrobial activity of tea tree oil characterised in this project indicates clearly that tea tree oil has the potential to become a clinically useful product. The in vitro data suggest that tea tree oil products may have efficacy in the treatment of many cutaneous skin infections. These data also provide a sound basis from which to proceed to in vivo work. What is required now is in vivo data from well-designed, randomised, controlled clinical trials.

11.3 Intellectual Property

Apart from the impact of the project upon the profile and marketability of tea tree oil, there are no commercially significant developments arising from the project.

12.0 Communications Strategy

Since one of the aims of the project was to address the paucity of information available in the literature, a substantial effort was put into publishing the results of the project in

peer-reviewed medical and scientific journals. A significant effort also went in to presenting project results at scientific and industry conferences as well as in the popular press.

Results were published as they became available. As a result of the project, the following papers have been submitted to or published in peer-review journals:

Carson, C. F. and Riley, T. V. (1995) Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. *Journal of Applied Bacteriology* **78:**264-269.

Carson, C. F., Cookson, B. D., Farrelly, H. D. and Riley, T. V. (1995) Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia*. *Journal of Antimicrobial Chemotherapy* **35:**421-424.

Carson, C. F. and Riley, T. V. (1995) Toxicity of the essential oil of *Melaleuca alternifolia* or tea tree oil (Letter). *Journal of Toxicology - Clinical Toxicology* **33:**193-194.

Carson, C. F., Hammer, K. A. and Riley, T. V. (1995) Broth micro-dilution method for determining the susceptibility of *Escherichia coli* and *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Microbios* **82:**181-185.

Carson, C. F., Hammer, K. A. and Riley, T. V. (1996) *In vitro* activity of the essential oil of *Melaleuca alternifolia* against *Streptococcus* spp. (Letter). *Journal of Antimicrobial Chemotherapy* **37:**1177-1178.

Hammer, K .A., Carson, C. F. and Riley, T. V. (1996) Susceptibility of transient and commensal skin flora to the essential oil of *Melaleuca alternifolia* (tea tree oil). *American Journal of Infection Control* **24:**186-189**.**

Carson, C. F., Hammer, K. A. and Riley, T. V. (1997) Use of the essential oil of *Melaleuca alternifolia* (tea tree oil) in cutaneous fungal infections (Letter). *Journal of British Podiatric Medicine* **52:**iv-v.

Hammer, K .A., Carson, C. F. and Riley, T. V. In vitro susceptibility of lactobacilli and organisms associated with bacterial vaginosis to *Melaleuca alternifolia* (tea tree) oil. **Submitted for publication**

There are two more manuscripts in preparation; one discussing the interaction of tea tree oil with various product excipients and the other detailing the mechanism of action work.

In addition to the above work, that was supported directly by RIRDC, two other publications on tea tree oil were produced by members of our laboratory:

Hammer, K .A., Carson, C. F. and Riley, T. V. In vitro susceptibility of *Malassezia furfur* to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Medical and Veterinary Mycology* **35:**375-377.

Hammer, K .A., Carson, C. F. and Riley, T. V. In vitro activity of *Melaleuca alternifolia* (tea tree) oil, intra-vaginal tea tree oil products and other essential oils against *Candida* spp. S**ubmitted for publication**

As well as papers published in medical and scientific journals, several oral and poster presentations were published as conference abstracts. These were as follows:

Carson, C. F. (1995) Evaluating the antibacterial activity of the essential oil of *Melaleuca alternifolia* and its major components. Proceedings of the National Conference on Tea Tree Oil, 24-25 August 1995, Sydney, Australia.

Carson, C. F. (1996) Potential uses of *Melaleuca alternifolia* oil – the acne removal company. Proceedings of the National Conference on Tea Tree Oil, 26 October 1996, Sydney, Australia.

Carson, C. F. and Riley, T. V. (1996) Working with and against tea tree oil – issues of synergy and antagonism. Proceedings of the 19th IFSCC Congress, Sydney, October 1996.

Hammer, K. A., Carson, C. F. and Riley, T. V. (1995) Susceptibility of transient and commensal flora to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Microbiology Australia* **16:** P30.4.

Riley, T. V. (1995) The anti-staphylococcal activity of tea tree oil and other research opportunities. Proceedings of the National Conference on Tea Tree Oil, 24-25 August 1995, Sydney, Australia.

Riley, T. V. (1996) Antiseptic properties of tea tree oil and MRSA. Proceedings of the National Conference on Tea Tree Oil, 26 October 1996, Sydney, Australia.

In addition, the following abstracts were submitted for presentation at the annual meeting of the Australian Society for Microbiology held in Adelaide in September, 1997:

Carson, C. F. and Riley, T. V. Effect of the essential oil of *Melaleuca alternifolia* (tea tree oil) on bacterial and yeast cell membranes.

Hammer, K. A., Carson, C. F. and Riley, T. V. Effect of organic matter, surfactants and cations on the antimicrobial activity of *Melaleuca alternifolia* (tea tree) oil.

Other talks on aspects of the work with tea tree oil were as follows:

- A/Prof Riley presented results of the group's work at the Australian Society for Microbiology (WA Branch) Country Scientific Weekend Meeting in May, 1996.
- A talk entitled "Tea tree and golden staph" was delivered by Ms Christine Carson at the July 1995 meeting of the Western Australian Essential Oils forum.
- Ms Christine F. Carson presented a talk entitled "Tea tree oil and product formulations" to the Bacteriology Research Group in the Department of Microbiology in November, 1994 .
- Ms C Carson delivered a lecture entitled "The antimicrobial activity of tea tree oil" to the West Australian branch of the Australian Society for Microbiology in December, 1994.

Throughout the course of the project numerous interviews for newspaper, radio and television were conducted. They are listed below.

- Television interviews for Australian evening news' programmes were conducted in October 1994.

- A/Prof Thomas V. Riley addressed several international television and newspaper groups and tea tree oil industry representatives in November 1994.
- Television interviews for Australian evening news' programmes were conducted in January 1995.
- An article describing aspects of our work with tea tree oil appeared in *The West Australian* (21/1/95) and resulted in many enquiries.
- An article entitled "Taking the 'quackery' out of tea tree oil" appeared in June 1995 issue of *Agriculture Today* and resulted in several enquiries from primary producers.
- The tea tree oil research group was featured on the ABC's programme, "The 7.30 Report" (28/6/95). This report discussed the susceptibility of "golden staph" to tea tree oil and resulted in several enquiries from the public and other media groups.
- An article discussing our work with tea tree oil appeared in the *Albany Advertiser* in July 1995.
- A/Prof Riley was interviewed for radio and newspaper articles in September 1995 at the annual Microbiology conference in Canberra.
- A radio interview discussing the recent findings about tea tree oil was given for the Victorian ABC on October 5, 1995 by Ms Carson.
- *GEO* magazine prepared a story regarding tea tree oil and our work in November 1995.
- On December 2 1995, a group of Scandinavian journalists and medical practitioners who had been brought to Australia by Australian Bodycare Pty. Ltd., visited our research group for a series of lectures on the properties of tea tree oil and the results of our work. Several interviews were prepared for national television and radio in Sweden and Denmark.
- A radio interview was conducted by Ms Carson on Thursday 7 March for Radio Australia (Melbourne).
- An article discussing our work with tea tree oil in the treatment of vaginal thrush appeared in the March edition of *Australian Pharmacist* (**15:**104,109).
- A series of lectures and interviews on tea tree oil were given to a group of journalists and medical practitioners from Denmark and the Czech Republic on 27 March 1996. The group had been brought to Australia by the Bodycare Corporation Pty. Ltd.

- *The West Australian* (3 April 1996) published a story about our work with tea tree oil.
- A radio interview was conducted by Ms Carson on Thursday 11 April for ABC Regional Radio (WA).
- A radio interview was conducted by Ms Carson on Thursday 9 May for 6NR (national).
- On Wednesday 29 May 1996, an article appeared in *The West Australian* newspaper outlining A/Prof Riley's research interests including the work on tea tree oil.
- In June 1996, Assoc/Prof Riley and Ms Carson visited the United Kingdom and met with representatives from several British hospitals regarding tea tree oil and MRSA and discussed collaborative clinical trials.
- Several interviews with UK print media journalists were conducted during the June 1996 visit. As a result, a multi-page supplement about tea tree oil was published by the *Daily Mail* newspaper. This paper has a circulation of approximately 1 million. Finally, scientific presentations were made in Copenhagen for a range of journalists and medical professionals.
- Ms Carson was interviewed on Tuesday 18 June for ABC 6WF (WA).
- On Thursday 27 June, Ms Carson was interviewed for 6PR (WA).
- An article discussing our work appeared in the June/August edition of *Earth Garden*
- *You* magazine published a piece on our work in the 14 July, 1996 issue.
- Interviews for the BBC television programme "Tomorrow's World" were conducted with Assoc/Prof Riley and Ms Carson on 1 and 3 August 1996 and a segment dealing with tea tree oil and methicillin-resistant *Staphylococcus aureus* was aired in the United Kingdom in early October. Subsequently, approximately 100 enquiries regarding tea tree oil were received from the UK, which have resulted in collaborative research.
- On Saturday 1 February 1997, an article appeared in the *West Australian* newspaper outlining our work on tea tree oil, in particular with MRSA.
- A/Prof Riley was interviewed for a South African magazine, *Fair Lady*, and an article was published in May, 1997.
- A/Prof Riley gave several interviews in Poland in May 1997.
- Ms Christine Carson was invited to speak at a by the WA Venereology Society seminar on June 8 1997.

13.0 References

Altman PM. Australian tea tree oil. *Aust J Pharm* 1988;69: 276-278.

Altman PM. Summary of safety studies concerning Australian tea tree oil. In *Modern Phytotherapy - The Clinical Significance of Tree Oil and other Essential Oils.* Proceedings of a Conference on December 1-2, 1990 in Sydney and a Symposium on December 8, 1990 in Surfers Paradise, II, 21-22.

Apted JH. Contact dermatitis associated with the use of tea tree oil (Letter). *Australas J Dermatol* 1991;32: 177.

Atkinson N, Brice HE. Antibacterial substances produced by flowering plants. 2. The antibacterial action of essential oils from some Australian plants. *Aust J Exp Biol 1995*;33: 547-554.

Balows A, ed. *Manual of Clinical Microbiology*. (5th ed.) American Society for Microbiology, Washington D.C. 1991.

Barnett BO, Frieden IJ. Streptococcal skin diseases in children. *Sem Dermatol* 1992;11: 3-10.

Bassett IB, Pannowitz DL, Barnetson RStC. A comparative study of tea tree oil versus benzoylperoxide in the treatment of acne. *Med J Aust* 1990;153: 455-458.

Belaiche P. Traitement des infections vaginales a *Candida albicans* par l'huile essentielle de *Melaleuca alternifolia* Cheel. *Phytother* 1985;15: 13-14.

Beylier MF. Bacteriostatic activity of some Australian essential oils. *Perfum Flavorist* 1979;4: 23-25.

Blackwell AL. Tea tree oil and anaerobic (bacterial) vaginosis.(Letter) *Lancet* 1990;337: 300.

Boon P, Johnstone L. Organic matter decay in coastal wetlands: an inhibitory role for essential oil from *Melaleuca alternifolia* leaves? *Arch Hydrobiol* 1997;138: 433-449.

Brophy JJ, Davies NW, Southwell IA, Stiff IA, Williams LR. Gas chromatographic quality control for oil of *Melaleuca* terpinen-4-ol type (Australian tea tree). *J Ag Food Chem* 1989; 37: 1330-1335.

Buchbauer G. Über das teebaumöl. *Eurocosmetics* 1997 i: 21-24.

Buck DS, Nidorf DM, Addino JG. Comparison of two topical preparations for the treatment of onychomycosis: *Melaleuca alternifolia* (tea tree) oil and clotrimazole. *J Fam Pract* 1994;38: 601-605.

Carson CF, Hammer KA, Riley T V. In vitro activity of the essential oil of *Melaleuca alternifolia* against *Streptococcus* spp. *J Antimicrob Chemother 1996;*37: 1177-1178.

Carson CF, Riley TV. Antimicrobial activity of the essential oil of *Melaleuca alternifolia*. *Letts Appl Microbiol* 1993;16: 49-55.

Carson CF, Riley TV. Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. *J Appl Bacteriol* 1995;78: 264-269.

Carson CF, Cookson BD, Farrelly HD, Riley TV. Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of Melaleuca alternifolia. *J Antimicrob Chemother* 1995;35: 421-424.

Carson CF, Hammer KA, Riley TV. Broth micro-dilution method for determining the susceptibility of *Escherichia coli* and *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Microbios* 1995;82: 181-185.

Carson CF, Riley TV. The antimicrobial activity of tea tree oil. *Med J Aust* 1994a;160: 236.

Carson CF, Riley TV. Susceptibility of *Propionibacterium acnes* to the essential oil of *Melaleuca alternifolia*. *Letts Appl Microbiol* 1994b;19: 24-25.

Carson CF, Riley TV. Toxicity of the essential oil of *Melaleuca alternifolia* or tea tree oil. (Letter). *J Toxicol Clin Toxicol* 1995;33: 193-194.

Carson CF, Hammer KA, Riley TV. Use of the essential oil of *Melaleuca alternifolia* (tea tree oil) in cutaneous fungal infections. (Letter). *J Brit Pod Med* 1997;52: iv-v.

Cook RL, Redondo-Lopez V, Schmitt C, Meriwether C, Sobel JL. Clinical, microbiological and biochemical factors in recurrent bacterial vaginosis. *J Clin Microbiol* 1992;30: 870-877.

Cruz T, Cabo MP, Cabo MM, Jimenez J, Cabo J, Ruiz C. *In vitro* effect of the essential oil of *Thymus longiflorus* Boiss. *Microbios* 1989;60: 59-61.

de Groot AC, Weyland JW. Contact allergy to tea tree oil. (Letter). *Contact Dermatitis* 1993;28: 309.

de Groot AC, Weyland JW. Systemic contact dermatitis from tea tree oil. *Contact Dermatitis* 1992;27: 279-280.

Dyke KGH, Curnock SP, Golding M,Noble WC. Cloning of the gene conferring resistance to mupirocin in *Staphylococcus aureus*. *FEMS Microbiol Letts* 1991;77: 195-198.

Elliott C. Tea tree oil poisoning. *Med J Aust* 1993;159: 830-831.

Faoagali J, George N, Leditschke JF. Does tea tree oil have a place in the topical treatment of burns? *Burns* 1997;23: 349-351.

Feinblatt HM. Cajeput-type oil for the treatment of furunculosis. *J Natl Med Assoc* 1960;52: 32-34.

Hammer KA, Carson CF, Riley TV. In vitro susceptibility of *Malassezia furfur* to the essential oil of *Melaleuca alternifolia* (tea tree oil). Proceedings of the 19th International Federation of the Societies of Cosmetic Chemists Congress, Sydney, Australia, 1996.

Hammer KA, Carson CF, Riley TV. Susceptibility of transient and commensal skin flora to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Amer J Infect Cont* 1996;24: 186-189.

Hammer KA, Carson CF, Riley TV. Effect of organic matter, surfactants and cations on the antimicrobial activity of *Melaleuca alternifolia* (tea tree) oil. Australian Society for Microbiology Annual Scientific Meeting and Exhibition, Adelaide 1997. Abstract P02.49, p. A111.

Hayes AJ, Markham JL, Leach DN, Southwell IA. Relationship between chemical composition and antimicrobial activity of Australian tea tree oil. Australian Society for Microbiology, Melbourne, Australia. In *Program and Abstracts of the Annual Scientific Meeting of the Australian Society for Microbiology, Perth Western Australia*, 1993.. Abstract P2.2, p. A-4.

Humphery EM. A new Australian germicide. *Med J Aust* 1930;1: 417-418.

Jacobs MR, Hornfeldt CS. *Melaleuca* oil poisoning. *J Toxicol Clin Toxicol* 1994;32: 461-464.

Janssen DA, Zarins LT, Schaberg DR, Bradley SF, Terpenning MS, Kauffman CA. Detection and characterisation of mupirocin resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1993;37: 2003-2006.

Joesoef MR, Schmid GP. Bacterial vaginosis: review of treatment options and potential clinical indications for therapy. *Clin Infect Dis* 1995;20(Suppl 1): S72-79.

Kauffman CA, Terpenning MS, Xiaogong H, Zarins LT, Ramsey MA, Jorgensen KA. *et al.* Attempts to eradicate methicillin resistant *Staphylococcus aureus* from a long term care facility with the use of mupirocin ointment. *Amer J Med* 1993;94**:** 371-8.

Kerr S, Kerr GE, MacKintosh CA, Marples RR. A survey of methicillin-resistant *Staphylococcus aureus* affecting patients in England and Wales. *J Hosp Infect* 1990;16: 35-48.

Knight TE, Hausen BM. Melaleuca oil (tea tree oil) dermatitis. *J Amer Acad Dermatol* 1994;30: 423-427.

Kristofferson SS, Atkin PA, Shenfield GM. Uptake of alternative medicine. *Lancet* 1996;347: 972.

Lassak EV, McCarthy T. *Australian Medicinal Plants*. Sydney : Methuen.1983.

Low D, Rawal BD, Griffin WJ. Antibacterial action of the essential oils of some Australian Myrtaceae with special references to the activity of chromatographic fractions of oil of *Eucalyptus citriodora*. *Planta Medica* 1974;26**:** 184-189.

Maple PAC, Hamilton-Miller JMT, Brumfitt W. Comparison of the in-vitro activities of the topical antimicrobials azelaic acid, nitrofurazone, silver sulphadiazine and mupirocin against methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 1992;29: 661-8.

Maruzzella JC, Chiaramonte JS, Garofalo MM. Effects of vapors of aromatic chemicals on fungi. *J Pharmaceut Sci* 1961;50: 665-668.

McKern HHG, Parnell IW. The larvicidal effect of various chemical compounds and plant products on the free-living stages of *Haemonchus contortus* Rud. (Nematoda). *J Helminthol* 1964;38: 223-244.

Moss A. Tea tree oil poisoning. *Med J Aust* 1994;160: 236.

Naguib MH, Naguib MT, Flournoy DJ. Mupirocin resistance in methicillin resistant *Staphylococcus aureus* from a veterans hospital. *Chemother* 1993;39: 400-4.

National Committee for Clinical and Laboratory Standards. Methods for dilution, antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard M7-A4. NCCLS, Wayne, PA. 1997.

National Committee for Clinical Laboratory Standards. . Methods for antimicrobial susceptibility testing of aerobic bacteria. M7-A2 (2nd ed.) NCCLS, Villanova, PA. 1990

Nenoff P, Haustein U–F, Brandt W. Antifungal activity of the essential oil of *Melaleuca alternifolia* (tea tree oil) against pathogenic fungi in vitro. *Skin Pharmacol* 1996;9: 388-394.

Nyirjesy P, Weitz MV, Grody MHT,. Lorber B. Over-the-counter and alternative medicines in the treatment of chronic vaginal symptoms. *Obstet Gynecol* 1997;90: 50-53.

Obata Y, Takayama K, Machida Y, Nagai T, Combined effect of cyclic monoterpenes and ethanol on percutaneous absorption of diclofenac sodium. *Drug Design and Delivery* 1991;8: 137-144.

Onawunmi GO, Yisak W-A, Ogunlana EO. Antibacterial constituents in the essential oil of *Cymbopogon citratus* (DC.) Stapf. *J Ethnopharmacol* 1984;12: 279-286.

Peña EF. *Melaleuca alternifolia* oil. Its use for trichomonal vaginitis and other vaginal infections. *Obstet Gynecol* 1962;19: 793-795.

Penfold AR, Grant R. The germicidal values of some Australian essential oils and their pure constituents. *J Proc Royal Soc NSW* 1925;60: 167-70.

Penfold AR, Grant R. The germicidal values of some Australian essential oils and their pure constituents. Together with those for some essential oil isolates, and synthetics. Part III. *Journal and Proceedings of the Royal Society of New South Wales* 1925;59: 346-350.

Penfold AR, Morrison FR. Some notes on the essential oil of *Melaleuca alternifolia*. *Australas J Pharm* 1937;18: 274-275.

Rahman M, Noble WC, Cookson B. Mupirocin-resistant *Staphylococcus aureus*. *Lancet* 1987;2: 387.

Raman A, Weir U, Bloomfield SF. Antimicrobial effects of tea-tree oil and its major components on *Staphylococcus aureus*, *Staph. epidermidis* and *Propionibacterium acnes*. *Letts Appl Microbiol* 1995;21: 242-245.

Reuveni R, Fleischer A, Putievsky E. Fungistatic activity of essential oils from *Ocimum basilicum* chemotypes. *Phytopathologische Zeitschrift* 1984;110: 20-22.

Reynolds JEF. (Ed) (1993) *Martindale - the extra pharmacopoeia*, 30th edn, pp. 1385. The Pharmaceutical Press, London.

Riley TV, Carson CF, Bowman RA, Mulgrave L, Golledge CL, Pearman JW, Grubb WB. Mupirocin-resistant methicillin-resistant *Staphylococcus aureus* in Western Australia. *Med J Aust* 1994;161: 397-398.

Rodger E. ‘Chronic mucocutaneous candidiasis - a case study’. *J Brit Pod Med* 1997;52: 9-10.

Ross SA, El-Keltawi NE, Megalla SE. Antimicrobial activity of some Egyptian aromatic plants. *Fitoterapia* 1980;51: 201-205.

Seawright A. Comment: Tea tree oil poisoning. *Med J Aust* 1993;159: 831.

Selvaag E, Erikson B, Thune P. Contact allergy to tea tree oil and cross-sensitisation to colophony. *Contact Dermatitis* 1994;31:124-125.

Shapiro S, Meier A, Guggenheim B. The antimicrobial activity of essential oils and essential oil components towards oral bacteria. *Oral Microbiol Immunol* 1994;9: 202-208.

Shemesh A, Mayo WL. Australian tea tree oil: a natural antiseptic and fungicidal agent. *Aust J Pharm* 1991;72: 802-3.

Shriner DL, Schwartz RA, Janniger CK. Impetigo. *Cutis* 1995;56: 30-32.

Söderberg TA, Johansson A, Gref R. Toxic effects of some conifer resin acids and tea tree oil on human epithelial and fibroblast cells. *Toxicol* 1996;107**:** 99-109.

Southwell IA, Freeman S, Rubel D. Skin irritancy of tea tree oil. *J Essent Oil Research* 1997;9: 47-52.

Standards Association of Australia. Essential oils - oils of *Melaleuca*, terpinen-4-ol type. Standards Australia, Sydney, 1985;2782.

Summanen P, Baron EJ, Citron DM, Strong CA., Wexler HM, Finegold SM. (1993) Wadsworth Anaerobic Bacteriology Manual 5th ed. Star Publishing Company, Belmont, California.

Swords G, Hunter GLK. Composition of Australian tea tree oil (*Melaleuca alternifolia*). *J Ag Food Chemistry* 1978;26: 734-737.

Tong MM, Altman PM, Barnetson RStC, ‘Tea tree oil in the treatment of tinea pedis’. *Australas J Dermatol* 1992;33: 145-149.

Udo EE, Pearman, JW, Grubb WB. Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. *J Hosp Infect* 1993;25: 97-108.

Udo EE, Pearman JW, Grubb WB. Emergence of high-level mupirocin resistance in methicillin-resistant *Staphylococcus aureus* in Western Australia. *J Hosp Infect* 1994;26: 101-9.

Van der Valk PGM, de Groot AC, Bruynzeel DP, Coenraads PJ, Weijland JW. Allergic contact dermatitis from 'tea tree' oil. *Ned Tijdschr Geneeskd* 1994;138: 823-825.

Villar D, Knight MJ, Hansen SR, Buck WB. Toxicity of *Melaleuca* oil and related essential oils applied topically on dogs and cats. *Vet Human Toxicol* 1994;36: 139-142.

Walker M, ‘Clinical investigation of Australian *Melaleuca alternifolia* oil for a variety of common foot problems’. *Curr Pod* 1972;April: 7-15.

Walsh LJ, Longstaff J. The antimicrobial effects of an essential oil on selected oral pathogens. *Periodontol* 1987;8: 11-15.

Williams LR, Home VN, Asre S. Antimicrobial activity of oil of *Melaleuca* (tea tree oil). Its potential use in cosmetics and toiletries. *Cosmetics, Aerosols and Toiletries in Australia* 1990;4: 12-3, 16-8, 22.

Williams LR, Home VN. (1989) Plantations of *Melaleuca alternifolia* - a revitalized Australian tea tree industry. *Proceedings of the 11th International Congress of Essential Oils, Fragrances and Flavours* 49-53.