Candidosis Management : Antifungal, Cytotoxic and Immunomodulatory Properties of Tea tree Oil and its Derivative Components

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ABBREVIATIONS

AIDS:	Acquired immuno-deficiency syndrome
ALS:	Agglutinin-like sequence
AMB:	Amphotericin B
ANOVA:	Analysis of variance
APECED:	Autoimmune polyendocrinopathy-candidiasis-ectodermal Dystrophy
BPE:	Bovine pituitary extract
BSA:	Bovine serum albumin
cDNA:	Complementary deoxyribonucleic acid
CHX:	Chlorhexidine gluconate
CLSI:	Clinical Laboratory Standards Institute
CSP:	Caspofungin
dH_2O:	Distilled water
dNTP:	Deoxynucleoside triphosphate mixture (A,T,G,C)
DMEM:	Dulbecco's modified Eagles medium
DMSO:	Dimethyl sulphoxide
DNA:	Deoxyribonucleic acid
DTT:	1,4-Dithiothreitol
ECM:	Extracellular matrix
EDTA:	Ethylenediaminetetraacetic acid
EGF:	Epidermal growth factor
ELISA:	Enzyme-linked immunosorbent assay
EPS:	Exopolymeric substance
FCS:	Foetal calf serum
g:	relative centrifugal force
G-CSF:	Granulocyte colony-stimulating factor-3
GM-CSF:	Granulocyte-macrophages colony-stimulating factor
GRO-α:	Growth-regulated oncogene CXCL-1
HBSS:	Hanks' balanced salt solution
HIV:	Human immunodeficiency virus
HRP:	Horseradish peroxidase
IC_{50}:	50% Inhibitory concentration
IFN-γ:	Interferon gamma
IL:	Interleukin
IL-1ra:	Interleukin-1 receptor antagonist
ISO:	International standards organization

KSFM:	Keratinocyte serum-free medium
KSFMT:	Keratinocyte serum-free medium with 0.25% Tween®80
LD_{50}:	Lethal dose 50%
mg:	milligram
ml:	millilitre
MIC:	Minimal inhibitory concentration
MIF:	Macrophage migration inhibitory factor
OKF6-TERT2:	Oral mucosal immortalised keratinocyte cell line
OPC:	Oropharyngeal candidosis
PBMC:	Peripheral blood mononuclear cells
PHA:	Phytohaemagglutinin
PMMA:	Polymethylmethacrylate
PMNL:	Polymorphonuclear leukocytes
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PMIC:	Minimal inhibitory concentration of planktonic cells
PRD:	Periradicular disease
RNA:	Ribonucleic acid
rpm:	Revolutions per minute
RPMI:	RPMI 1640 medium
RT-PCR:	Reverse transcriptase polymerase chain reaction
SAB:	Sabouraud dextrose agar
SCCP:	Scientific Committee on Consumer Products
SEM:	Scanning electron microscopy
SMIC:	Minimal inhibitory concentration of sessile cells
spp.:	Species
TNF-α:	Tumour necrosis factor alpha
TTO:	Tea tree oil
T4-ol:	Terpinen-4-ol
TMB:	3,3′,5,5’-tetra-methylbenzidine
Tween®:	Polyoxyethylene sorbitan monooleate
VRZ:	Voriconazole
v/v:	Volume / volume
w/v:	Weight / volume
XTT:	2,3 bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide
YPD:	Yeast peptone dextrose broth
zym:	Zymosan A from *Saccharomyces cerevisiae* cell wall

Declaration of originality

This thesis is the original work of the author unless otherwise stated.

Steven Milligan, November 2010

SUMMARY

Oropharyngeal candidosis (OPC) is a common opportunistic yeast infection in elderly and immuno-compromised populations, caused by *Candida albicans* and other *Candida* spp. forming biofilms on the oral epithelium or artificial denture surfaces. Oral thrush (pseudomembranous candidosis) is the most common type of OPC occurring when a biofilm grows on oral mucosal surfaces, while growth on dentures commonly causes denture stomatitis in denture-wearers. OPC causes significant morbidity with symptoms including inflammation, pain, burning, eating difficulties and alteration of taste sensation. Conventional antifungal treatments have limited success due to biofilm resistance mechanisms, with recurring infections promoting development of azole resistance. Other problems with current antifungal drugs include toxicity, drug interactions and unpleasant taste. Therefore, alternative methods for prophylactic or therapeutic management of C*andida* spp*.* biofilms are desirable.

This study aimed firstly to evaluate the efficacy of tea tree oil (TTO) and its derivatives against biofilms formed by a clinically-diverse panel of *C. albicans* isolates; and secondly to assess the toxicological effects of TTO exposure using a clinically relevant oral cell line. Thirdly, this study aimed to further investigate previously reported anti-inflammatory effects of TTO.

TTO is a complex mixture of essential oils; however, individual components of TTO are commercially available. TTO has broad spectrum antimicrobial activity and TTO oral products are currently available. However, evidence for antifungal efficacy is limited and there are concerns regarding safety of long-term use of TTO products.

The data presented demonstrate TTO and its derivatives are effective antifungal agents. Minimal inhibitory concentrations (MIC) of TTO and seven components

were determined for planktonic *C. albicans* cells (PMIC) using the standard CLSI dilution technique. The $PMIC_{50}$ value for TTO was 0.5%, with lower values for two components - 0.25% for both terpinen-4-ol (T4-ol) and *α*-terpineol. Growth of all 100 strains was inhibited by 1% TTO, 0.5% terpinen-4-ol and 0.5% *α*-terpineol. A pilot study found no decrease in TTO sensitivity with multiple TTO exposure. Sessile susceptibilities (SMFC) were determined using a metabolic assay on *C. albicans* cells after 24 h treatment of pre-formed biofilms, to determine the most effective anti-biofilm components. T4-ol and *α*-terpineol were potent biofilm inhibitors, which could inhibit biofilm metabolism by 50% at $PMIC_{50}$ concentrations ($SMFC_{50}$ = 0.25%), exhibiting significantly greater anti-biofilm activity than TTO ($SMFC_{50}$ = 1%). Strains isolated from different patient groups had similar biofilm susceptibilities. Other components tested had little effect on biofilm metabolism ($SMFC_{50}$ of 2% to >4%). Shorter treatments modelling a 'mouthwash' exposure time produced moderate inhibition (50%) of pre-formed biofilm metabolism after 2 min in 1% *α*-terpineol, while longer exposures with 1% T4-ol (15 min) and 2% TTO (60 min) were required to give this level of inhibition. A time-dependent treatment effect for TTO and the single components was also seen at these concentrations, with longer exposures giving better inhibition of biofilm metabolism.

Inhibition of biofilm formation and morphogenesis was also investigated to define effective components, concentrations and exposure times for prophylactic use. Presence of TTO, T4-ol or *α*-terpineol could prevent morphogenesis of *C. albicans*, and therefore block biofilm formation, if present within 2 hours of adherence of cells to a surface. One hour treatments with $PMIC_{50}$ levels of TTO (0.5%) or the 2 components (0.25%) could effectively prevent biofilm formation. Pre-coating a plastic well with 1% TTO prior to inoculation resulted in strong inhibition (>50%) of biofilm formation.

Cellular cytotoxicity studies demonstrated that antifungal concentrations of TTO and T4-ol were cytotoxic to human cells *in vitro*. Investigations using a human oral epithelial cell line (OKF6-TERT2) and primary oral fibroblasts indicated that 2 min exposures to TTO and T4-ol showed cytotoxic effects at 0.25%, comparable with 0.12% chlorhexidine, with 0.125% TTO / T4-ol being non-toxic. Previously reported immunomodulatory effects were investigated using non-toxic concentrations of TTO / T4-ol (0.125%). The cytokine response of oral epithelial cells following TTO / T4-ol treatment was monitored using quantitative PCR, protein arrays and an IL-8 ELISA. TTO did not exhibit any clear immunomodulatory effects, but T4-ol pre-treatment of zymosan-activated cells resulted in reduced IL-8 protein in ELISA assays, indicating a potential to reduce inflammation. Although inflammation is a major symptom of OPC infections, it is also an important part of the host response to control the yeast pathogen. An anti-inflammatory agent may help to control candidosis symptoms, but may cause problems in controlling the infection.

These studies demonstrate that T4-ol could be suitable for use in prophylactic oral hygiene products such as mouthrinses and denture cleansers, and also as a novel treatment for established OPC infections. The use of T4-ol, a single component from TTO, has advantages over the complete essential oil in terms of product safety and consistency. Preclinical and clinical trials of mouthwashes or denture cleansers, containing the range of T4-ol concentrations (0.125 - 0.5%) investigated in these studies, would be required to validate the clinical use of such a product.

In conclusion, TTO-derived mouthwashes and denture cleansers may offer both a suitable alternative to conventional azole treatment of OPC and also a safe prophylactic alternative for inhibiting microbial biofilms, as they exhibit potent antifungal activity.

Chapter 1: *Introduction*

Oral Candidosis – Conventional Therapies and Natural Alternatives

A healthy oral cavity can normally be maintained by regular brushing of teeth and dentures to remove microbial biofilms. Patient non-compliance, environmental and genetic factors result in this microbial biofilm causing diseases such as caries, periodontal disease and candidosis. The oral healthcare sector has produced a wide range of antimicrobial products including toothpastes, mouthwashes and denture cleansers. However, many of these products have side-effects associated with them, such as tooth staining, unpleasant aftertaste, tissue irritation and resistance problems (Choo *et al.*, 2001). Antiseptic mouthwashes do not kill all oral micro-organisms, but those containing chlorhexidine are considered the most effective (Adams & Addy, 1994). However, chlorhexidine also has the aforementioned side-effects. Yeasts, particularly *Candida* species, are commonly isolated from the oral cavity, and given the opportunity can form biofilms resulting in various forms of oral candidosis. Candida biofilms can resist current antifungal treatments, so development of novel, more effective prophylactic measures, with fewer side-effects, would be of great clinical benefit. This is particularly true for immuno-compromised and elderly individuals, who are more likely to develop serious oral candida infections. The following section describes the role of *Candida* yeasts in oral candidosis, their ability to exist within complex biofilm communities and conventional and novel methods of chemotherapeutic intervention.

1.1 Oropharyngeal Candidosis

When the hosts' innate and adaptive immunological defences are weakened, *Candida* species (spp.) can become opportunistic fungal pathogens, resulting in yeast overgrowth and penetration of the oral tissues (Akpan & Morgan, 2002; Webb *et al.*, 1998a). This results in significant morbidity including oral pain and burning, altered taste sensation, and eating difficulties, leading to nutritional problems (Finlay & Davies, 2005; Rossie & Guggenheimer, 1997; Wray & Bagg,

1997). Acute oropharyngeal candidosis (OPC) is rare in healthy adults but occurs in up to 5% of newborn infants, who become infected during birth from mothers with vaginal candidiasis, and are susceptible due to their immature immune system (Epstein & Polsky, 1998). Around 10% of the elderly population are affected by OPC, often due to a poor immune system, but also because denture-wearers are susceptible to denture stomatitis, one of the major types of oral candidosis. Immunological impairment also increases OPC rates in patients with diabetes mellitus (Soysa *et al.*, 2006), malignancies such as leukaemia and lymphoma (Davies *et al.*, 2008; Rodu *et al.*, 1988), neutropenia, or HIV infection where OPC indicates possible clinical progression to AIDS (Vazquez, 2007). Ninety percent of patients with advanced AIDS develop oral candidosis, demonstrating the crucial role that the immune system plays in controlling growth of *Candida* spp. (Akpan & Morgan, 2002). Genetic autoimmune diseases such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) also cause chronic candidosis which is implicated in development of oral cancer (Rautemaa *et al.*, 2007). Immuno-suppressed patients develop OPC due to compromised host recognition of the pathogen and problems with activation of antifungal defence mechanisms (Villar & Dongari-Bagtzoglou, 2008). Other causes are use of broad-spectrum antibiotics which alter the normal oral microbial flora allowing overgrowth of yeast (Soysa *et al.*, 2008), immuno-suppressive therapies (corticosteroids or cytotoxic drugs) and radiation therapy (Soysa *et al.*, 2004).

Saliva is important in helping to control yeast growth in the oral cavity (Epstein, 1990; Peterson, 1992). Salivary flow helps remove organisms from the oral mucosa and dilutes any organisms. Reduced saliva causes a decrease in the pH of the mouth, which encourages increased yeast proliferation (Lundstrom *et al.*, 1984). Saliva also contains antimicrobial proteins (lactoferrin, sialoperoxidase,

lysozyme), anti-candidal antibodies (Nikawa *et al.*, 1994) and phagocytes which can regulate growth of *Candida* spp.. Salivary gland hypofunction, commonly known as xerostomia or 'dry mouth', reduces salivary flow and is therefore a risk factor for OPC (Guggenheimer & Moore, 2003). Xerostomia is a common symptom in the elderly (Turner & Ship, 2007) and patients with diabetes (Davies *et al.*, 2006), Sjögren's syndrome (Radfar *et al.*, 2003), and those receiving head/neck radiation therapy or many medications (Peterson, 1992). Other risk factors include dentures, smoking (Soysa & Ellepola, 2005) and a high sugar diet. Glucose in saliva enhances yeast growth, as it can be utilised as an energy source, and also improves the adherence of candida cells to oral epithelial cells (Ohman & Jontell, 1988).

There are several different types of oral candidosis, illustrated in Figure 1.1 (Akpan & Morgan, 2002; Odds, 1988; Samaranayake *et al.*, 2009). These include:

- Pseudomembranous (thrush)
- Atrophic and denture-induced stomatitis (erythematous)
- Angular cheilitis (stomatitis)
- Chronic hyperplastic (candidal leukoplakia)
- Median rhomboid glossitis

The commonest form of acute candidosis is pseudomembranous candidosis, characterised by yellowish-white plaques on the surface of buccal and labial mucosa and also sometimes on the tongue, gums, palate or pharynx. This is commonly known as oral thrush. These plaques are composed of desquamated epithelial cells, fibrin and fungal hyphae. They can normally be scraped away to leave red or bleeding sites (Wray & Bagg, 1997). Symptoms, if present, include burning or dryness of the mouth, loss of taste and pain from swallowing. Acute

erythematous candidosis normally gives a burning sensation in the mouth, with a bright red tongue and patchy red changes on the palate. The erythematous mucosa is sensitive and painful, and can be difficult to diagnose. It often develops in patients taking antibiotics or steroid inhalers, and also in HIV-infected patients (Lewis *et al.*, 1991).

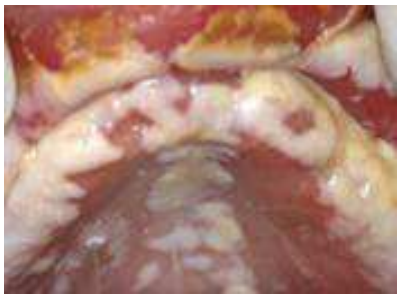
Chronic candidosis, including denture-induced candidosis, or denture stomatitis, occurs when the yeast grows as a biofilm on the denture surface, often co-aggregating with oral streptococci between the denture surface and the palatal mucosa, causing inflammation and pain (Budtz-Jorgensen, 1990a; Douglas, 2003). Movement of poorly-fitting dentures can irritate and damage the oral mucosa, allowing access of the yeast to the mucosa and subsequent inflammation from the host response. Colonisation with *C. albicans* can be very high in denture-wearers, 78% compared to 37% for dentate patients (Abu-Elteen & Abu-Alteen, 1998), and denture stomatitis incidence rates of up to 65% have been reported in denture wearers (Dreizen, 1984). With over 15 million people in the UK wearing dentures (Coulthwaite & Verran, 2007), the problem is certainly significant. Rates can also be underestimated since patients often experience no symptoms and diagnosis requires removal of dentures and careful inspection. Regular cleaning of dentures and good oral hygiene are the main ways to prevent denture stomatitis. Treatment includes correcting denture faults, cleaning dentures carefully and antifungal therapy (Jose *et al.*, 2010; Samaranayake *et al.*, 2009).

Denture wearers are also susceptible to angular cheilitis, which presents as an erythematous fissuring at one or both corners of the mouth (Budtz-Jorgensen, 1990b; Cross & Short, 2008; MacFarlane & Helnarska, 1976). This inflammatory reaction is often caused by *C. albicans*, but may also involve bacteria, particularly

Staphylococcus aureus and streptococci (Ohman *et al.*, 1986). These infections can become severe in immuno-compromised individuals (Epstein & Polsky, 1998).

Chronic hyperplastic candidosis, or leukoplakia, presents as bilateral white lesions on the buccal mucosa, tongue and floor of the oral cavity. *Candida* spp. are often cultured from these lesions, but it is not clear if the yeast is the cause or simply an opportunistic invader of damaged tissue. A small proportion of the lesions are pre-malignant and will develop into cancer (van der Waal *et al.*, 1997). The main risk factor for leukoplakia is smoking (Freitas *et al.*, 2006).

Median rhomboid glossitis is a fairly rare condition presenting as a chronic symmetrical lesion on the tongue. It is linked with smoking and inhaled steroids, and candida can be isolated from around 85% of cases (Budtz-Jorgenson, 1990a). There are a number of excellent reviews on the causes, types and treatment of oral candidosis (Epstein & Polsky, 1998; Pappas *et al.*, 2009; Samaranayake *et al.*, 2009; Webb *et al.*, 1998a, b, and c).



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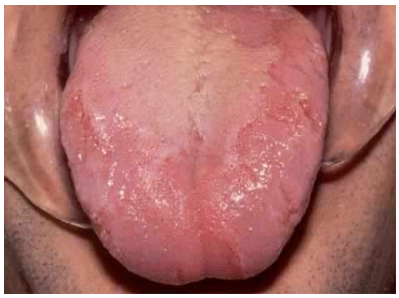

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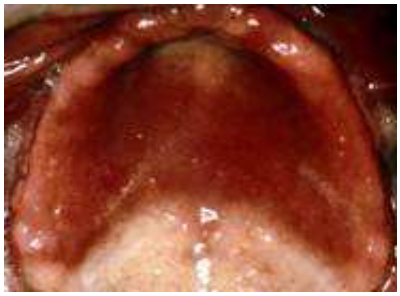

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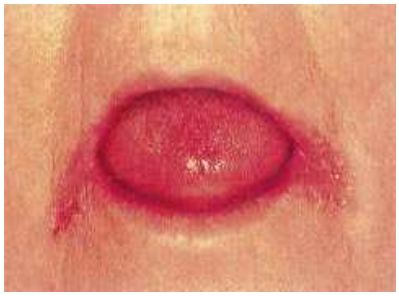

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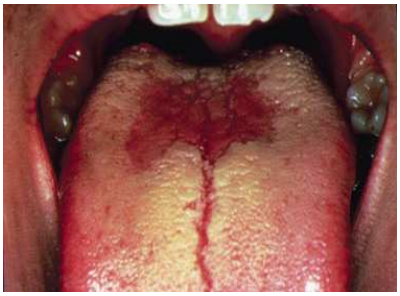

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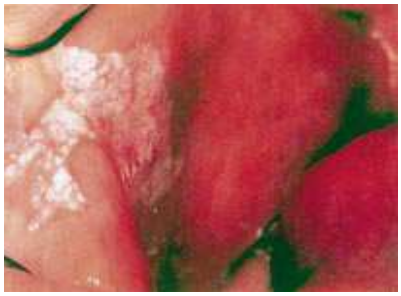

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Figure 1.1: Types of oral candidosis:

(**1**) pseudomembranous (thrush), (**2**) erythematous, (**3**) denture stomatitis, (**4**) angular cheilitis, (**5**) median rhomboid glossitis, (**6**) chronic hyperplastic (leukoplakia) [Akpan & Morgan, 2002; Samaranayake *et al.*, 2009].

1.2 Secondary effects of oral candidosis

1.2.1 Oral cancer

Consumption of high quantities of alcohol is known to increase oral cancer rates. Certain yeast and bacterial species in the oral flora possess alcohol dehydrogenase enzyme activity (Meurman & Uittamo, 2008). This results in production of acetaldehyde, a known carcinogen (Yokoyama *et al.*, 2007). Many *Candida* spp. (Nieminen *et al.*, 2009) and oral streptococci (Kurkivuori *et al.*, 2007) can produce acetaldehyde from ethanol metabolism. *C. albicans* and *C. glabrata* can also produce acetaldehyde from glucose fermentation (Nieminen *et al.*, 2009; Uittamo *et al.*, 2009). Patients with poor oral health status have been shown to have higher salivary acetaldehyde concentrations than those with better oral health (Homann *et al.*, 1997; Homann *et al.*, 2001). Therefore, it is plausible that poor oral hygiene may contribute to an increased risk of oral cancers. In contrast, improving oral hygiene with alcohol-containing mouthrinse may also have associated problems, as it has been shown that these mouthwashes can raise localised salivary acetaldehyde to concentrations associated with carcinogenic effects (Lachenmeier *et al.*, 2009). However, the evidence for this link is inconclusive at present.

A recent study in Finland found high levels of oral cancer in a group of APECED patients, a rare genetic autoimmune disorder (Rautemaa *et al.*, 2007). These patients experience recurring oral candidosis which has to be controlled with repeated antifungal drug therapy. 10% of the patients over 25 years of age developed oral or oesophageal squamous cell carcinoma. The production of acetaldehyde by the yeast was suggested to be the cause of the cancer (Uittamo, 2009).

1.2.2 Systemic candidiasis (candidaemia)

A major concern is for the haematogenous spreading potential of *Candida* spp. from the oral cavity through the bloodstream to distal organ sites, resulting in severe systemic disease. This is a particular problem in immuno-compromised patients, where systemic candidiasis is associated with mortality rates in excess of 70% (Fraser *et al.*, 1992; Migliorati & Madrid, 2007). The increased use of indwelling biomaterials, such as catheters, has inadvertently assisted in candidaemias by providing substrates on which *Candida* spp. can grow and proliferate as biofilm structures (Ramage *et al.*, 2006b). A recent Scottish survey of candidaemia patients found a rate of 4.8 candidaemia cases per 100,000 of the population per year, which was higher than many other European countries (Odds *et al.*, 2007). The prevalence of each species isolated is shown in Table 1.1. The two predominant species were *C. albicans* and *C. glabrata*, representing 75% of all isolates.

Table 1.1: *Candida* species isolated from blood cultures in Scotland
Adapted from Odds and colleagues (Odds *et al.*, 2007)

Species	Total no. of isolations	No. of patients
C. albicans	156 (52%)	121 (50%)
C. glabrata	68 (22.7%)	50 (20.7%)
C. parapsilosis	35 (11.7%)	28 (11.6%)
C. guilliermondii	10 (3.3%)	7 (2.9%)
C. dubliniensis	9 (3%)	7 (2.9%)
C. tropicalis	6 (2%)	6 (2.5%)
C. lusitaniae	6 (2%)	6 (2.5%)
C. krusei	3 (1%)	3 (1.2%)
Other species	7 (2.3%)	2 (0.8%)
Mixed species		12 (5%)

1.3 *Candida* species associated with OPC

The genus *Candida* is a collection of some 150 yeast species, in the class *Deuteromycetes* (Lodder, 1970). Seven *Candida* species are of major medical importance, with *C. albicans*, *C. glabrata* and *C. tropicalis* being the most frequently isolated, representing over 80% of isolates from medical specimens (Scully *et al.*, 1994). The frequency of infections by *Candida* spp. is increasing worldwide, with the risk of infection being particularly high in immuno-compromised and hospitalised patients (Sims *et al.*, 2005). These susceptible populations are growing due to increasing life span, development of better treatments for cancer, and also conditions associated with AIDS. Yeasts (mainly *C. albicans*) are the third leading cause of catheter-related infections, with the second highest colonisation to infection rate and the overall highest crude mortality (Crump & Collignon, 2000). *Candida* spp. are part of the normal commensal microflora of the skin, mouth, intestinal tract and vagina (Epstein & Polsky, 1998). They are frequently isolated from the oral cavity, with between 40 to 60% of the population being carriers (Epstein & Polsky, 1998), but few of these carriers develop clinical symptoms (Soysa *et al.*, 2008). Modern sequencing techniques have recently shown that a wide range of candidal species are found in healthy individuals (Ghannoum *et al.*, 2010). *Candida* spp. are present in saliva as planktonic cells (free-floating), which act as a reservoir for subsequent biofilm formation on the soft tissues, hard tissues and dentures.

C. albicans is the leading opportunistic pathogen associated with oral candidosis, and is known to be one of the most successful species at forming biofilms (Hawser & Douglas, 1994). Other species that can cause disease include *C. glabrata, C. parapsilosis, C. tropicalis, C. guilliermondii, C. krusei, C. lusitaniae and C. dubliniensis* (Kennedy *et al.*, 2006; Odds *et al.*, 1998). Mixed infections are common, both multiple yeast species and yeast / bacteria combinations

(Bagg *et al.*, 2003; Coco *et al.*, 2008; Davies *et al.*, 2002; Sweeney *et al.*, 1998; Thein *et al.*, 2009). A recent study in Glasgow isolated 7 yeast species from 37 denture-wearers, as shown in Table 1.2 (Coco *et al.*, 2008). Yeast was cultured from 92% of the denture-wearers. The main species isolated were *C. albicans* and *C. glabrata*, the same as observed for the Scottish candidaemia study (Table 1.1). These two species were found in combination in 25% of patients. *C krusei*, *C. parapsilosis* and *C. tropicalis* and *Saccharomyces cerevisiae* were also identified. Note that percentage figures total over 100% due to isolation of multiple species in some patients.

Table 1.2: Yeast species isolated from 37 denture-wearers in Scotland
Adapted from Coco and colleagues (Coco *et al.*, 2008).

Species	No. of patients
C. albicans	28 (75%)
C. glabrata	11 (29.7%)
S. cerevisiae	4 (10.8%)
C. krusei	2 (5.4%)
C. parapsilosis	1 (2.7%)
C. tropicalis	1 (2.7%)
C. famata	1 (2.7%)
Mixed species	13 (35%)
No yeast isolated	3 (8%)

C. albicans is the predominant pathogenic yeast species and is therefore the most well studied. It has an arsenal of virulence determinants including its cell wall, adhesins (agglutinins), degradative enzymes (proteolytic, haemolytic and lipolytic enzymes) (Naglik *et al.*, 2008), dimorphic behaviour (Whiteway & Oberholzer, 2004), thigmotropism and phenotypic switching (Calderone & Fonzi, 2001). These phenotypic characteristics give *C. albicans* a competitive

advantage in the oral cavity, but it is the immune-competence of the host that is crucial in determining whether clearance, colonisation, or candidosis will occur (Cannon *et al.*, 1995; Whiteway & Oberholzer, 2004). However, local factors such as the presence of a denture, salivary flow and compliance with oral hygiene also play a key role. The pathogenesis of *C. albicans* is beyond the scope of this thesis. However, a key phenotypic trait associated with pathogenicity which has received an increasing amount of attention by the research field has been in relation to biofilm formation and the resulting recalcitrance to antimicrobial treatment (Ramage *et al.*, 2009). *C. albicans* biofilms play a key role in the oral cavity with respect to OPC and inflammation of the oral mucosa (Ramage *et al.*, 2004).

1.4 Candida biofilms

OPC is caused predominantly by yeasts growing as biofilms on oral mucosa or denture surfaces, with *C. albicans* being the most frequently isolated species (Ramage *et al.*, 2005). Denture stomatitis is the most common condition caused by biofilm growth on a prosthetic device (Douglas 2003). *C. albicans* biofilm growth on biomaterial surfaces is increasingly becoming a major source of infection (Douglas, 2002; Kojic & Darouiche, 2004). Central venous catheters, plastic tubes used for the administration of intravenous fluids, are the most commonly infected surgically-implanted medical devices (Donlan *et al.*, 2001; Donlan, 2008; Ramage *et al.*, 2006b). Infections can be endogenous, when *C. albicans* is transferred to the catheter tip due to the intestinal mucosa being punctured, or exogenous, from contamination by hospital staff (Douglas, 2003).

Biofilms can be defined as a community of microorganisms attached to a surface, which is embedded in a complex extracellular matrix of polymers (Donlan & Costerton, 2002; Ramage *et al.*, 2009). Mature biofilms can more easily form on

non-renewing surfaces, such as teeth, dentures and catheters, rather than on rapidly shedding oral mucosa. Dental plaque is an excellent example of a bacterial biofilm, being composed of streptococci, particularly *Streptococcus mutans*, and a diverse range of other bacteria. The matrix is generally slimy and 'glue-like', aiding attachment to a surface, and is very important in protecting the microbes from environmental factors, such as antibiotics (natural, as well as man-made) or disinfectants (Ramage *et al.*, 2005; Ramage *et al.*, 2009). The synthesis of matrix products, or exopolymeric substance (EPS), increases when a developing biofilm is exposed to a liquid flow (Baillie & Douglas, 2000), as demonstrated in Figure 1.2 (Ramage *et al.*, 2001b). This allows the microbe to attach more strongly to a surface to avoid being detached and washed away. It has been shown that the biofilm can become denser, but with a reduced thickness, when exposed to greater shear stress (Mukherjee *et al.*, 2009). An interesting potential way to treat biofilms is to use enzymes which can digest the EPS, to allow better penetration of antimicrobials (Johansen *et al.*, 1997).

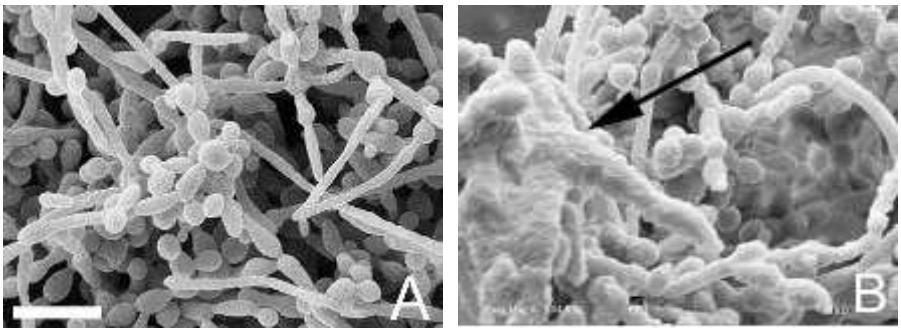


Figure 1.2: *Candida albicans* biofilm with EPS from static and continuous flow models. (**A**) biofilm grown under static conditions, (**B**) biofilm grown in a continuous media flow model (250x magnification). Arrow indicates increase of EPS resulting in a cloudy film over the biofilm (Ramage *et al.*, 2001b).

Growth rate within a biofilm can be very slow, partially due to a lack of nutrients (Douglas, 2003). This results in reduced uptake of antimicrobials, making these agents much less effective. As *Candida* biofilms are difficult to treat, preventative chemotherapeutic measures which stop biofilm formation are desirable (Ramage *et al.*, 2006a).

As biofilms have high intrinsic resistance to antifungal therapies and immune cell assault (Ramage *et al.*, 2001c), their characteristics and organisation are of increasing interest and concern. *C. albicans* biofilms normally contain a mixture of morphological forms, as this species is polymorphic. A mature biofilm will be composed of a dense network of oval, budding yeast cells (blastospores), pseudohyphae and elongated hyphae (Hawser & Douglas, 1994). The hyphae are formed when the blastospores form germ tubes and begin to elongate, a process which allows the yeast to form a mature biofilm to cover a surface. Polymorphism is an important virulence factor, affecting adherence to epithelial cells (Villar *et al.*, 2004). It has been shown that some other species, particularly *C. glabrata* and *C. parapsilosis*, do not produce true hyphae, and so produce an inferior biofilm (Kuhn *et al.*, 2002a; Mukherjee *et al.*, 2005). Numerous changes in gene expression occur as *C. albicans* develops into a biofilm (Yeater *et al.*, 2007), i.e. cells in the biofilm have a very different phenotype to their planktonic forms. This is caused by changes in expression of genes involved in hyphal morphogenesis such as *EGF1, CPH1, TEC1* and *BCR1* transcription factors (Lo *et al.*, 1997; Nobile & Mitchell, 2006; Ramage *et al.*, 2002d), upregulation of methionine and cysteine biosynthetic genes (Garcia-Sanchez *et al.*, 2004), upregulation of azole efflux-pump genes *CDR1* and *MDR1* (Mukherjee *et al.*, 2003; Ramage *et al.*, 2002a) and upregulation of the *ALS* (agglutinin-like sequence) gene family of adhesins (Chandra *et al.*, 2001a; Dranginis *et al.*, 2007; Green *et al.*, 2004; Nobile *et al.*, 2008). Nobile and Mitchell have published an

excellent review which tackles this burgeoning field (Nobile & Mitchell, 2006). A recent study used microarray analysis of RNA to compare gene expression levels within an *in vivo* catheter biofilm to expression in planktonic cells (Nett *et al.*, 2009). 124 transcripts were up-regulated in the biofilm and it was shown that cells at early stages of biofilm formation expressed particular adherence genes not seen in mature biofilms.

C. albicans biofilm formation can be considered as three separate phases (Chandra *et al.*, 2001a):

1) Attachment and colonisation of yeast cells to a surface
2) Proliferation of cells to form a basal layer of anchoring cells
3) Growth of pseudohyphae and hyphae, along with production of EPS, to produce a complex, mature biofilm

(Nobile & Mitchell, 2006; Ramage *et al.*, 2001b).

Figure 1.3 illustrates the main stages of *C. albicans* biofilm formation. Microbial biofilm model systems have recently been reviewed (Coenye, T., 2010).

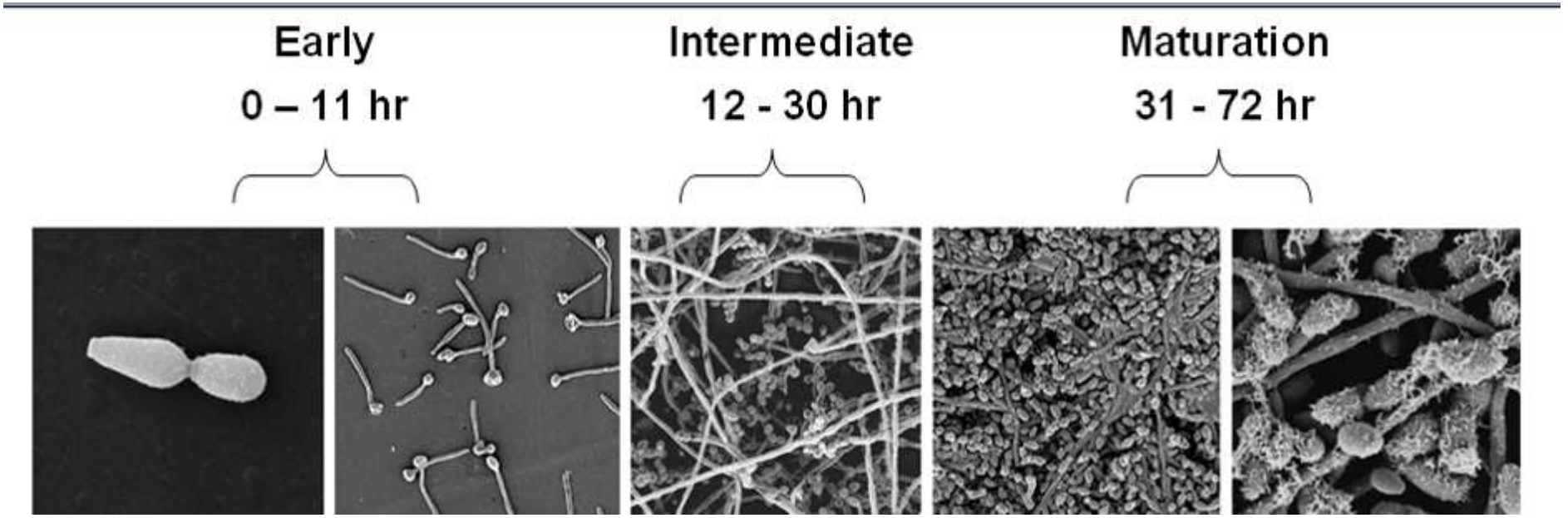


Figure 1.3: Biofilm maturation. Overview of main aspects of *Candida albicans* biofilm development, from scanning electron micrographs (SEM) of Thermanox™ coverslips. The three main phases of biofilm development are illustrated, as previously described (Chandra *et al.*, 2001a). The SEM images demonstrate initial adhesion, filamentation, hyphal and yeast proliferation, with maturation and associated extracellular matrix (ECM) production. The key development phases are indicated, along with respective timelines. (Ramage *et al.*, 2009).

1.5 Treatment of OPC

Candida species commonly colonise the oral cavity at low levels, and given the opportunity can form biofilms resulting in various forms of oral candidosis. Biofilms formed by *Candida* spp. can resist current antifungal treatments, so development of novel, more effective prophylactic measures, with fewer side-effects, would be of great clinical benefit. This is particularly true for immuno-compromised and elderly individuals, who are more likely to develop serious oral candidal infections. The following describes conventional and novel methods of chemotherapeutic intervention.

1.5.1 Dentifrices, mouthrinses and gels

Oral hygiene products such as dentifrices (toothpastes), mouthrinses and gels have been available for many years to improve oral health. They have been developed to augment mechanical removal of microbial biofilms by tooth-brushing and flossing, particularly to reduce dental plaque, and hence prevent excessive dental caries. Most conventional toothpastes have few active ingredients other than detergents (surfactant), such as sodium dodecyl sulphate (SDS), and fluorides which have strong anti-caries effects. Fluoride has several beneficial properties, including reducing acid demineralization of enamel and enhancing enamel remineralisation which together help to reduce caries (ten Cate & Featherstone 1991). Fluoride also has multiple effects on microbial metabolism, including inhibition of acid production by plaque bacteria, and can sensitize biofilm organisms to acid killing (Marquis *et al*., 2003; Bradshaw *et al*., 2002). Some products also contain other antimicrobial ingredients, including chlorhexidine (Lie & Enersen, 1986), triclosan (Russell, 2004), cetylpyridinium chloride (Davies, 2007) and essential oils (Ciancio, 2003; Fine *et al.*, 2007).

Antimicrobial mouthwashes are particularly useful as their active ingredients can access hard-to-reach areas such as interproximal plaque, which can help to reduce gingivitis (Charles *et al.*, 2000a). It should be noted that commercial mouthrinses also contain many other ingredients which are often not specified by the manufacturers. In particular, ethanol is often present at high levels to aid solubility of ingredients. The antiseptic chlorhexidine (CHX) has been used in oral products for 50 years, is considered by dentists as the 'gold standard' bactericidal agent and also has fungicidal properties (Ellepola & Samaranayake, 2001; Hiom *et al.*, 1992). A 0.2% solution can prevent development of the dental plaque biofilm if used regularly (Al-Tannir & Goodman, 1994) and chlorhexidine gluconate has been accepted by the American Dental Association for gingivitis treatment (Fischman, 1994). It can adsorb to tooth surfaces, remains highly protein-bound, and disrupts bacterial cell membranes (Russell & Day, 1993). However, long-term use can cause yellow-brown staining, affects taste, promotes calculus formation and can cause mucosal desquamation, thus reducing patient acceptance (McCoy *et al.*, 2008). 0.2% CHX has been shown to be cytotoxic *in vitro,* in keratinocyte and fibroblast skin cells (Boyce *et al.*, 1995) and also in gingival fibroblasts (Flemingson *et al.*, 2008). CHX can also cause hypersensitivity reactions including contact dermatitis (Calnan, 1962; Ebo *et al.*, 1998), and there have been safety concerns as immediate systemic hypersensitivity (anaphylaxis) has been reported; in 1984 the Japanese Ministry of Health recommended avoiding chlorhexidine use on mucous membranes (Krautheim *et al.*, 2004).

It has been suggested that Gram-negative bacteria (particularly *Pseudomonas* spp.) can develop CHX resistance (Thomas *et al.*, 2000). *In vitro* studies have shown that there are less-susceptible bacteria within mixed biofilms which become more common with CHX use (McBain *et al.*, 2003). However,

longitudinal clinical studies have not found significant changes in susceptibility of oral flora with CHX use (Maynard *et al.*, 1993; Schiott *et al.*, 1976a and b; Sreenivasan & Gaffar, 2002).

The antiseptic mouthrinse Listerine™ (Johnson & Johnson Healthcare Products) contains the phenolic essential oils eucalyptol, thymol, menthol and methyl salicylate as active ingredients (Jackson, 1997). It disrupts microbial cell walls and inhibits bacterial enzymes, but is less effective than chlorhexidine at preventing plaque and gingivitis. It has similar adverse effects to chlorhexidine, including burning sensations, a bitter medicinal taste, occasional staining and potential tissue irritation. Burning sensation is caused by high alcohol content, which can be up to 28%. Listerine™ does not disrupt the normal oral flora (Fischman, 1994). Essential oil dentifrices (toothpastes) are also available (Charles *et al.*, 2000b).

Triclosan is a chlorinated phenol with broad-spectrum antibacterial properties. It has been used as a dentifrice in toothpaste in combination with a copolymer, polyvinylmethyl ether maleic acid (Volpe *et al.*, 1996), which increases the retention of the triclosan on oral surfaces, and also with zinc (Davies, 2007; Gilbert *et al.*, 2007; Saxton, 1986). It has also been shown that triclosan has an anti-inflammatory effect (Ramberg *et al.*, 1995), which may be useful for controlling some of the symptoms associated with candidosis. Triclosan/copolymer has also been used successfully in mouthwashes (Ayad *et al.*, 1995).

Cetylpyridinium chloride, used in mouthwashes since 1940 (Davies, 2007), is a quaternary ammonium compound which, like CHX, binds to oral tissues (Radford *et al.*, 1997). It also binds to negatively-charged phosphates in bacterial cell membranes, which can disrupt the cell wall and increase permeability.

1.5.2 Denture cleansers

The recommended method for cleaning dentures is regular mechanical brushing, which removes thicker biofilms from the denture surface (Jagger & Harrison, 1995; Nikawa *et al.*, 1999). This is an important first step, as biofilms can resist antiseptic treatments (Gilbert *et al.*,1997). Ultrasonic devices are also available as cleaning aids. However, mechanical cleansing often does not remove the biofilm completely, and can damage the denture surface. Brushing can increase surface roughness on the dentures (Oliveira *et al.*, 2007), which aids attachment and colonisation by *Candida* sp (Young *et al.*, 2009). Biofilms can form in cracks or imperfections in the denture bases, and these are difficult to remove by brushing (Ramage *et al.*, 2002d). Microwave irradiation is an excellent alternative which damages yeast cell membranes (Campanha *et al.*, 2007; Polyzois *et al.*, 1995; Sanita *et al.*, 2008). However, there can be problems with shrinkage of denture-lining (Dixon *et al.*, 1999; Seo *et al.*, 2007). Chemical denture cleansers are often preferred by elderly and handicapped patients (Buergers *et al.*, 2008). There are a wide variety of cleansing agents and disinfection regimens available to remove biofilms from dentures, ranging from chemicals such as sodium hypochlorite, glutaraldehyde, hydrogen peroxide and enzymes (Budtz-Jorgensen & Kelstrup, 1977), to commercial mouth rinses (Schwartz *et al.*, 1988), effervescent cleansing tablets (e.g. Steradent), and even vinegar. The evidence for the effectiveness of these chemicals is conflicting (Basson *et al.*, 1992; Buergers *et al.*, 2008; Schwartz *et al.*, 1988). They can also cause weakening of the dentures and undesirable colour changes. Therefore, the search for alternative prophylactic and therapeutic strategies to reduce denture stomatitis and other forms of OPC is ongoing.

1.6 Conventional antifungal therapy

When candidosis is unresponsive to basic oral hygiene, pharmaceutical-based treatment commences with a small number of antifungal drugs, including polyenes (nystatin and amphotericin B) or azoles (fluconazole, itraconazole, and more recently voriconazole and posaconazole) (Laudenbach & Epstein, 2009; Scheinfeld, 2007; Spreghini *et al.*, 2008; Webb *et al.*, 1998b). The echinocandin caspofungin is used primarily for systemic infection, but is highly active against *C. albicans* biofilms (Bachmann *et al.*, 2002; Denning, 2003; Kauffman & Carver, 2008; Keating & Figgitt, 2003). Whilst these drugs are often effective, some strains show multi-drug resistance, particularly non-*albicans Candida* isolates (Li *et al.*, 2007; Niimi *et al.*, 2010). An earlier study in Glasgow looked at azole susceptibility in 300 yeast strains isolated from oral cancer patients and found 13% of strains were resistant to both fluconazole and itraconazole (Bagg *et al.*, 2006). Strains that seem sensitive to drugs *in vitro*, i.e. planktonic CLSI MIC testing, can be resistant *in vivo*, as the biofilm gives them considerable protection (Chandra *et al.*, 2001b; Hawser & Douglas, 1995; Ramage *et al.*, 2002c). Resistant *C. glabrata* isolates are most common, and such resistance may well become more of a problem in the future, as patients with candidosis often have recurring infections which require repeated drug treatments (Bagg *et al.*, 2005). These repeat exposures may encourage changes in the fungal population, selecting for more drug-resistant species like *C. glabrata*. Guidelines have recently been issued for use of these drugs in both systemic and mucosal infections (Pappas *et al.*, 2009). Many of these drugs are systemic and there are issues with drug interactions, particularly in patients with cancer or AIDS. The interaction of azoles with agents such as antacids, insulin, oral anticoagulants and corticosteroids influences the level of these agents in blood, which can affect their efficacy or toxicity (van der Bijl & Arendorf, 1993). These antifungal drugs can also adversely affect the immune response of epithelial cells (Lilly *et al.*,

2005). Finally, many antifungals have a very unpleasant taste which results in considerable patient non-compliance with recommended treatments. In summary, failure of OPC treatment is caused by biofilm resistance to antiseptics and antifungal drugs, in combination with poor patient compliance, often due to side-effects. Therefore, alternative methods for suppressing biofilm formation or treating severe candidosis are desirable.

1.7 Alternative therapeutic strategies from nature

Early civilisations used plants as their main source of medicines, and most of the world's population still rely on them today. Plants have evolved a multitude of components to attract pollinating insects, avoid mechanical damage and give protection from microbial and parasitic diseases. These have been exploited to prevent and treat human infections. In oral hygiene, chewing sticks from various plants have been traditionally used for thousands of years for brushing teeth, like a primitive toothbrush (Wu *et al.*, 2001). In many Muslim countries, these are known as miswak (Arabic for tooth cleaning stick), and are made from the roots and stems of *Salvadora persica* (Al-Otaibi & Angmar-Mansson, 2004). Clinical studies have shown that miswak extract could inhibit *C. albicans* (al-Bagieh *et al.*, 1994) and also the periodontal bacterial pathogen *Actinobacillus actinomycetemcomitans* (al-Otaibi, 2004) which resulted in reductions in plaque and gingival indices compared to tooth-brushing. Recent scientific research has shown extracts from plants used for African chewing sticks to have antibacterial properties (More *et al.*, 2008), while the leaves and twigs of *Azadirachta indica* (Neem) are widely used in India for cleaning teeth (Patel & Venkatakrishna-Bhatt, 1988). Chewing sticks are still recommended as oral hygiene tools in developing countries as they are easily available, cheap and simple to use.

Aromatic plants contain essential oils which have been extracted and used for centuries in herbal medicine, perfumes and food flavourings. These oils are composed of a vast variety of components, particularly terpenes, which have a multitude of properties including anti-cancer and antimicrobial effects (Edris, 2007). Fungi are common plant pathogens and essential oils can protect plants from fungal diseases (Terzi *et al.*, 2007). Plants used for their essential oils include garlic, lavender, thyme, mint, eucalyptus and lemon balm.

1.7.1 Tea tree oil

Another of these plants is Australian tea tree, *Melaleuca alternifolia*, in the Myrtaceae family, commonly called narrow-leaved paperbark tree (Figure 1.4), which provides tea tree oil (TTO), one of the most widely used essential oils. TTO is an aboriginal traditional medicine for bruises, insect bites and skin infections (Altman, 1988; Low, 1990). This aromatic essential oil is a complex mixture of over 100 terpene hydrocarbons extracted by steam distillation from the leaves and terminal branches of *M. alternifolia* and related species. The antimicrobial properties of TTO and its components were first investigated in the 1920s (Penfold & Grant, 1925), and mentioned in the British Medical Journal as early as 1933 (Anonymous, 1933a; Anonymous, 1933b). It has been used as a topical antiseptic since these early publications and also as a veterinary antiseptic for many years (Mozelsio *et al.*, 2003; Reichling *et al.*, 2004). *M. alternifolia* Cheel (name of major chemotype) has been grown in commercial plantations since 1970, mainly in New South Wales (Carson *et al.*, 2006). Currently it is used in skin, nail, hair care and oral products, particularly mouthwashes (SCCP, 2008). TTO has antimicrobial activity against a broad spectrum of organisms including *Escherichia coli*, *Staphylococcus aureus* (Caelli *et al.*, 2000; Carson *et al.*, 1995; Carson *et al.*, 2002), oral bacteria (Hammer *et al.*, 2003b), fungi (Hammer *et al.*, 2004; Nenoff *et al.*, 1996), viruses (Schnitzler *et al.*, 2001; Timpanaro *et al.*,

2007) and also protozoa (Mikus *et al.*, 2000). The antimicrobial properties of individual TTO components have also been investigated (Carson & Riley, 1995). Much more research is required to understand the mode of action and safety of TTO, before it is fully accepted as an alternative prophylactic or therapeutic agent (SCCP, 2004 and 2008).

Figure 1.4: *Melaleuca alternifolia*, the source of tea tree oil

1.7.2 Composition of TTO

TTO is a complex mixture of mono-terpenes, sesquiterpenes and other aromatic molecules, with around 100 components being identified (Brophy *et al.*, 1989). The composition is influenced by genetic variation (chemotype of plant), growing conditions (habitat and climate), age of leaves and length of distillation. The best product is obtained from plants growing in native, swampy habitat of New South Wales. There is an International Standard ISO 4730:2004, which sets maxima and / or minima for several of the components (ISO, 2004). Terpinen-4-ol (T4-ol), the major component, has to be at a minimum of 30% and maximum of 48%. The main components of TTO and their proposed chemical structures are illustrated in Figure 1.5.

Component	%
terpinen-4-ol	**40**
γ-terpinene	23
***α*-terpinene**	10
1, 8-cineole	5
terpinolene	3
***p*-cymene**	3
***α*-terpineol**	2.5
Others	13

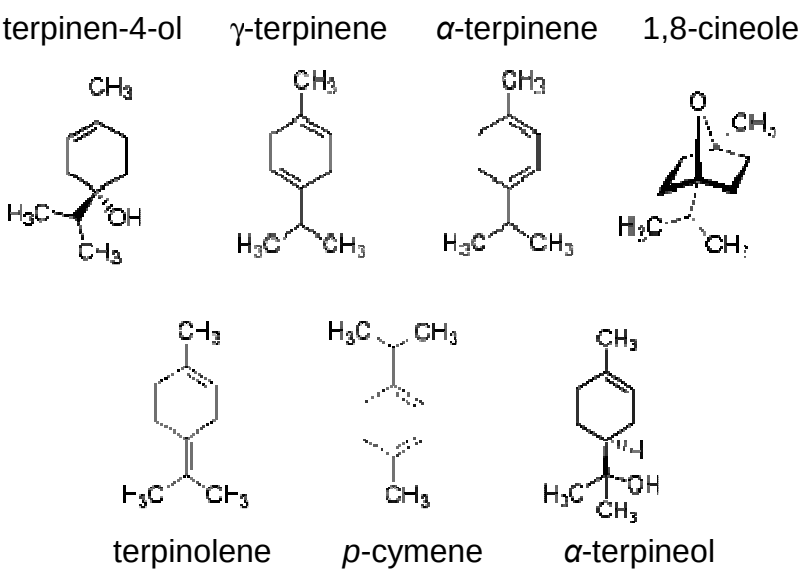


Figure 1.5: Typical tea tree oil composition and chemical structures of components

1.7.3 Oral TTO products

Essential oil mouthwashes, such as Listerine™ (section 1.5.1), are in common use (Ciancio, 2003), and TTO is now also widely used in mouthwashes at a concentration of around 0.2% (Vazquez & Zawawi, 2002). Many *in vitro* studies have reported its inhibition of *C. albicans* (Bagg *et al.*, 2006; Banes-Marshall *et al.*, 2001; D'Auria *et al.*, 2001; Hammer *et al.*, 1998; Hammer *et al.*, 2000). Previous research in Glasgow Dental Hospital has shown sensitivity of azole-resistant yeasts to TTO at concentrations used in oral care products (Bagg *et al.*, 2006). Additionally, TTO has been used successfully in palliative care patients, to treat severe OPC infections that do not responded to conventional antifungals (Dr. M.P. Sweeney, personal communication). TTO has also recently been successfully used to reduce inflammation in denture stomatitis (Catalan *et al.*, 2008).

A small clinical trial evaluated an alcohol-based TTO mouthwash as a treatment of OPC in 12 HIV-positive patients who had been treated unsuccessfully with oral fluconazole (Jandourek *et al.*, 1998). Six patients improved after 2 weeks treatment, while 2 patients' infections were resolved and 6 others had reduced symptoms after 4 weeks treatment. In a follow-up study, the alcohol-based TTO was compared with an alcohol-free solution in an attempt to reduce the oral burning side-effects commonly seen in the first study (Vazquez & Zawawi, 2002). Sixty percent of patients demonstrated a clinical response to the Melaleuca oral solutions (7 patient's infections resolved and 8 patients improved clinically) at the 4-week evaluation. The alcohol-free solution produced fewer side-effects and cured 5 patients, compared to 2 for the alcohol-containing solution. A TTO gel has been investigated in a clinical trial for chronic gingivitis (Soukoulis & Hirsch, 2004). A 2.5% TTO gel was compared with a 0.2% chlorhexidine gel and a placebo gel. The TTO gel reduced gingival inflammation more than chlorhexidine, but did not inhibit plaque formation. Chlorhexidine mouthwash has been shown to reduce plaque formation (Lennon & Davies, 1975).

1.7.4 Mode of Action

TTO is a broad-spectrum, membrane-active disinfectant similar to chlorhexidine. It denatures proteins and disrupts normal cellular membrane structure and function, which causes cytoplasmic leakage, cell lysis and death (Cox *et al.*, 1998; Cox *et al.*, 2000; Giordani *et al.*, 2006; Hammer *et al.*, 2004; Soderberg *et al.*, 1996). TTO components are lipophilic hydrocarbons, which accumulate in the membrane lipid bilayer and alter both the structural and functional properties of the membranes. This causes loss of chemiosmotic control which results in swelling of the bilayer. The cell membrane loses its integrity, increasing its permeability to protons and ions, which disrupts the intracellular pH. Membrane proteins are also significantly affected by the terpenes (Sikkema *et al.*, 1995).

Stationary phase microbial cells are more tolerant to TTO than exponentially growing cells, as their membranes have different lipid composition (Cox *et al.*, 1998). Respiration of bacteria and *Candida* spp. is significantly inhibited by TTO (Cox *et al.*, 2000), again due to membrane disruption. It has been shown that 0.5% TTO damages membranes of *Escherichia coli*, *Staphylococcus aureus* and *C. albicans*, allowing entry of the fluorescent stain propidium iodide (PI) (Cox *et al.*, 2001). However, *C. albicans* was less permeable to PI than both the bacterial species, while leakage of potassium ions (K^+ efflux) was substantial in *E.coli*, moderate in *S.aureus* and not detected from the yeast cells. This demonstrates differences in TTO effects, due to variation in cell membrane structure between Gram-negative and Gram-positive bacteria and between bacteria and yeasts.

In *C. albicans*, 0.125% TTO has been shown to block morphogenesis to filamentous growth by inhibiting germ-tube formation (D'Auria *et al.*, 2001; Hammer *et al.*, 2000). This can potentially block biofilm formation, but the effect is reversible with removal of TTO, resulting in filamentation. T4-ol is considered the principal antimicrobial component (Carson & Riley, 1995) and it has recently been shown that it is a more potent antibacterial agent against methicillin-resistant *S. aureus* (MRSA) and coagulase-negative staphylococci isolates than TTO, with neither agent exhibiting toxicity to fibroblast cells at the concentrations tested (Loughlin *et al.*, 2008). Therefore, based on the data from these studies there is the potential of using purified components in mouthwashes and denture-cleansing agents. Unfortunately the action of TTO is not specific to micro-organisms, as it can also disrupt human cell membranes.

1.8 Effects of Tea tree oil on Human Cells

In addition to antimicrobial activity, TTO has been applied to skin for many years for its proposed anti-inflammatory activity. However, TTO is documented to exert both cytotoxic and immuno-modulatory effects on mammalian cells (Carson *et al.*, 2006). Data on safety and toxicity of TTO is relatively limited (SCCP, 2008), but for it to be used as an oral hygiene product it should conform to the EC Cosmetics Directive 76/768/EEC – application to toxicity testing of oral hygiene products and TTO (updated by EU Regulation 1223/2009).

The membrane-disrupting properties of TTO are non-specific, affecting mammalian cells as well as micro-organisms. Yeast cells are eukaryotic and so have many similarities to human cells. Cytotoxicity is therefore a potential problem in development of clinical treatments containing TTO.

1.8.1 *In vitro* studies

A number of studies have investigated the effects of TTO and its constituents on a variety of cell types. *In vitro* testing of cell lines is considered an economical and ethical adjunct, or potential alternative to animal testing (Hammer *et al.*, 2006).

i) Cytotoxic effects

The simplest *in vitro* studies use various cell lines grown as a monolayer or in suspension. A Swedish study determined cytotoxic concentrations of TTO to fibroblasts and epithelial cells isolated from human gingival tissue (Soderberg *et al.*, 1996). They calculated the IC_{50}, i.e. the concentration giving a 50% reduction in cell viability after a 1 h treatment. Both cell lines had similar IC_{50} values around 0.03%, with epithelial cells being slightly more sensitive. They simultaneously established that TTO demonstrates an MIC_{50} around 0.014% against *Staphylococcus aureus*, suggesting that the bacteria are more sensitive than the

human cells to TTO. An Australian study investigated the effects of TTO and three water-soluble constituents (terpinen-4-ol, *α*-terpineol and 1,8 cineole) on a variety of cell lines (Hayes *et al.*, 1997). HeLa cells (epithelial) had an IC_{50} of 0.28% TTO, some cell lines showed similar susceptibilities (K562 chronic myelogenous leukaemia, HepG2 hepatocellular carcinoma), while others (CTVR-1 and MOLT-4 leukaemia cells) were more susceptible (IC_{50} 0.06%), similar to the Swedish study described above. It was also shown that TTO was extremely toxic to monocytes and neutrophils, with a 20 h exposure of TTO giving an IC_{50} of around 0.016% (Hart *et al.*, 2000). This toxic effect could be reduced significantly by using the water-soluble fraction, containing terpinen-4-ol, *α*-terpineol and 1,8-cineole. Preparation of the TTO dilutions in plastic tubes in RPMI media without serum resulted in the most toxic components adsorbing to the plastic. This method made a 0.125% TTO dilution non-toxic. Finally, another study demonstrated that exposure to 1% TTO for 60 min was toxic for total leukocytes, whereas 0.1% TTO for 30 min was toxic to peripheral blood mononuclear cells (Caldefie-Chezet *et al.*, 2006).

In summary, relatively low concentrations of TTO (0.1% or less) can be toxic to a wide variety of cells, but cytotoxic effects depend on cell type, and the length of exposure and concentration. There can be technical difficulties working with TTO due to adsorption of some components to plastic, particularly the more toxic ones, and results are also influenced by the technique used to measure viability. There can also be significant variation in the composition of oil from different sources, which can make comparing studies difficult. In comparison, chlorhexidine gluconate, a routine antimicrobial found in many mouthwashes, has been shown to inhibit growth of epithelial cells and fibroblasts at concentrations of 0.005%, below the antibacterial concentration of 0.05% (Boyce *et al.*, 1995).

This study suggested that topical use of chlorhexidine in wounds was not advisable, due to its cytotoxicity. A recent study confirmed *in vitro* cytotoxicity in human gingival fibroblasts (Flemingson *et al.*, 2008). A summary of some key findings from the literature is shown in Table 1.3.

Table 1.3: Summary of inhibitory concentrations of TTO in human cells

Cell type	IC_{50} %TTO	Treatment Time (h)	Comments
Primary gingival epithelial / fibroblasts (Soderberg *et al.*, 1996)	0.03	1	MIC_{50} 0.014% *S. aureus*
HeLa (epithelial) HepG2 (liver carcinoma) K562 (leukaemia) (Hayes *et al.*, 1997)	0.28	4	
CTVR-1 / MOLT-4 (leukaemia) (Hayes *et al.*, 1997)	0.06	4	
Monocytes / Neutrophils (Hart *et al.*, 2000)	0.016	20	0.125% water-soluble fraction is non-toxic
Total leukocytes (Caldefie-Chezet *et al.*, 2006)	1	1	
Peripheral blood mononuclear cells (Caldefie-Chezet *et al.*, 2006)	0.1	0.5	

Monolayer cell cultures of individual populations of cells are simplified models of mucosal cell surfaces. Organotypic models provide a more sophisticated system, comprising a multilayered epithelial structure with similar organization to the cells in native mucosal surfaces. Three-dimensional models of oral mucosa have recently been developed using immortalised oral keratinocytes (OKF6-TERT2) and 3T3 mouse fibroblasts (Dongari-Bagtzoglou & Kashleva, 2006; Klausner *et al.*, 2007). Fibroblasts are important components of these models, promoting keratinocyte growth and differentiation into stratified squamous epithelia. These 3-D models resemble the commercially available system from SkinEthic (de Brugerolle de *et al.*, 1999), which is based on TR146, a buccal carcinoma cell line.

A study in Japan used a skin model comprising keratinocytes and dermal fibroblasts within a collagen gel to test the cytotoxicity of cyclic monoterpenes (Kitahara *et al.*, 1993). Terpenes were shown to prevent proliferation of keratinocytes and also inhibited fibroblast growth, which blocked contraction of the collagen lattice. There are limited studies investigating terpene toxicity in organotypic models.

ii) Immunomodulatory effects

There is now strong evidence from *in vitro* studies, mainly using human monocytes and polymorphonuclear neutrophils, to support the hypothesis that TTO is anti-inflammatory (Carson *et al.*, 2006). Hart *et al* found 0.125% TTO significantly inhibited production of TNF-α, IL-1β and IL-10, but not the chemokine IL-8 in LPS-induced monocytes (Hart *et al.*, 2000). They also showed similar inhibition by 0.05% terpinen-4-ol, though IL-8 was also suppressed slightly. This same group investigated the effects of TTO on superoxide production by human phagocytic cells (Brand *et al.*, 2001). They found that T4-ol

suppressed production of oxygen derived reactive species in monocytes, but not in neutrophils. These cells produce superoxide when stimulated *in vivo*, and this is involved in host defence. The authors somewhat speculatively surmised that inappropriate or excessive activation of monocytes and neutrophils, leading to release of reactive oxygen species, can cause bystander tissue damage, therefore suppression by terpinen-4-ol can be advantageous. In addition, they hypothesise that neutrophils are still able to eliminate any foreign antigens, but the monocyte suppression can limit tissue damage. Another study showed a 3-fold induction in IL-4 from phytohaemagglutinin (PHA) lectin-stimulated peripheral blood mononuclear cells (PBMC) by 0.1% TTO and a 2-fold IL-10 induction with 0.01% TTO. This therefore indicates a possible simultaneous allergic response through IL-4 induction with an anti-inflammatory effect associated with IL-10, making the overall interpretation of the data problematic. Moreover, a reduction in IL-2 was observed in cells treated with 0.01% TTO (Caldefie-Chezet *et al.*, 2006). This study also found that 30 min in 0.1% TTO was highly toxic to PBMC. Therefore, the IL-4 induction is significant as the cell viability is low. This study adds to the evidence for the anti-inflammatory properties of TTO, but this comes with certain caveats, *i.e*. other immune-induced pathologies.

To date there has been no *in vitro* research published on the immunomodulatory effects of TTO on epithelial cells, the first cells exposed on skin or oral mucosa. Epithelial cells act as an infection barrier but also produce cytokines which are crucial in regulating inflammation (Dongari-Bagtzoglou & Fidel, 2005). Oral epithelial cells produce pro-inflammatory IL-6 and IL-8 in response to bacterial infections (Yumoto *et al.*, 1999) and also a strong IL-8 response to *C. albicans (*Dongari-Bagtzoglou & Kashleva, 2003; Schaller *et al.*, 2002*)*. *C. albicans* infection of human oral epithelial cells induces strong expression of the

chemokine IL-8 and the cytokine granulocyte-macrophages colony-stimulating factor (GM-CSF), as well as moderate induction of IL-1α, IL1β, IL-6, IFN-γ and TNF-α (Schaller *et al.*, 2004). This leads to the chemo-attraction of polymorphonuclear leukocytes (PMNLs) to the site of infection, which results in reduced growth of the pathogen and hence less tissue damage. This cytokine response is strongest when the yeast strain is highly invasive (Villar *et al.*, 2005).

1.8.2. *In vivo* studies

i) Dermal effects

The most common use for TTO is in skin and hair care products and for the treatment of acne and skin infections. Therefore most *in vivo* data relates to dermal exposure, either in human users or in animal studies. TTO can be a skin irritant at higher concentrations, and allergies have been reported (de Groot & Weyland, 1992; Rutherford *et al.*, 2007). However, rates have been very low in the 80 years of its commercial use (Carson *et al.*, 2006). A recent study in Australia screened 2320 individuals using TTO patch testing and found 41 (1.8%) gave positive reactions (Rutherford *et al.*, 2007). Only 17 (0.7%) of these people had allergic contact dermatitis. However, this study deliberately used TTO which had been allowed to oxidise, as they found that freshly-opened TTO produced very weak or no reaction. It is thought that degradation products of mono-terpenes are the main allergens in TTO (Hausen *et al.*, 1999). Another study using higher quality TTO in patch-testing found only 1 positive reaction in 217 patients (<0.5%) (Veien *et al.*, 2004). The same group tested four different 5% TTO lotions in 160 patients, resulting in no allergies, but with irritation in 3% of patients. This suggests that a 5% solution of TTO produces skin irritation in a significant number of individuals. TTO is therefore a skin sensitiser and can induce contact allergy (SCCP, 2008). Allergic skin reactions have also been reported after oral intake of TTO. A patient who was using undiluted oil for

external treatment of dermatitis subsequently had more severe dermatitis after ingesting some of the oil (de Groot & Weyland, 1992).

Other plant essential oil products are known to have irritant and allergic effects. Contact dermatitis was seen in 14% of patients given lavender oil in a Japanese study (Sugiura *et al.*, 2000). There have been isolated reports of immediate systemic hypersensitivity (anaphylaxis) to TTO (Mozelsio *et al.*, 2003). For comparison, chlorhexidine exposure can also cause contact dermatitis and anaphylaxis (Evans, 1992; Krautheim *et al.*, 2004).

Several murine studies have shown inhibitory effects on inflammatory processes, including reduced contact hypersensitivity to a chemical hapten (Brand *et al.*, 2002a), reduced histamine-induced oedema (Brand *et al.*, 2002b) and blocking of zymosan-induced inflammation by inhaled TTO (Golab & Skwarlo-Sonta, 2007). In humans, nickel-induced contact hypersensitivity can be reduced by the topical application of 100% TTO (Pearce *et al.*, 2005). Both TTO (Koh *et al.*, 2002) and terpinen-4-ol (Khalil *et al.*, 2004) have been shown to reduce histamine-induced weal and flare reaction in human skin. This contrasts with the studies mentioned above which found immuno-stimulatory effects, such as allergies/contact dermatitis, from TTO used at higher concentrations (Rutherford *et al.*, 2007). This highlights the very complex nature of the immune system and also the complexity of TTO. Studies have also been performed with individual TTO components (T4-ol, *α*-terpineol and *α*-terpinene) in research focusing on other essential oils. These *in vivo* studies showed that T4-ol, *α*-terpineol and *α*-terpinene had direct or indirect anti-inflammatory activity (Moretti *et al.*, 1997; Pongprayoon *et al.*, 1997). Table 1.4 summarises some of these *in vivo* findings.

Table 1.4: *In vivo* immuno-modulatory effects of TTO and components

	Treatment	Effects
Human studies	100% TTO	Reduction in nickel-induced contact hypersensitivity (Pearce *et al.*, 2005)
	100% TTO/ T4-ol	Reduced histamine-induced wheal/flare reaction (Khalil *et al.*, 2004; Koh *et al.*, 2002)
Murine studies	TTO	Reduction in contact hypersensitivity (Brand *et al.*, 2002a), histamine-induced oedema (Brand *et al.*, 2002b) and zymosan-induced inflammation (Golab & Skwarlo-Sonta, 2007)
	T4-ol, *α*-terpineol, *α*-terpinene	Anti-inflammatory effects (Pongprayoon *et al.*, 1997)

ii) Systemic effects of oral exposure

A large number of cases of human intoxication with TTO have been reported, often accidentally in young children and usually with no long term problems. The American Association of Poison Control Center reported 787 incidents in 2003 associated with TTO ingestion (Watson *et al.*, 2004). 66% of these cases occurred in children less than 6 years old, while in a minority of cases (28) adverse reactions occurred after individuals followed the recommendations for the product. However, in the majority of cases outcomes were either no effect, or minor effects such as skin irritation. No deaths have been reported in the literature due to TTO ingestion.

Symptoms following TTO ingestion range from ataxia, drowsiness, nausea, stomach pain and skin rashes, to an extreme case of one individual who ingested half a cup of pure tea tree oil, resulting in a 12 h coma followed by hallucinations then abdominal pain and diarrhoea for 6 weeks (Hammer *et al.*, 2006; SCCP, 2008). TTO is categorised as a Schedule 6 poison in Australia. Substances within this category have " a moderate potential for causing harm, the extent of which can be reduced through the use of distinctive packaging with strong warnings and safety directions on the label " (Hammer *et al.*, 2006). The LD_{50} oral dose of TTO in rats was found to be 2300 mg/kg body weight (Russell, 1999). LD_{50} is the lethal dose that kills 50% of individuals.

1.9 Hypothesis

It is our hypothesis that derivative compounds extracted from *Melaleuca alternifolia* (tea-tree) have powerful antimicrobial properties which could be used as novel mouthwashes and denture-cleansing agents, or to augment current treatments.

1.10 Aims

C. albicans is the predominant yeast associated with oropharyngeal candidosis (OPC), which presents most often clinically as thrush or denture stomatitis. These infections are characterised by multispecies biofilms forming on the oral epithelium or artificial denture surfaces. Current antifungal treatments have limited success due to resistance of biofilms, with recurring infections common. Therefore, alternative methods for suppressing or eradicating such biofilms are desirable. The main aims of this investigation are as follows:

1. Examine the effects of *M. alternifolia* derivative compounds on inhibition and killing of planktonic and adherent populations of *C. albicans.*

2. Examine the toxicological effects of *M. alternifolia* derivative compounds using an oral epithelial cell model.

3. Examine the immuno-modulatory capacity of these compounds.

Chapter 2:
Materials and Methods

2.1 Antifungal susceptibility testing

2.1.1 Chemicals and antifungal compounds

All chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Tea tree oil (TTO) and seven HPLC-grade derivatives of the oil were used in the study. The derivatives used were terpinen-4-ol, *α*-terpineol, 1,8-cineole, terpinolene, *α*-terpinene, γ-terpinene and *p*-cymene. Stock concentrations of each component were freshly prepared for each experiment in RPMI-1640 medium AutoMod™ (RPMI, R7755), containing 0.5% (vol/vol) Tween®80 (ICI Americas Inc.). Tween®80, or polyoxyethylene sorbitan monooleate, is a non-anionic surfactant and emulsifier that aids the dispersal of the TTO and derivatives, some of which are not water soluble. The final concentration of Tween®80 in all susceptibility tests was 0.25% v/v. All stock solutions were vortex mixed thoroughly in plastic bijoux prior to aliquoting for dilutions. Care was taken to ensure consistency when preparing serial dilutions in 96-well plates. For MIC assays, serial dilutions of TTO, T4-ol and *α*-terpineol were prepared in advance in 96-well plates and stored at -80ºC, as this minimised differences in preparing serial dilutions and was shown not to affect antifungal activity of these solutions. Fresh dilutions were used for all other derivatives in MIC testing, while dilutions of TTO and derivatives were prepared freshly each time for sessile experiments (Section 2.1.3). For conventional antifungal drug testing, voriconazole (VRZ, [VFend®] Pfizer Pharmaceuticals, New York, NY, USA) was obtained in a 10 mg/ml vial of clinical formulation, amphotericin B (AMB, [AmBisome®] Gilead Sciences, Foster City, CA, USA) was obtained in a 50 mg vial of clinical formulation, and caspofungin (CSP, [Cancidas®] Merck Sharp Dohme Ltd, White House Station, NJ, USA) was obtained in a 50 mg vial of clinical formulation. Each antifungal drug (VRZ, AMB and CSP) was prepared at stock concentrations of 10 mg/ml in molecular biology grade double distilled sterile water (Invitrogen, Paisley, Scotland) and used within

24 h of reconstitution. Fresh ampoules of antifungal drug were used for each experimental procedure.

2.1.2 *Candida albicans* isolates

One hundred clinical *C. albicans* isolates were used in the course of this study, from a variety of clinical sources. These included isolates from denture stomatitis patients at the Glasgow Dental Hospital and School (n = 26) (Coco *et al.*, 2008), elderly palliative care patients from the Accord Hospice, Paisley (n = 30), neonates from the Royal Hospital for Sick Children (Yorkhill Division) (n = 36), and strains from a Scottish bloodstream infection study (n = 8) (Odds *et al.*, 2007). All strains were maintained on Sabouraud dextrose (SAB) agar (Oxoid, Basingstoke, UK) at 4^{o}C and stored indefinitely on Microbank beads (Prolab Diagnostics) at -80^{o}C. The type strain *C. albicans* ATCC 90028 was used as a control strain.

2.1.3 CLSI broth microdilution susceptibility testing

Antifungal testing to determine the minimal inhibitory concentrations (MIC) of TTO and constituent components in planktonic *C. albicans* cells was performed using the Clinical Laboratory Standards Institute M27-A3 broth microdilution method for yeasts (CLSI, 2008). All preparations of microtitre plates were performed in a Microflow laminar flow biological safety cabinet (Bioquell UK Limited, Andover, UK). 100 µl aliquots of RPMI containing 0.5% v/v Tween® 80 were dispensed into columns 2 to 8 on 96-well microtitre plates (round-bottomed wells), using a multichannel pipette. 200 µl of each stock TTO component was dispensed into column 1 and then serially double diluted to give a final concentration range of 2% to 0.03% (columns 1 to 7) after the addition of yeast suspensions. Note that the final Tween®80 concentration was 0.25%, as each well contained 100 µl of the dilution of the test solution and 100 µl of the yeast

preparation. Column 8 served as a positive control with a final concentration of 0.25% Tween® 80 (100 µl of RPMI / 0.5% Tween®80 added with no TTO components to an equal volume of yeast solution). The plates were sealed with an adhesive lid (Mylar plate sealers, Fisher Scientific, Loughborough, UK) to prevent evaporation of the volatile TTO chemicals, stored at 4°C, and used within 24 h. After initial testing of the first 16 strains, it was clear that lower concentrations of terpinen-4-ol (T4-ol) and α-terpineol ought to be used, as their MIC values were lower than TTO. Also, the other derivatives were ineffective at the lower concentrations, so a more limited dilution range was selected (Table 2.1). For TTO, T4-ol and *α*-terpineol, serial double dilution plates were prepared in advance and stored at -80°C, then thawed as required. This was shown not to diminish the activity of each component, and improved consistency of test solutions.

Each clinical strain was propagated on SAB agar overnight at 30°C. A loopful of culture was taken and a yeast suspension prepared in sterile phosphate buffered saline (PBS: 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 [Oxoid, Cambridge, UK]). This suspension was further diluted 1:10 in PBS to enable enumeration of cells/ml in a Neubauer haemocytometer (Fisher Scientific, UK). Each strain was subsequently adjusted to a density of 10^5 cells / ml in RPMI. 100 µl (10^4 cells) of suspension was added to microtitre wells containing 100 µl TTO components, including the antifungal-free positive control. The microtitre plates were then sealed with adhesive lids and incubated at 37°C for 24 to 48 h. The MIC was determined by directly visualising yeast growth within the microtitre plate and selecting the lowest concentration of each derivative that inhibited growth of the test isolate, compared to the positive control.

Table 2.1: TTO and derivatives, with range of concentrations tested

Component	Planktonic (%)	Biofilm (%)
Pure Tea tree oil	**0.03 - 2**	**0.5 – 4**
terpinen-4-ol, *α*-terpineol	**0.05 - 1**	**0.25 - 2**
1,8-cineole, terpinolene	**0.5 - 1**	**2**
***α*-terpinene, *γ*-terpinene, *ρ*-cymene**	**2**	**4**

2.1.4 Repeat exposure MIC testing

C. albicans strains from 8 palliative care patients were selected to investigate the potential influence of multiple exposures to TTO on the susceptibility to TTO and conventional antifungal agents. MIC testing was performed as described above in a 96-well plate using a more sensitive range of TTO of 0 - 1.2% v/v, in incremental steps of 0.1% (all containing RPMI with 0.25% Tween®80). Subcultures were taken from wells using a 48-pin replica plating tool (Boekel Scientific, Feasterville, PA, USA) and grown on SAB agar overnight to determine the minimum fungicidal concentrations (MFCs). Colonies were then sub-cultured (from the highest TTO concentration giving growth) onto another SAB plate to be used in the subsequent MIC test. CLSI MIC testing was also performed on each strain at the initiation of the experiment and after the final challenge with TTO using amphotericin B, voriconazole and caspofungin at a clinically relevant range (0.0625 to 32 mg/L). This provided an indication of the potential for TTO-derived mouthwashes to alter susceptibility of *C. albicans* strains to these conventional antifungal drugs (Section 2.1.1).

2.1.5 Sessile susceptibility testing

Antifungal susceptibility testing of sessile cells was performed as described previously (Ramage *et al.*, 2001a). Isolates were propagated overnight in yeast peptone dextrose broth (YPD: 1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose [Oxoid]). 10 ml YPD was dispensed into 50 ml centrifuge tubes, then inoculated with a loopful of cells from SAB agar plates containing freshly grown isolates. These were incubated at 30^{o}C overnight in an orbital shaker at 400 revolutions per minute (rpm) [Vortemp1550, Labnet International Inc., Woodbridge, NJ, USA]. All strains grew in the budding–yeast phase. Cells were centrifuged at 3000 rpm for 10 min, and the pellet resuspended in 5 ml PBS. This was diluted 1:100 in PBS and enumerated as described in section 2.1.3. All isolates were adjusted to 1 × 10^6 cells/ml in RPMI 1640. Biofilms were formed on commercially available pre-sterilised, polystyrene, flat-bottomed, 96-well microtitre plates (CLS3628 Corning, Sigma-Aldrich) by pipetting standardised cell suspensions (200 µl of 1 × 10^6 cells/ml) into each well of the microtitre plate (final inoculum of 2 × 10^5 cells/well), with a multichannel pipette, and incubating for 24 h at 37^{o}C. Following biofilm growth, media was removed and TTO components serially double-diluted in RPMI containing 0.25% Tween®80 (final concentration of 0.25% Tween®80, as all serial dilutions were added directly to biofilm in well without any further dilution), to give final concentration ranges, within the microtitre plate, for TTO, T4-ol and α-terpineol as indicated in Table 2.1. Only one concentration was tested for the other components, as lower concentrations were shown to be ineffective against the biofilms. These were incubated at 37^{o}C for 24 h. Antifungal-free wells (with RPMI / 0.25% Tween®80) and biofilm-free wells were included to act as positive and negative controls, respectively. Sessile minimal fungicidal concentrations ($SMFC_{50}$ and $SMFC_{80}$) were determined as the lowest concentration of antifungal giving a 50% or 80% reduction in biofilm metabolic activity relative to the antifungal-free positive

control, using the XTT reduction assay, as described below (Pierce *et al.*, 2008; Ramage *et al.*, 2001a; Ramage & Lopez-Ribot, 2005). Testing of these isolates was performed in duplicate.

2.1.6 XTT- reduction metabolic assay

XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide) was prepared in a saturated solution at 0.5 mg/ml in PBS. The solution was filter-sterilised through a 0.2 μm filter and 11 ml aliquots stored at -80^{o}C. Prior to each assay, an aliquot of stock XTT was thawed, and menadione (10 mM in acetone) added to a final concentration of 1 μM. XTT/menadione solution (100 μl) was then added to each biofilm in a 96-well microtitre plate, and to the control wells to measure background XTT-reduction levels. The plates were then incubated in the dark for up to 3 h at 37^{o}C and the colour change quantified in an automated microtitre plate reader (Tecan Sunrise, Jencons, East Grinstead, West Sussex, UK) at 490 nm. The colorimetric change (colourless to orange) in the XTT-reduction assay directly correlates with the metabolic activity of the biofilm, as previously described (Ramage *et al.*, 2001a). The assay is based on the reduction of XTT tetrazolium to tetrazolium formazan by active mitochondria in the presence of menadione, an electron-coupling agent. XTT was also used in a cytotoxicity assay to measure epithelial cell metabolism, using an optimised 0.25 mg/ml solution in appropriate cell medium (Section 2.2.4.1)

2.1.7 Quantification of biofilm formation

Biofilm biomass was assessed using a crystal violet assay (Mowat *et al.*, 2007). Following biofilm formation, media was removed and the biofilms were air dried at 37^{o}C in an incubator. Crystal violet solution (100 μl 0.5% w/v) was added to each well for 5 min to stain the biofilm. The solution was then removed by carefully rinsing the biofilms under slow-running tap water, holding the plate vertically, until

all unbound stain was washed off. All liquid was carefully removed before the biofilms were destained by adding 100 µl of 95% ethanol into each well. The ethanol was then gently pipetted several times to extract the dye from the cells; each well being treated in an identical manner with a multichannel pipette. 80 µl of stained ethanol was transferred to a clean 96-well microtitre plate and its absorbance read at 570 nm in an automated microtitre plate reader (Tecan Sunrise). The absorbance values are proportional to the quantity of biofilm biomass.

2.1.8 Inhibition of biofilm formation

The effects of TTO and its most efficacious antifungal components on the early stages of biofilm formation were investigated. Four *C. albicans* strains were plated on 96-well plates at 2×10^5 cells/well, as described in section 2.1.5. Cells were treated at 0, 1, 2 or 4 h after plating with TTO (0.5, 0.25, 0.125%), T4-ol (0.25, 0.125, 0.0625%), and α-terpineol (0.25, 0.125, 0.0625%). The cells were then incubated for the remainder of the 24 h period at 37^{o}C. The levels of biofilm inhibition were compared to an untreated control (treated with RPMI / 0.25% Tween®80) using the crystal violet assay, and morphological effects assessed microscopically, *i.e*. blocking of hyphae formation as a mechanism of biofilm inhibition.

In addition, to model a short 1 h denture soak, cells were plated as above and allowed to attach for an hour. Solutions of TTO and derivatives, at the same concentrations as above, were then added to cells for 1 h only. TTO solutions were then removed, biofilm cells washed in PBS and fresh RPMI added for 24 h at 37^{o}C. Biofilms were assessed using crystal violet to quantify biomass.

2.1.9 Effect of adsorbing (coating) substrate with TTO

In order to assess the effect of coating a surface with TTO prior to cells attaching to a surface, 96-well plates were coated with various dilutions of TTO prior to inoculation with *C. albicans*, in order to examine the ability of TTO to inhibit fungal growth and biofilm formation. TTO doubling dilutions were prepared from 2% to 0.015%, in RPMI / 0.25% Tween®80, and 40 µl added to individual wells. Wells with only 0.25% Tween®80 in RPMI were used as controls. Plates were sealed with adhesive lids and stored overnight in the fridge to allow adsorption to the polystyrene plate, then solution was removed and excess liquid allowed to evaporate in a laminar flow cabinet. Four *C.albicans* strains (DS18, 20, 22, 23 - denture stomatitis isolates) were selected to be plated in replicates at 2 x 10^5 cells/well. Plates were sealed with parafilm and incubated overnight at 37^{o}C. Biofilms were examined microscopically to examine morphology, and biofilm was quantified using the crystal violet assay.

2.1.10 Effect of short exposures to TTO and derivative components on pre-formed *C. albicans* biofilm metabolism

Two denture stomatitis *C. albicans* strains (DS19 and DS23 – isolated from denture stomatitis cases) were grown as biofilms on 96-well plate for 24 h (see section 2.1.5). The strains were chosen to represent a weak biofilm former (DS19) and a strong biofilm former (DS23). These biofilms were then treated with 2% TTO, 1% T4-ol or 1% *α*-terpineol for 2, 5, 15 or 60 min. Control biofilms were treated with RPMI / 0.25% Tween®80 for appropriate times. Experiments were performed in quadruplicate for each strain. The test compound was removed after exposure and RPMI used to rinse biofilm briefly. XTT was immediately added as described in Section 2.3.4, to determine relative inhibition of biofilm metabolism compared to untreated biofilm cells.

2.1.11 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the morphology of a 24 h *C. albicans* biofilm, comparing untreated cells with TTO or T4-ol exposed cells. Strain YH28 (neonate throat swab), which forms a strongly adherent *in vitro* biofilm as assessed by metabolic and biomass assays, was prepared as in Section 2.1.5, and then inoculated in a 24-well flat bottom plate at 10^6 cells/ml, each well containing a Thermanox™ coverslip (Nunc Inc, Thermo Fisher Scientific). Cells were allowed to attach to coverslips for 2 h, washed briefly in PBS and then TTO (0.25%), T4-ol (0.125%) or control media (RPMI / 0.25% Tween®80) was added to wells for overnight incubation at 37ºC. Treatment solutions were then removed, coverslips washed carefully in PBS to detach any loosely adherent cells from the biofilm, and processed for SEM.

An additional pilot experiment was designed to test if TTO or T4-ol could prevent formation of a yeast biofilm on denture material. Denture acrylic discs (a kind gift of Dr. Donald Cameron, Department of Restorative Dentistry, University of Glasgow) made from polymethylmethacrylate (PMMA), were pre-treated by soaking for 60 min in 0.5% TTO, 0.5% T4-ol or PBS (control), then inoculated with 1×10^5 *C. albicans* cells (BC071), as described previously, and incubated for 24 h at 37°C. Discs were briefly rinsed in PBS to remove non-adherent cells before processing.

SEM Processing: Biofilms were fixed for 2 h on coverslips or denture acrylic using 2% para-formaldehyde, 2% gluteraldehyde, 0.15 M sodium cacodylate and 0.15% Alcian Blue, pH 7.4 (Erlandsen *et al.*, 2004). The fixative solution was removed and 0.15 M sodium cacodylate buffer added to the samples, which were then stored at 4°C. Cacodylate has an arsenic component that prevents growth of contaminating organisms.

After fixation, the cacodylate buffer was removed and biofilms washed 3 × 5 min with 300 µl fresh buffer to remove any remaining gluteraldehyde. Following this, a solution of 1% Osmium tetroxide (OsO_4) prepared 1:1 with 0.15 M sodium cacodylate buffer was added to the samples and incubated for 1 h. Samples were then washed with distilled water 3 × 10 min to remove osmium. 0.5% aqueous uranyl acetate was added to wells and incubated in the dark for 30 min at room temperature. This step improves the conductivity of the sample. Uranyl acetate was then removed and samples quickly rinsed with distilled water. Samples were then dehydrated 2 × 5 min each in an ascending ethanol series using 30%, 50%, 70%, 90% ethanol, then 4 × 5 min absolute alcohol (100%) followed by 2 × 5 min dried absolute alcohol. Coverslips and denture discs were then placed in a glass petri dish of hexamethyldisilazane (HMDS) for 5 min, then transferred to another dish of HMDS for 5 min before placing in a 24-well plate lined with filter paper (HMDS will corrode plastic) to dry overnight in a dessicator. Critical point drying and attachment of samples to stubs with double-sided conductive tape and sputter coating were performed by Margaret Mullin at the electron microscopy suite (Integrated Microscopy Facility, Joseph Black Building, University of Glasgow) and viewed under a JEOL JSM-6400 scanning electron microscope. It should be noted that SEM dehydrates samples which results in complexity of biofilm structure being lost.

2.2 Mammalian cell interactions with antifungal compounds

Epithelial and fibroblast cell lines were grown as adherent monolayers in 5% CO_2, 37^{o}C. All culture media and supplements were obtained from Invitrogen (Paisley, UK), unless otherwise indicated.

2.2.1 Primary periradicular fibroblasts (PRD)

Primary periradicular disease (PRD) fibroblasts (kind gift of Professor Colin Murray, University of Glasgow), were chosen initially to screen a selection of TTO components to discover potential cytotoxicity issues, as once they are isolated they are easy and inexpensive to culture (Osorio *et al.*, 1998). They were cultured for approximately 12 passages in standard Dulbecco's Modified Eagles Medium (DMEM) with 10% Foetal calf serum (FCS) [F9665, Sigma-Aldrich UK], 2 mM L-glutamine, 100 IU penicillin and 100 μg/ml streptomycin in a 75 cm^2 flask (658170, Greiner). PRD fibroblasts were isolated from tissue specimens obtained from patients undergoing periradicular surgery within the Department of Restorative Dentistry, or routine dental extractions within the Oral Surgery Department. Patients were in good health and not taking long-term medication for chronic conditions. Specimens were collected in DMEM on ice and processed within three hours. Periradicular tissues obtained upon dental extraction were carefully dissected off the dental root apex with a sterile Swan-Morton® scalpel (Swann Morton, Sheffield, UK), taking care to avoid contamination from adjacent periodontal ligament cells or attached periodontal tissue. PRD tissue was subsequently minced and placed in DMEM supplemented with 10% FCS, penicillin/streptomycin/amphotericin B in T25 CellStar® flasks (Greiner Bio-One GmbH, Germany) and placed in a humidified 5% CO_2 incubator at 37^{o}C. Fibroblasts were allowed to outgrow from the PRD tissue fragments over a three-week period and the culture medium changed every 3-4 days.

At sub-confluence, fibroblasts were washed twice with PBS then detached with 0.025% trypsin and 0.05% EDTA (Sigma, Poole, UK) at 37^{o}C for 5 min and seeded into a T75 CellStar® flask (Greiner Bio-One GmbH, Germany). Once PRD fibroblasts had reached 80% confluence, this procedure was repeated to prepare larger quantities of cells for freezing and subsequent experiments. All fibroblasts cultures used in experiments were at passage 6 to 10.

2.2.2 OKF6-TERT2 oral epithelial cell line

OKF6-TERT2 cells (kind gift of the Rheinwald laboratory, Brigham and Woman's Hospital, Boston) are an immortalized human oral keratinocyte cell line. These cells have been immortalized by forced expression of telomerase, and resemble primary oral keratinocytes in studies of cytotoxicity or inducible cytokine and beta-defensin expression (Dongari-Bagtzoglou & Kashleva, 2003). These cells were initially used in the *in vitro* cytotoxicity assay described below and then for immunomodulation studies. OKF6-TERT2 cells were cultured in keratinocyte serum-free medium (KSFM) [37010-022 Invitrogen] supplemented with 100 IU penicillin, and 100 μg/ml streptomycin, 25 μg/ml bovine pituitary extract (BPE), 0.2 ng/ml epidermal growth factor (EGF) and 0.3 mM $CaCl_2$ (0.4 mM total Ca^{2+}). BPE and EGF were filter-sterilised (0.2 μm). Cells were seeded at 5 x 10^3 / ml in a 75 cm^2 flask (10^5 cells / flask). At approximately 30% confluence, media was changed to a mixture of 50% KSFM, 25% DMEM, 25% Ham's F12 with 1.5 mM glutamine, supplemented as above with penicillin / streptomycin, BPE and EGF. Cells were passaged at approximately 90% confluence, using 0.05% Trypsin EDTA. For cytokine assays, cells were cultured in defined-KSFM (10744-019, Invitrogen) to remove batch variability found in BPE. In defined-KSFM, BPE is replaced with defined growth-promoting additives, including insulin, EGF and fibroblast growth factor (proprietary solution – concentrations not given). Also,

BPE only has around one month stability in medium, compared to 3 months stability of defined-KSFM.

2.2.3 TR146 human squamous cell carcinoma cell line

TR146 (Cancer Research Technology, London, UK) is a human epithelial cell line derived from squamous cell carcinomas of the head and neck (Rupniak *et al.*, 1985). These were cultured in DMEM (D5671, Sigma-Aldrich UK) containing 4.5 g/L of glucose and supplemented with 10% foetal calf serum (F9665, Sigma-Aldrich UK), 2 mM L-glutamine, 100 IU penicillin and 100 μg/ml streptomycin in a 75 cm^2 flask (658170, Greiner). These were used in cytotoxicity assays as another epithelial cell line to compare with OKF6-TERT2.

2.2.4 Cytotoxicity assays

The above human cells were used to determine cytotoxic concentrations of TTO and various components. TTO and component dilutions were prepared in KSFM with 0.25% Tween®80 (KSFMT). 0.12% chlorhexidine gluconate [C9394, 20% aqueous solution, Sigma-Aldrich] (CHX) in KSFMT was used as a positive control, as this has been previously demonstrated to exhibit cytotoxic effects on epithelial cells (Eren *et al.*, 2002; Shakespeare *et al.*, 1988)**.** KSFMT was also used for the negative (untreated) control cells, *i.e*. control cells were exposed to KSFMT without TTO or component, which had no adverse effects on cellular viability and morphology.

2.2.4.1 Modified XTT-reduction assay

Cells were added to a 96-well plate at 2 x 10^4 cells / well in KSFM (or DMEM / 10% FCS for fibroblasts) and incubated for 18 h, or until cells were 70 to 80% confluent. Media was then removed and cells treated with 50 μl test compound for 2 min. Test compound was removed and cells washed gently 3 times in 150 μl Hanks' balanced salt solution (HBSS, Sigma-Aldrich UK). A modified XTT

reduction assay was used to assess metabolism of treated cells (Section 2.1.6). 100 µl of 0.25 mg/ml XTT in KSFM (or DMEM / 10% FCS for fibroblasts) was added to each well and plates incubated in 5% CO_2 at 37^{o}C for 2 to 3 h. The colour change was then quantified in an automated microtitre plate reader at 490 nm (Tecan Sunrise, Jencons, UK).

2.2.5 Assessing interleukin-8 production by zymosan-stimulated OKF6 cells

OKF6-TERT2 cells were inoculated into 96-well plates, at 2 x 10^4 cells / well in defined-KSFM and incubated for 18 h in 5% CO_2 at 37^{o}C. Yeast-derived zymosan A (*S. cerevisiae* cell wall component Z4250, Sigma-Aldrich) was used as a positive control which activates inflammatory mediators (Huber & Weiss, 1989). A stock solution was prepared in dimethyl sulphoxide (DMSO >99.5%, 60153, Sigma-Aldrich) at 30 mg/ml, which was then diluted in cell-culture medium (defined-KSFM) to final concentrations between 10 and 100 µg/ml (maximum final concentration of 1:300 DMSO). This concentration of DMSO was compared with cell culture medium alone and found to have no impact on mRNA or protein expression (data not shown). Zymosan A (zym) is a glucan with repeating glucose units connected by β-1,3-glycosidic linkages, which closely resembles glucans found in candida cell wall. Zym binds to Toll-like receptor 2 (TLR2), activating transcription of inflammatory cytokine genes (Netea *et al.*, 2002; Roeder *et al.*, 2004). A dose response curve was initially performed with various concentrations (0, 10, 100 µg/ml, Figure 6.1). Supernatants were subsequently harvested at 4 and 24 h, stored at -80^{o}C, then processed for detection and quantification of extracellular interleukin 8 (IL-8) protein using enzyme-linked immunosorbent assays (ELISA), as described in Section 2.4.2. Experiments were performed in triplicate on 2 independent cell preparations.

2.2.6 Assessing IL-8 production by zymosan-stimulated OKF6 cells pre-treated with TTO and derivative components

OKF6-TERT2 cells were plated and grown in 1.6 ml defined-KSFM in a 12-well cell culture dish, at a density of 2 x 10^5 cells / well and incubated for 18 h in 5% CO_2 at 37^{o}C to give cells at around 80% confluency. Media was then removed prior to a 2 min treatment with 400 µl 0.125% TTO, 0.125% T4-ol or control media, all in defined-KSFMT. Solutions were then removed and cells gently washed 3 times with 2 ml HBSS. Finally, 2 ml of 50 µg/ml zym (containing1/600 DMSO, *i.e.* 0.167% DMSO) was added to half the wells, while defined-KSFM with the equivalent concentration of DMSO (0.167%) was added to control wells, followed by incubation for 4 or 24 h. Supernatants were harvested and frozen at -80^{o}C for subsequent analysis by cytokine protein array (Section 2.4.1) and ELISA to quantify IL-8 protein (Section 2.4.2). In addition, cells were harvested from the 12-well plate into 1 ml TRIzol® (Invitrogen, Paisley, UK), according to the manufacturer's instructions. The TRIzol® cellular suspension was then stored at -80^{o}C. RNA was subsequently extracted for use in PCR to quantify gene expression (Section 2.3).

2.3 Gene expression studies

2.3.1 RNA purification

TRIzol® samples were removed from the -80^{o}C freezer, thawed to room temperature and processed for RNA purification according to the manufacturer's instructions (Invitrogen, Paisley, UK). Briefly, 100 µl bromochloropropane (Fisher Scientific) was added, the samples mixed by vortexing for 30 seconds then centrifuged at ×13000 g (relative centrifugal force) for 15 min at 4^{o}C. The upper aqueous clear layer was then removed and placed into a clean microcentrifuge tube and an equal volume of isopropanol (100%) added. This was then stored overnight at -20^{o}C to precipitate the RNA, before centrifugation at ×13000 g for

15 min at 4^{o}C. The resulting pellet was washed with 1 ml of 70% ethanol, air dried and resuspended in 15 µl of RNase-free water at 60^{o}C for 10 min. RNA was then treated with 2 units RQ1 Dnase I (Promega, Southampton, UK) at 37°C for 30 min to remove contaminating genomic DNA, with a further 40 units RNase OUT Ribonuclease inhibitor (Invitrogen) added to minimise RNA degradation. Dnase I was then inactivated by adding 2 µl stop solution and incubating at 65^{o}C for 10 min. RNA concentration and quality was then assessed using a NanoDrop™ spectrophotometer ND-1000 (Labtech International Ltd, Ringmer, East Sussex, UK), prior to reverse transcription (RT) to make cDNA.

2.3.2 RT-PCR (Reverse transcription polymerase chain reaction)

cDNA was prepared using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Briefly, 500 ng RNA was incubated in RNase-free water with 0.5 µg Oligo(dT)$_{12\text{-}18}$ (Invitrogen) and 10 mM dNTP mixture at 65^{o}C for 5 min, quick chilled, then reverse transcribed using 100 U Superscript II RT (Invitrogen) at 42^{o}C for 50 min in the presence of the manufacturer's buffer (50 mM Tris HCL (pH 8.3), 75 mM KCL, 3 mM $MgCl_2$) with 0.01 M DTT and 1 µl (40 U) RNase OUT Ribonuclease inhibitor (Invitrogen) in a total volume of 25 µl. Finally, the Superscript was inactivated by heating to 70^{o}C for 15 min. Oligo (dT) binds to the poly-A tail of messenger RNA (mRNA), producing cDNA from any RNA transcripts.

The presence of genomic DNA contamination of the final synthesised cDNA was evaluated using PCR with primers known to amplify both genomic and cDNA, on no-RT control RNA, performed in the absence of Superscript. Any RNA sample associated with a visible band was deemed to be contaminated, re-treated with DNase and re-examined for residual DNA contamination. cDNA was used immediately in PCR or stored at -20^{o}C. To amplify the gene of interest, 0.5 µl of

cDNA was added to 47 µl of 1.1× ReddyMix™ PCR Mastermix (ABgene, Epsom, UK, containing 1.25U Thermoprime *Taq* DNA polymerase, 75 mM tris-HCl, 20 mM $(NH_4)_2$ SO_4, 1.5 mM $MgCl_2$, 0.01% Tween®20, 0.2 mM each dNTP, plus precipitant and red dye for loading into gel) and 1 µl each of 10 mM forward and reverse primers for the interleukin genes IL-1β, IL-6, IL-8 and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are shown in Table 2.2.

The PCR conditions were as follows:

94 ºC / 2 min

94 ºC / 30 sec
50 ºC / 30 sec x 35 cycles
72 ºC / 30 sec

72 ºC / 10 min

Standard PCR analysis was performed to determine relative end-product amounts, after all cycles were completed. PCR products were visualised on an agarose gel as described below.

2.3.3 Horizontal gel electrophoresis

The PCR products were run on a 1.8% (w/v) agarose gel (Invitrogen), prepared with 0.5 × Tris borate buffer (1 litre 0.5 × TBE contains: 5.4 g tris base [Fisher Scientific, UK]; 2.75 g boric acid [Fisher Scientific, UK], 2 ml 0.5 M EDTA pH 8.0 in distilled H_2O). 50 ml agarose solution (Sigma-Aldrich, UK) was heated in a microwave oven until it was molten, cooled to 50°C and 5 µl of ethidium bromide (10 mg/ml) added and mixed. This was then poured into a casting tray and allowed to solidify at room temperature. 15 µl of PCR product was loaded into each well. 500 ng (1 µl) of 100 bp DNA ladder (New England Biolabs, UK) was mixed with loading dye and loaded into a separate well to allow estimation of

PCR product size. The tank was connected to the electric source and run at 65 V for 1 h. The gel was observed under the UV light excitation and the image digitally photographed (Bio-Rad Gel Doc 2000, Bio-Rad Life Sciences, Hemel Hempstead, UK).

2.3.4 Real-time quantitative PCR

A better method for accurate measurement of gene expression (mRNA) is real-time PCR. This measures the DNA product at each cycle, which in turn is relative to amount of mRNA in sample template, and allows calculation of changes in gene expression caused by various treatments.

A series of preliminary experiments were performed to optimise the quantitative PCR reactions. This helped prevent both inhibition problems from using excess cDNA and also sensitivity / reproducibility issues from using too little cDNA (data not shown). Serial dilution of cDNA was performed, demonstrating consistent linear amplification of cDNA using an MxPro MX3000P Quantitative PCR machine and analysed using the Mx3000P software (Stratagene, Amsterdam, Netherlands). Standard curve analysis was undertaken for all primer pairs detailed in Table 2.2. Standard curves were then analysed, with R Squared (R^2) and amplification efficiency values exceeding 0.9 and 90% deemed acceptable. Each 50 µl real-time PCR reaction contained the following: 0.5 µl of 1 µg/µl cDNA, 0.5 µl forward and reverse primer (10 nmol) (Invitrogen), 0.25 µl ROX reference dye (Invitrogen), 12.5 µl SYBR® Green (Invitrogen) and 10.5 µl of molecular biology grade dH_2O.

PCR reaction conditions were as follows:

95 ºC / 10 min

94 ºC / 30 sec
58 ºC / 30 sec x 40 cycles
72 ºC / 30 sec

72 ºC / 10 min

For each condition 1 in 20 dilutions of cDNA samples were prepared, and 2 μl of this was PCR-amplified using 200 nM primers (0.5 μl forward and reverse primers at 10 μM) and SYBR green (Invitrogen), as described above. Reactions were performed in duplicate in 0.2 ml optical tube strips (Agilent Technologies, South Queensferry, West Lothian, UK) using the Mx3000P QPCR machine (Agilent technologies). A standard dissociation curve protocol was included after the 40 amplification cycles to confirm that only one product was made and this was also confirmed in initial experiments by running products on a 2% agarose gel. The threshold cycle (C_t) was automatically determined and verified manually. Amplification plots were observed at the logarithmic scale and threshold selected to be above any baseline fluorescence, in the region where amplification was exponential (steep curve) and where the majority of replicates gave very similar C_t values, *i.e*. where duplicate amplification plots were parallel. IL-6 and IL-8 values were normalised using GAPDH as a housekeeping gene. No-RT controls were included to check for DNA contamination and no template controls, with water, were run to rule out other contamination problems.

Table 2.2: PCR primer sequences, showing location and product size

Target	Sequence of primer	Binding site	Product size (base pairs)
IL-1β F	TCCCCAGCCCTTTTGTTGA	1103 -1121	90
IL-1β R	TTAGAACCAAATGTGGCCGTG	1173 -1193	
IL-6 F	CAATCTGGATTCAATGAGGAGAC	373 - 393	115
IL-6 R	CTCTGGCTTGTTCCTCACTACTC	466 - 488	
IL-8 F	CAGAGACAGCAGAGCACACAA	21 - 41	170
IL-8 R	TTAGCACTCCTTGGCAAAAC	171 - 190	
GAPDH F	CAAGGCTGAGAACGGGAAG	282 - 300	133
GAPDH R	GGTGGTGAAGACGCCAGT	397 - 414	

2.4 Protein expression studies

2.4.1 Cytokine array

A human cytokine protein array kit, Proteome Profiler panel A (ARY005, R&D Systems, Abingdon, UK), was used to measure cytokine production from OKF6-TERT epithelial cells in response to TTO and terpinen-4-ol, both in the presence and absence of zymosan A (zym). The kit contains nitrocellulose membranes pre-blotted with duplicates of 36 capture antibodies to human cytokine proteins (Figure 2.1A). Cell supernatants can be mixed with a cocktail of biotinylated detection antibodies and incubated with the membrane. Any cytokine/detection antibody complex will bind to the membrane via the capture antibody. Proteins are detected using Streptavidin-horseradish peroxidase and chemiluminescent detection reagents. The level of light produced at each spot is proportional to the amount of bound cytokine. This is a very useful way to simultaneously look at the relative levels of different cytokines within a sample.

A

+ve	C5a	CD40	G-CFS	GM-CSF	GROα	I-309	sICAM-1	IFNγ	+ve
	IL-1α	IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-8	
	IL-10	IL-12p70	IL-13	IL-16	IL-17	IL-17E	IL-23	IL-27	
	Il-32α	IP-10	I-TAC	MCP-1	MIF	MIP-1α	MIP-1β	Serpin E1	
+ve	RANTES	SDF-1	TNF-α	sTREM-1	-ve				

B

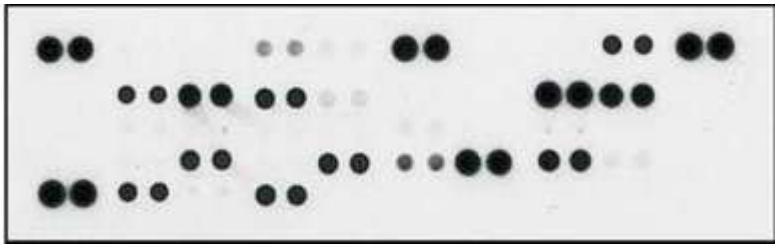


Figure 2.1: Cytokine array membrane.

A) schematic of the membrane layout

B) example of a processed membrane

Kits were used following manufacturer's instructions, with all incubations done at room temperature (20℃), all buffers were supplied by the manufacturer (formulation undisclosed). Briefly, membranes were blocked in 2 ml array buffer 4 for 1 h on rocking platform. Supernatants were briefly centrifuged to remove particulates then 1 ml was mixed with 500 µl array buffer 4, 15 µl detection antibody cocktail and this mixture incubated for 1 h. Buffer was then removed from membranes, sample mixture added and membranes incubated overnight on rocking platform at 4^{o}C.

Membranes were washed 3 times in 20 ml wash buffer, then 2 ml of 1/2000 Streptavidin-HRP antibody was incubated with membrane for 30 min on rocker, followed by 5 x 15 min washes in 30 ml wash buffer. Extensive washing was used to minimise background. 500 µl immobilon western chemiluminescent detection reagent (Millipore) was put onto membrane, carefully covering the whole surface, and incubated for 5 min. Excess solution was drained off the membrane, blots put in clingfilm and exposed to X-ray film. Digital images were taken of the developed films using a Bio-Rad Gel Doc 2000 (Bio-Rad Life

Sciences, Hemel Hempstead, UK) and spot density analysed using Quantity One software (Biorad).

2.4.2 ELISA Assays

Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK) were used in accordance with the manufacturer's instructions. All assays were optimised and validated prior to use. Immulon 4 HBX flat-bottom 96-well microtitre plate (Fisher Scientific, UK) were coated with IL-8 capture antibody (4 μg/ml in PBS), sealed and incubated overnight at 4°C. All subsequent incubations were at room temperature. 200 μl blocking buffer was added for 1 h. Standards and samples were added in duplicate or triplicate, incubated for 2 h, and then biotinylated IL-8 detection antibody (20 ng/ml in TBS + 0.1% BSA + 0.05% Tween®20) was added and incubated for 2 h. Streptavidin-horseradish peroxidase (HRP) was added for 20 min, protected from light. Plates were developed with 100 μl 3,3′,5,5'-tetra-methylbenzidine (TMB, R&D Systems, UK). Blue colour was allowed to develop in the dark, and plates were read at 630 nm with an automated microtitre plate reader (Tecan Sunrise, Jencons, UK). Between each step described above, the plate was washed three times with wash buffer. For each wash, wells were filled with wash buffer and allowed to stand for at least 2 min prior to removal of buffer. After the third wash, remaining wash buffer was removed by pounding the plate on paper towels. The detection range for the assay was 60-2000 pg/ml. A standard curve was prepared to calculate concentration of IL-8 in samples.

ELISA Reagents

Blocking buffer: PBS +1% BSA

Wash buffer: PBS pH 7.2 with 0.05% Tween®20

TBS: Tris-buffered saline 20 mM Trizma base, 150 mM NaCl pH 7.2 - 7.4

Detection antibody buffer: TBS + 0.1% BSA / 0.05% Tween®20, 0.2 μm filtered

Substrate solution: 1:1 mix of colour reagent A (H_2O_2) + colour reagent B (tetra-methylbenzidine DY999, R&D Systems, UK).

2.5 Statistical analysis

Graphpad Prism, version 4.0 for Windows (Graphpad Software, CA, USA) and SPSS® software (Statistical Package for the Social Sciences, Chicago, USA) were used for statistical analysis to determine significant changes, p values less than 0.05. p is the probability of seeing a difference, by chance, as large as the observed difference, where there really is no difference (Altman,1991).

2.5.1 Independent data analysis

One-way analysis of variance (ANOVA) and Dunnett's t-tests were performed to compare untreated controls with all treatments of *C. albicans* strains, human cells (toxicity and gene expression studies), and pre-treated surfaces. In antifungal experiments, ANOVA Kruskal Wallis tests and Dunn's multiple comparison tests were performed on all datasets, to test for significant differences in patient groups, regarding sensitivity of strains to TTO and components. Time-course inhibition data was analysed using the Jonckheere-Terpstra non-parametric multiple comparison test with SPSS®, to test for ordered differences among groups treated with different drug doses at different time-points. This was followed by a one-way ANOVA with Bonferroni's correction for multiple comparisons to identify significant changes due to exposure time or antifungal concentration.

2.5.2 Non-independent data analysis (replicate data)

Using the General Linear model in SPSS® software, replicate data in single experiments were analysed by repeated measures ANOVA, with Bonferroni's correction for multiple comparisons, comparing all time-points/drug doses to no-exposure controls, to determine treatments giving statistical significant effects (Olsen, 2003).

Further details are given in results sections.

Chapter 3:
Antifungal Effects of TTO and Derivative Components

3.1 Introduction

Tea tree oil (TTO) has been shown to be an effective antibacterial and antifungal agent in a number of studies (Martin & Ernst, 2004; Willcox, 2005). It has activity against many bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa (*Papadopoulos *et al.,* 2006*)*, and has been investigated in relation to both microbial planktonic and biofilm growth. An earlier study from within our research group, investigating the effect of TTO on a wide variety of oral yeast species of varying antifungal sensitivity (Bagg *et al.*, 2006), showed that TTO was an effective *in vitro* antifungal agent (with MIC_{50} of 0.5% TTO), and both azole-resistant and azole-sensitive strains of planktonic *Candida albicans* and *Candida glabrata* were equally susceptible to TTO. 88% of the azole-resistant strains exhibited an MIC of 0.5% TTO or less. This current study focuses on *C. albicans*, as it is the main OPC pathogen, partly because it forms biofilms more readily than many other *Candida* spp.; this is also seen *in vitro*, where *C. glabrata* isolates generally do not form biofilms. Further studies have also reported that TTO has potent inhibitory effects against *C. albicans* using a variety of methodologies (Hammer *et al.*, 2004; Vazquez *et al.*, 2000). Whilst these studies have supported the evidence for the broad antimicrobial activity associated with TTO, its complex constituent nature has hindered its development as a drug, due to variation in the composition of the oil. Therefore, understanding the nature of the active component(s) of TTO is pivotal to optimizing TTO as an antifungal agent.

Mondello and colleagues (2006) previously observed that T4-ol was the most effective component, and that it exhibited activity against both azole-sensitive and azole-resistant *C. albicans* (Mondello *et al.*, 2006). Similarly, previous studies have reported that this component demonstrates both superior antifungal activity (Hammer *et al.*, 2003a; Terzi *et al.*, 2007) and antibacterial activity (Hammer *et*

al., 2003b; Raman *et al.*, 1995). Both T4-ol and α-terpineol have hydroxyl groups in their chemical structures, making them moderately water-soluble, which allows them to diffuse through water and enter cell membranes, unlike the water-insoluble derivatives tested in this study which were not effective at inhibiting growth of *C. albicans*, presumably due to their inert structure, *i.e.* lacking hydroxyl groups on their terpene ring.

Increasing use of conventional antifungals in parallel with larger groups of susceptible individuals (ageing population and immunosuppressive therapies more common), has resulted in the emergence of multidrug-resistant *Candida* strains (Akins, 2005; Cannon *et al.*, 2007; Niimi *et al.*, 2010; Sanglard & Odds, 2002), which require novel approaches to control. This risk is amplified by the rates of non-compliance for recommended drug treatment, caused by side-effect issues and an unpleasant taste. *Candida glabrata* has emerged in recent years as an oral pathogen, and azole-resistance has been shown to be more common in this species and other non-albicans *Candida* spp. (Bagg *et al.*, 2003; Li *et al.*, 2007). *C. glabrata* can be innately less susceptible to azoles than *C. albicans*, but can also develop resistance with repeated exposure to the drugs. One of the main mechanisms of azole resistance is over-expression of efflux-pump genes to allow increased removal of drug molecules from the cell (Cannon *et al.*, 2009; Niimi *et al.*, 2010). In addition to the drug-barrier effect of the biofilm lifestyle, there is the opportunity for transfer of these efflux-pump genes from *C. glabrata* to *C. albicans*, conferring drug resistance in these mixed populations. This can be seen as a symbiotic relationship, with the strong biofilm forming *C. albicans* giving protection to the *C. glabrata* cells, while *C. albicans* can potentially benefit from acquisition of beneficial genes.

TTO treatment may have value in controlling these problem infections, but because this is a complex mixture of derivative components it is difficult to develop as a defined antifungal agent. These studies sought to elucidate which components of TTO demonstrate highest antifungal activity (both on sessile and planktonic growth) and investigate the effects of repeated exposure to TTO on susceptibility of *C. albicans* to TTO and conventional drugs.

3.2 CLSI (M27-A) MIC susceptibility testing of *C. albicans*

The planktonic minimal inhibitory concentrations (PMIC) of tea tree oil (TTO) and 7 monoterpene derivatives were determined for one hundred clinical strains of *C. albicans*, as described in section 2.1.3. A summary of this data is shown in Table 3.1.

Table 3.1: Planktonic MIC values (%) for *C. albicans* isolates (n = 100)

	PMIC ATCC 90028	$PMIC_{50}$ range	$PMIC_{50}$	$PMIC_{90}$
Tea tree oil	**0.25**	**0.125 – 1.0**	**0.5**	**1.0**
terpinen-4-ol	**0.125**	**0.0625 – 0.5**	**0.25**	**0.5**
α-terpineol	**0.125**	**0.125 – 0.5**	**0.25**	**0.5**
1,8-cineole	**>1.0**	**0.5 – >1**	**1.0**	**>1.0**
terpinolene	**>1.0**	**0.5 – >1.0**	**>1.0**	**>1.0**
α-terpinene	**>2.0**	**2 – >2**	**>2.0**	**>2.0**
γ-terpinene	**>10**	**2 – >2**	**>2.0**	**>2.0**
ρ-cymene	**20**	**2 – >2**	**>2.0**	**>2.0**

The $PMIC_{50}$ and $PMIC_{90}$ are defined as the lowest concentrations required to inhibit growth of 50% and 90% respectively of the *C. albicans* strains tested; *i.e.* 50% of isolates growing in the planktonic phase of growth were inhibited by

0.5% TTO, and all strains tested were inhibited by 1% TTO. Terpinen-4-ol (T4-ol) and *α*-terpineol exhibited stronger inhibitory effects, with both showing activity at 0.25%, i.e. effective at half the $PMIC_{50}$ of TTO. 1,8-cineole and terpinolene were less active than TTO, both requiring at least 1% to inhibit at least 50% of strains. The other derivatives tested were ineffective at 2% v/v.

$PMIC_{50}$ concentrations for all strains in a specific patient group, *i.e*. denture (n=26), palliative (n=30), neonate (n=36) and candidaemia (n=8), were plotted together to compare the susceptibility of *C. albicans* strains isolated from different groups to the most active TTO components. The data indicated that the $PMIC_{50}$ for each group were within the same range for TTO, T4-ol and *α*-terpineol (Figure 3.1). Nevertheless, statistically significant differences between groups were observed, but only for TTO and *α*-terpineol with regards to their planktonic MICs when using a Kruskal-Wallis test ($p<0.0001$ and $p=0.0122$, respectively). In Dunn's multiple comparison tests, no statistical differences in $PMIC_{50}$ values between clinical groups were observed for T4-ol and *α*-terpineol treatment ($p>0.05$), whereas for TTO treatment the $PMIC_{50}$ for denture isolates was significantly higher than for both the palliative care group ($p<0.01$) and candidaemia group ($p<0.001$) (Figure 3.1, Table 3.2). Mann Whitney t-tests were subsequently performed for the α-terpineol MICs groups, based on earlier statistical analysis with the Kruskal-Wallis test that indicated significance, and it was shown that the $PMIC_{50}$ values for palliative candida strains were significantly lower than for the denture ($p=0.024$) and neonate ($p=0.026$) groups.

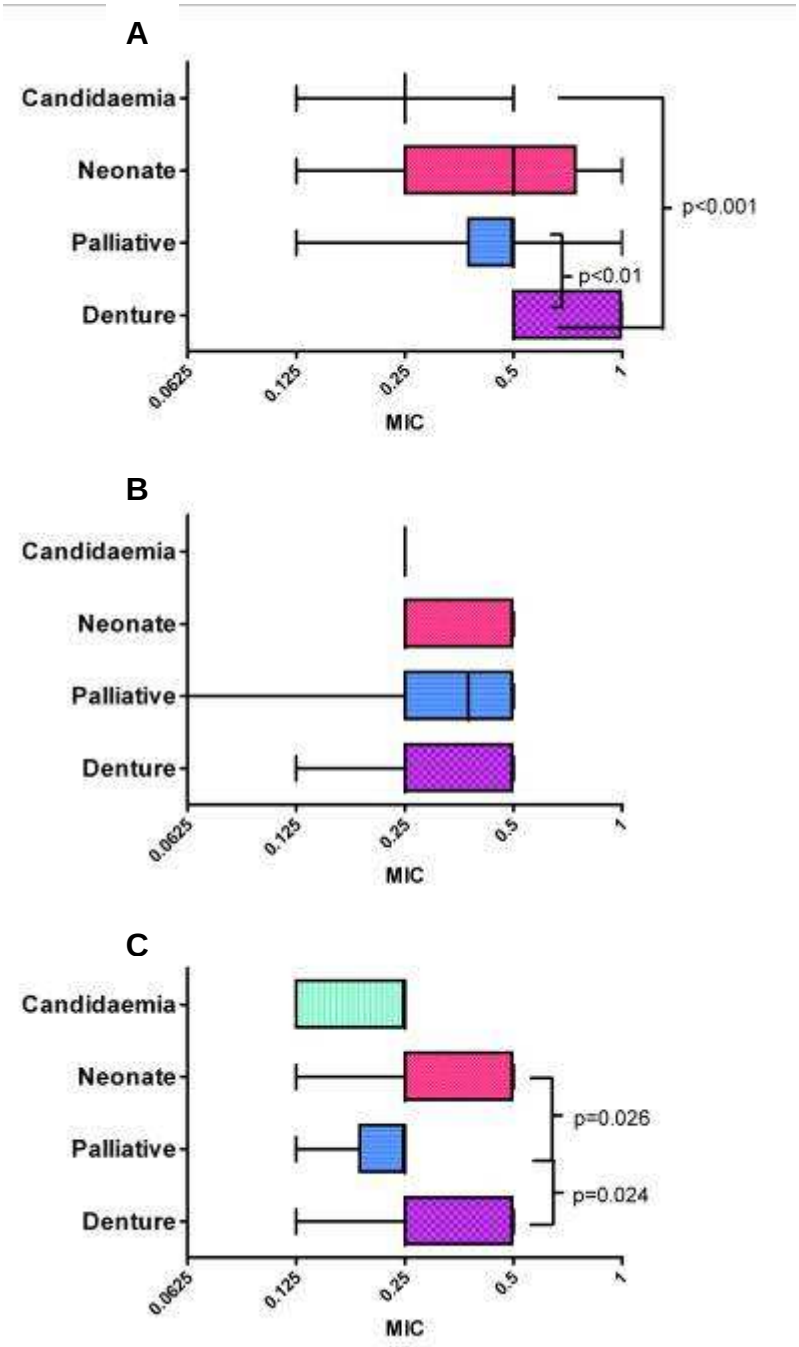


Figure 3.1: $PMIC_{50}$ of TTO and selected monoterpene components

Planktonic MIC's of *Candida albicans* isolates from different patient groups were determined for **A**) TTO, **B**) T4-ol and **C**) *α*-terpineol. Box and whisker plots show range of $PMIC_{50}$ values, with boxes indicating inner quartile range and median (long vertical line). Isolates: denture (n=26), palliative (n=30), neonate (n=36) and candidaemia (n=8). Differences between patient groups were assessed by a one-way Anova Kruskal-Wallis test, Dunn's multiple comparison tests (Table 3.2) and Mann Whitney u-test (where appropriate). Statistically significant p values are indicated in the figure.

Table 3.2: Dunn's multiple comparison tests on TTO $PMIC_{50}$

Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
Neonate vs Denture	-19.03	P < 0.05	*
Neonate vs Palliative	4.516	P > 0.05	ns
Neonate vs Candidaemia	30.33	P < 0.05	*
Denture vs Palliative	**23.55**	**P < 0.01**	**
Denture vs Candidaemia	**49.36**	**P < 0.001**	***
Palliative vs Candidaemia	25.82	P > 0.05	ns

Isolates: denture (n=26), palliative (n=30), neonate (n=36) and candidaemia (n=8)

3.3 Effects of repeated TTO exposure on *C. albicans* susceptibility

Given that TTO has potential as an oral hygiene product, it is likely that there would be repeated use of the product on a daily basis. To assess whether this may have an impact on sensitivity shifts to TTO a limited repeated exposure experiment was designed and performed, as described in section 2.3.2. Planktonic MIC testing for TTO was performed on 8 selected palliative care *C. albicans* strains using CLSI methodology, using a specifically defined decimal range of TTO (0 to 1.2% in incremental steps of 0.1%). The initial PMIC for TTO ranged from 0.125 to 0.5%. Subcultures were taken from wells using a replica plating tool and grown on Sabourauds agar overnight to determine MFC values. Colonies were then sub-cultured onto another SAB plate to be used in the next MIC test. After ten repeated exposures MIC values fluctuated for the majority of strains from 0.2% to 1% TTO (Figure 3.2). Strain 8 maintained a stable TTO PMIC at 0.8%, until the last pass (decreased to 0.6%). Any decreased sensitivity found during the experiment was not maintained to the endpoint. 1% TTO always

inhibited growth of every strain tested. No strain had decreased TTO sensitivity after 10 cycles of growth in TTO.

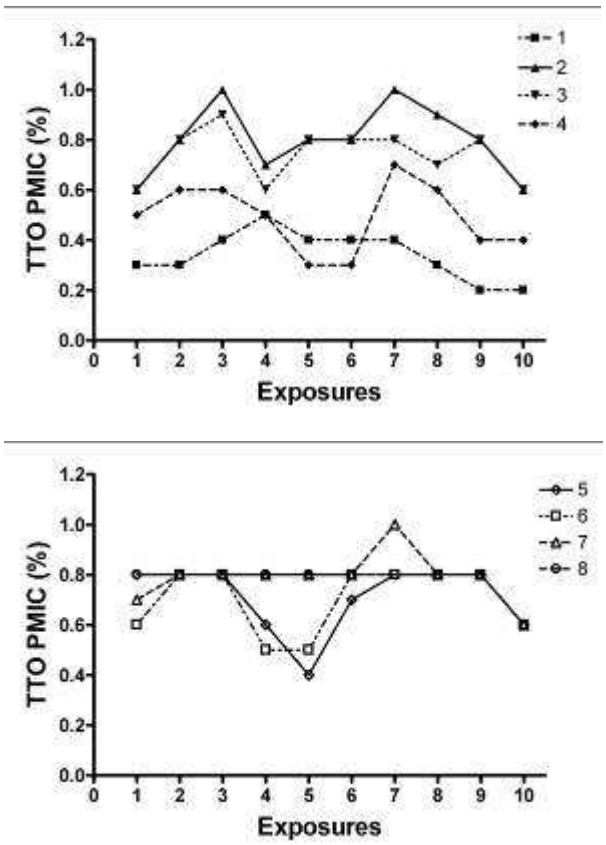


Figure 3.2: Changes in TTO PMIC after repeat TTO exposures of *C. albicans* isolates (1 to 8) from palliative care patients. PMIC values fluctuated by around 0.4% for each strain, while no PMIC above 1% TTO was found. Decreased TTO sensitivities were not maintained by any strains.

In addition, susceptibilities of these 8 strains to conventional antifungal drugs (amphotericin B, voriconazole and caspofungin) were determined after 1 and 10 exposures to TTO (Table 3.3). Decreases in susceptibility to amphotericin B were observed after 10 exposures in strain 7 (0.125 µg/ml compared to original of 0.06 µg/ml) and also to caspofungin in strain 3 and 8 (0.5 µg/ml compared to original 0.25 µg/ml). All other susceptibilities remained stable. Strain 5 was tested at intermediate exposures to determine if susceptibility to conventional antifungals changed during the experiment. There was no change in the antifungal susceptibility profiles (data not shown).

Table 3.3: Conventional antifungal PMIC values (mg/L) for 8 *C. albicans* strains after 1 and 10 exposures to TTO

	Strain	1	2	3	4	5	6	7	8
	Exposures								
AMB	1	0.03	0.06	0.06	0.06	0.06	0.06	0.06	0.06
	10	0.03	0.06	0.06	0.06	0.06	0.06	**0.125***	0.06
VRZ	1	< 16	16	512	62	512	256	256	512
	10	< 16	16	512	62	512	256	256	512
CSP	1	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25
	10	0.25	0.25	**0.5***	0.25	0.25	0.25	0.25	**0.5***

(**AMB**: amphotericin B, **VRZ**: voriconazole, **CSP**: caspofungin)

* bold denotes change in PMIC after 10 exposures to TTO

3.4 Sessile susceptibility testing of *C. albicans* pre-formed biofilms

Sessile susceptibility testing was performed as previously described (Ramage *et al.*, 2001a). The clinical isolates were grown for 24 h to form biofilms and then incubated with TTO or its derivatives (in RPMI / 0.25% Tween®80) for a further 24 h. Inhibition was calculated by comparing with control biofilms incubated for 24 h in RPMI / 0.25% Tween®80. The 50% and 80% sessile minimal fungicidal concentrations (SMFC) of TTO and 7 derivatives were determined using an XTT assay for all *C. albicans* strains, as shown in Table 3.4. Strains which formed poor biofilms were excluded from the analysis, leaving 69 isolates in this part of the study. An example of the XTT assay is shown in Figure 3.3.

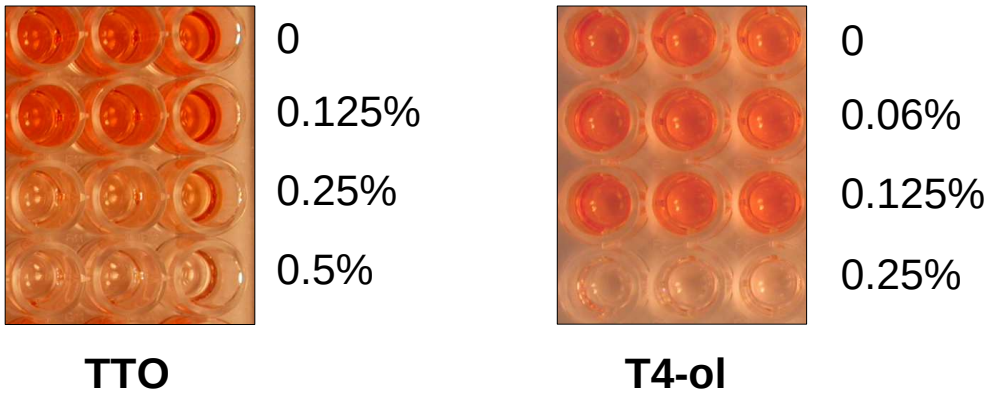


Figure 3.3: Treated *C. albicans* biofilms in wells of 96-well plates, showing orange colour produced by cell metabolism of XTT. Overnight treatment with 0.25% concentrations of TTO or T4-ol effectively kills cells, showing as pale orange wells. Lower concentrations have little effect.

Table 3.4: Sessile MFC values (%) for *C. albicans* isolates (n = 69)

	*$SMFC_{50}$ Range	*$SMFC_{50}$	$SMFC_{80}$
TTO	**1 – 4**	**1**	**2**
terpinen-4-ol	**<0.25 – >1**	**0.25**	**0.5**
α-terpineol	**<0.25 – 1**	**0.25**	**0.5**
1,8-cineole	**<2 – >2**	**2**	**>2**
terpinolene	**2 – >2**	**>2**	**>2**
α-terpinene	**4 – >4**	**4**	**>4**
γ-terpinene	**4 – >4**	**>4**	**>4**
ρ-cymene	**4 – >4**	**>4**	**>4**

*$SMFC_{50}$ and $SMFC_{80}$ are defined as the concentrations required to inhibit biofilm metabolism by 50% and 80% respectively, compared to the control biofilm in RPMI / 0.25% Tween®80, as quantified by the XTT assay.

1% TTO reduced biofilm metabolism by at least 50% in the majority of strains ($SMFC_{50}$ = 1%). T4-ol and *α*-terpineol were more active than TTO ($SMFC_{50}$ = 0.25%), effectively inhibiting biofilm metabolism at the planktonic MIC_{50} concentration. 1,8-cineole gave an $SMFC_{50}$ of 2%, double that of TTO. The remaining derivatives exhibited poor inhibition of biofilm metabolism ($SMFC_{50}$ > 2%). In summary, TTO was shown to have strong antifungal properties against a wide range of clinical *C. albicans* isolates and the two derivatives, T4-ol and *α*-terpineol, were both more potent than the TTO mixture.

$SMFC_{50}$ concentrations for all strains in a specific patient group, *i.e.* denture (n=12), palliative (n=25), neonate (n=24) and candidaemia (n=8), were plotted together to compare the susceptibility of *C. albicans* strains from different groups to the most active TTO components. $SMFC_{50}$ ranges for isolates from different clinical groups were statistically similar (Figure 3.4). No statistical differences were observed following one-way Anova Kruskal-Wallis analysis and Dunn's multiple comparison tests ($p>0.05$).

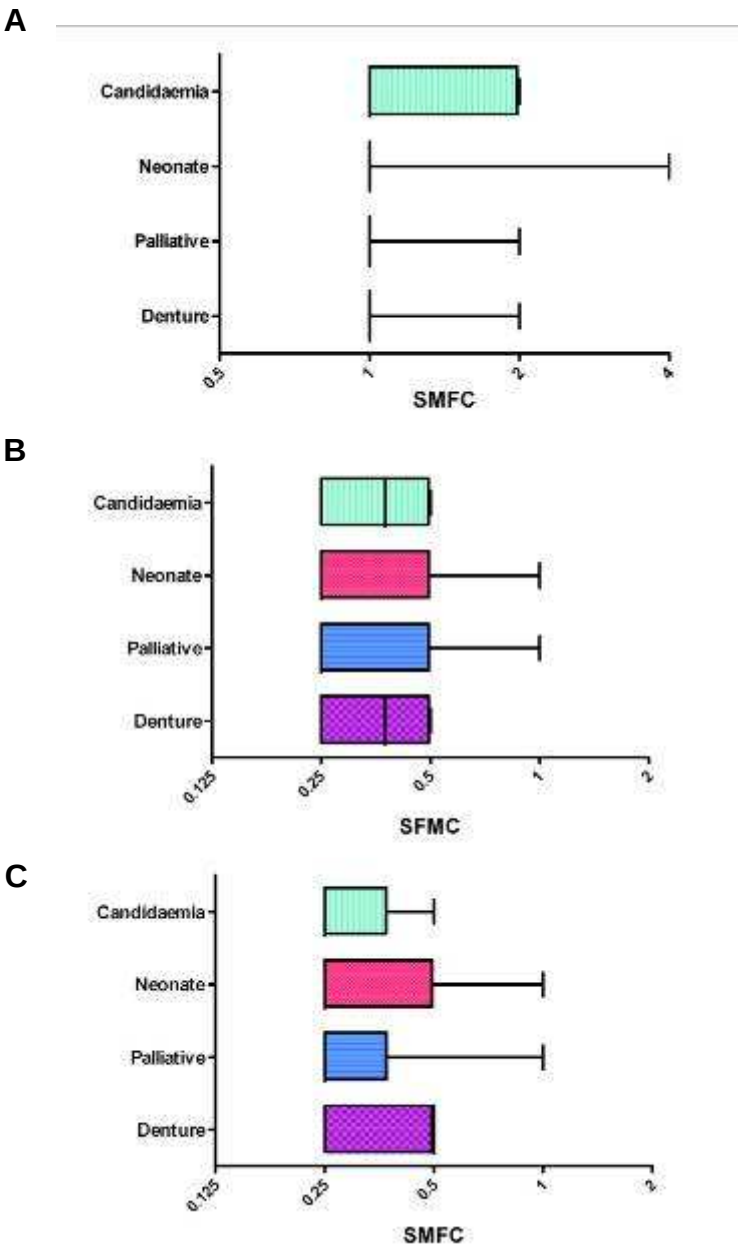


Figure 3.4: $SMFC_{50}$ values for selected treatments with TTO and components, plotted in different patient groups. Sessile MFC_{50} values of *C. albicans* isolates were assessed for **A**) TTO, **B**) T4-ol and **C**) *α*-terpineol. The XTT reduction assay was used to measure metabolic activity after 24 h treatment of mature biofilms. Box and whisker plots show range of $SMFC_{50}$ values, with boxes indicating inner quartile range and median (long vertical line). Differences between patient groups were assessed by a one-way Anova Kruskal-Wallis test and Dunn's multiple comparison tests. No statistical differences between groups were found for TTO, T4-ol or *α*-terpineol treatment ($p>0.05$). Strains in each group: Denture (n=12), palliative (n=25), neonate (n=24) and candidaemia (n=8).

3.5 Metabolic reduction of biofilms following $PMIC_{90}$ exposure

The previous data in Figure 3.4 illustrates inhibitory profiles at defined ranges for TTO, T4-ol and *α*-terpineol, but provides little quantitative data in relation to how these components perform in relation to killing the cells within the biofilm. This analysis examines the specific percentage of metabolic reduction of the biofilms when exposed to defined planktonic MIC_{90} levels of TTO or individual monoterpene components (Figure 3.5). Note how T4-ol and *α*-terpineol at these low concentrations maintain sustained activity within the biofilm, and are both significantly more effective than TTO (* p<0.001).

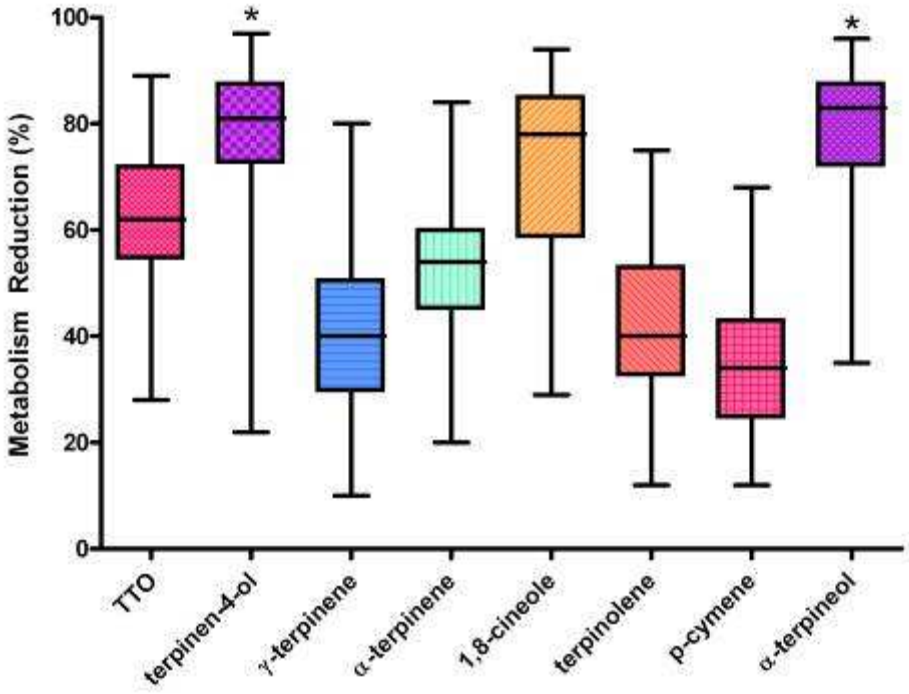


Figure 3.5: Reduction of biofilm metabolism on exposure to $PMIC_{90}$ levels of TTO components (n=69 strains). The concentrations of the various components, based on planktonic MIC_{90} levels, were TTO (1%), T4-ol (0.5%), γ-terpinene (4%), *α*-terpinene (4%), 1,8-cineole (2%), terpinolene (2%), *p*-cymene (4%) and *α*-terpineol (0.5%). Components are arranged in order of abundance in TTO, from left (most abundant) to right. Box and whisker plots show data range (outliers removed), with boxes indicating inner quartile range and median. T4-ol and α-terpineol at these low concentrations are significantly more effective than TTO (* p<0.001).

Figure 3.5 illustrates the difference in anti-biofilm potencies of the various components in relation to the effect of 1% TTO. For each treatment there were a small number of strains which were poorly inhibited, but the box and whisker plots illustrate that the majority of strains were inhibited metabolically by a similar proportion, within an approximate 20% range. Several derivatives, namely γ-terpinene, *α*-terpinene, terpinolene and *p*-cymene had minimal fungicidal activity on *C. albicans* biofilms. In contrast, T4-ol and α-terpineol (both in purple) were very effective; 0.5% of each giving higher rates of metabolic inhibition (mean 77%) than 1% TTO (mean 61%). Statistical analyses using a one-way Anova Kruskal-Wallis test and Dunn's multiple comparison tests were performed. Statistically significant differences were found in effectiveness of the different components at planktonic MIC_{90} levels. In particular, TTO was less inhibitory than T4-ol and *α*-terpineol ($p<0.001$), but significantly better than γ-terpinene ($p<0.001$), terpinolene ($p<0.001$) and ρ-cymene ($p<0.001$). No statistical difference was found between T4-ol and *α*-terpineol treatment at the concentrations used ($p>0.05$).

Subsequent analyses of the data were performed for TTO, T4-ol and *α*-terpineol by subdividing the strains into different patient groups (Figure 3.6). Susceptibility to each treatment was consistent between different groups. A comparison of the activity of compounds against planktonic and sessile cells is summarised in Table 3.5.

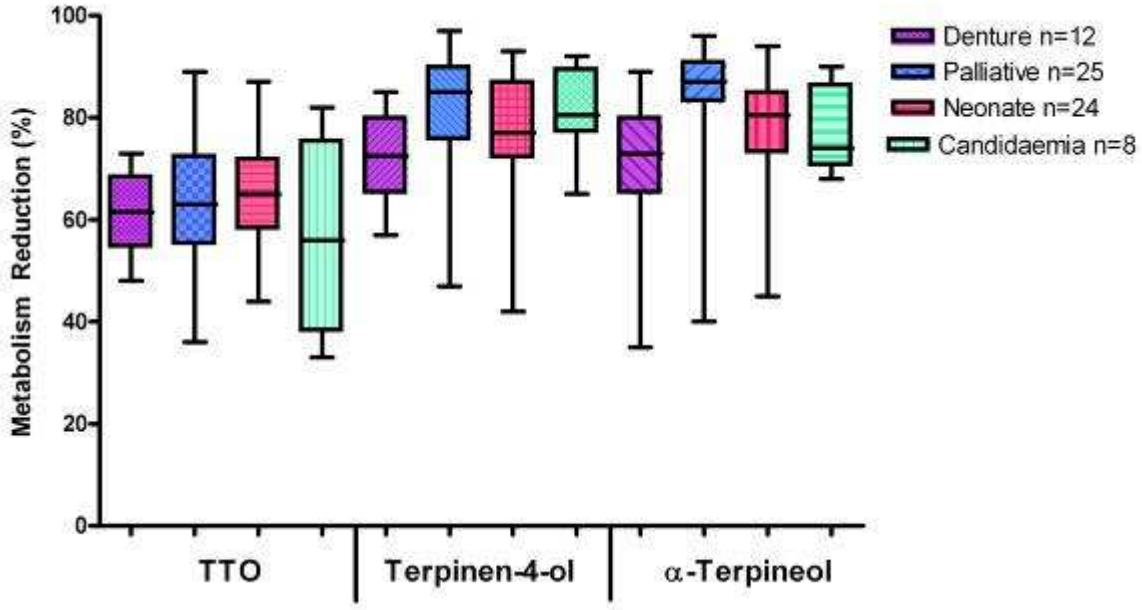


Figure 3.6: Reduction in biofilm metabolism for different clinical populations of *C. albicans* on exposure to $PMIC_{90}$ levels of the most efficacious TTO components (TTO [1% v/v], T4-ol and *α*-terpineol [0.5% v/v]). Box and whisker plots show data range, with boxes indicating inner quartile range and median. This illustrates the similar ranges of metabolic inhibition for each patient group of *C. albicans* clinical isolates. The two components gave similar results, each effective at 0.5%. Mean levels of inhibition were 61% for TTO, and 77% for T4-ol and *α*-terpineol. Statistically significant differences in treatment effects were found when comparing TTO with each component within groups of strains. No statistical difference was found between T4-ol and *α*-terpineol treatment ($p > 0.05$).

Table 3.5: Summary of the most effective anti-candidal TTO components

	PLANKTONIC (%)			SESSILE (%)		
	Range	**MIC_{50}**	**MIC_{90}**	**Range ($SMFC_{50}$)**	**$SMFC_{50}$**	**$SMFC_{80}$**
TTO	0.125 – 1.0	0.5	1.0	1.0 – 4	1.0	2.0
Terpinen-4-ol	0.0625 – 0.5	0.25	0.5	<0.25 – >1.0	0.25	0.5
α-Terpineol	0.125 – 0.5	0.25	0.5	<0.25 – 1.0	0.25	0.5

3.6 *C. albicans biofilm* survival after short TTO exposure

Previous sections investigated overnight exposure of biofilms to various treatments, to determine which of the TTO components exhibited the strongest antifungal properties, i.e. the components that inhibited biofilm metabolism at the lowest concentrations (lowest $SMFC_{50}$) However, a major aim of this study is to ascertain the antifungal potential of TTO components in a mouthwash, which therapeutically requires shorter exposure times. Therefore, this section aimed to examine the activity of short exposures of TTO and the two strongest antifungal components (T4-ol and *α*-terpineol) against two clinical isolates from denture stomatitis patients that represented effective and ineffective biofilm formers (Figure 3.7). These isolates were DS19 – an ineffective biofilm former (OD_{490} < 0.9) and DS23 – an effective biofilm former (OD_{490} >1). Pre-formed biofilms of both strains were exposed to TTO (2%), T4-ol (1%) or *α*-terpineol (1%) for 2, 5, 15 and 60 min (quadruplicate treatments and controls were used). Metabolism was measured immediately after treatment by XTT assay.

A 2 min exposure with 2% TTO reduced metabolism of DS19 by 48%, and DS23 by 31%. Longer exposures (5 and 15 min) produced greater inhibition. Nevertheless, 60 min exposure did not completely stop metabolism of either strain, with DS23 showing moderate metabolism (OD_{490} ~0.5) after exposure. Inhibition levels after short exposures to 1% T4-ol were equivalent to 2% TTO for DS19, but lower for DS23 (18% inhibition), whereas 1% *α*-terpineol produced stronger levels of inhibition than 2% TTO (DS19 69%, DS23 40%). When strains were exposed for 60 min to either component, their metabolism was reduced to near background levels. The DS19 controls showed considerable variation, as the weak biofilm had loose cells which could be dislodged in the washing step. Data were analysed by repeated measures ANOVA, with Bonferonni multiple comparison correction, using the General linear model in SPSS® software

comparing all time-points to no-exposure controls, to determine exposure times giving statistical significant effects (* $p<0.05$, ** $p<0.01$). Statistical analysis indicated that significant inhibition of the strong biofilm former (DS23) required 60 min exposure with 2% TTO, while the poor biofilm (DS19) was inhibited by a 15 min exposure ($p<0.05$). 1% T4-ol gave significant inhibition (70%) of DS23 after 15 min, while DS19 was inhibited after 2 min exposure, *i.e*. 1% T4-ol inhibited the *C. albicans* biofilms faster than 2% TTO. The 1% *α*-terpineol solution reduced biofilm metabolism of both strains after 2 min. It should be noted that these data are preliminary, as experiments were not repeated due to time constraints.

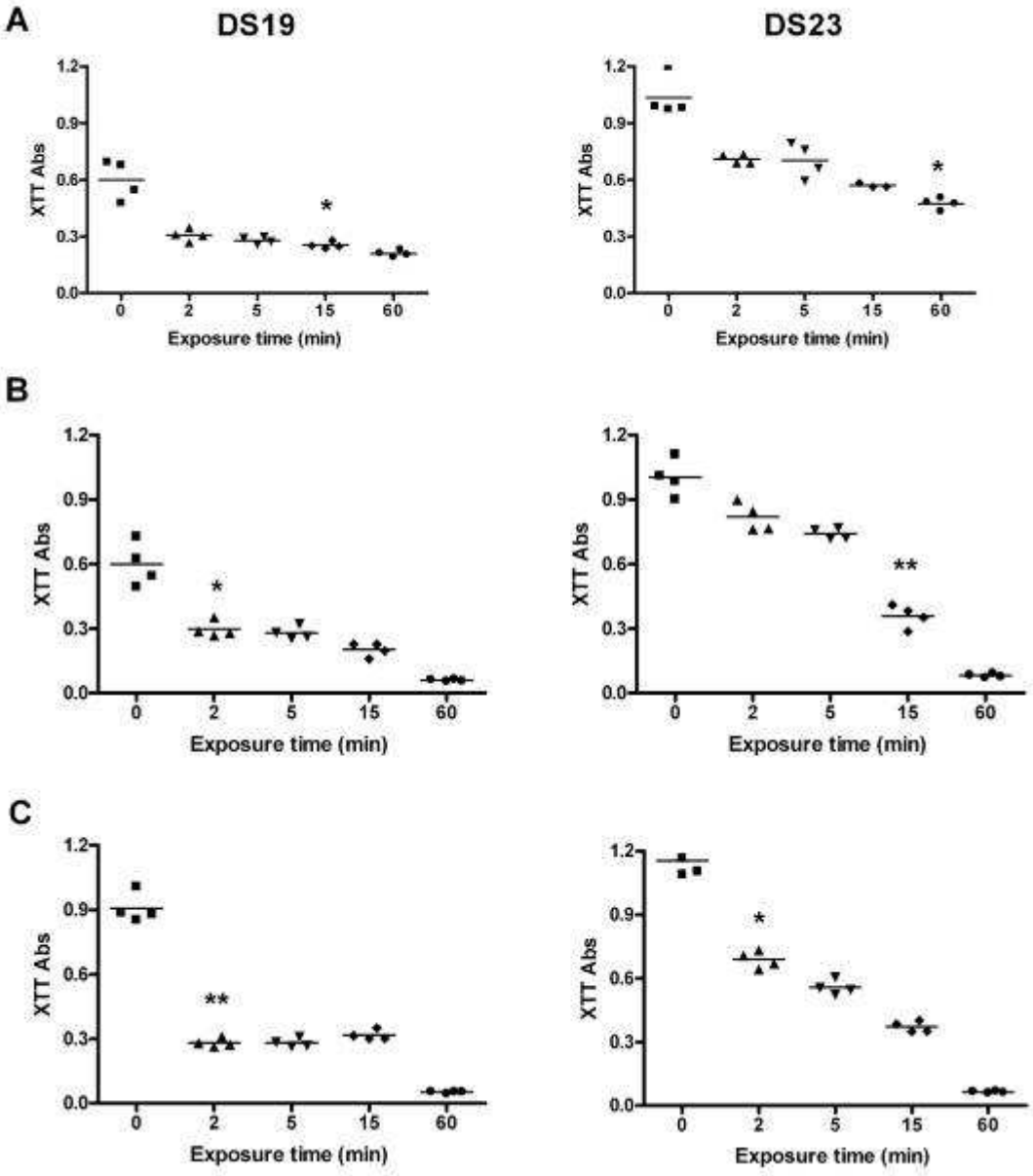


Figure 3.7: Time-kill studies of *C. albicans* with TTO components

Quadruplicate wells with pre-formed biofilms were exposed to **A**) TTO (2%), **B**) T4-ol (1%) or **C**) *α*-terpineol (1%) for 2, 5, 15 and 60 min (n=4 for each). Note the effectiveness of 60 min exposures of T4-ol and *α*-terpineol in comparison to TTO, particularly for strain DS23. Repeated measures ANOVA was performed using the General linear model in SPSS®, to determine statistical significance (* $p<0.05$, ** $p<0.01$). Inhibition of the strong biofilm former (DS23) required 60 min exposure with 2% TTO, 15 min 1% T4-ol or only 2 min in 1% *α*-terpineol. The poor biofilm (DS19) was inhibited by a 15 min exposure to 2% TTO while the components were effective after 2 min.

3.7 Summary and Discussion

The data presented in this component of the study clearly demonstrates that TTO is an effective inhibitor of *Candida albicans*, with a 1% TTO concentration (dissolved in RPMI / 0.25% Tween®80) consistently inhibiting growth of all the clinical strains tested and the $PMIC_{50}$ for all 100 strains being 0.5% (range 0.125% to 1% TTO). This is consistent with an earlier study from within our research group investigating the effect of TTO on a wide variety of oral yeast species (Bagg *et al.*, 2006). The aforementioned study did not examine the antifungal effects of individual TTO components, and a major aim was to determine which of these components had antifungal properties and also to discover if any component was more effective than the complete oil. The data indicated that T4-ol and *α*-terpineol exhibited the most effective antifungal activity, with $PMIC_{50}$ concentrations of 0.25%. Many of the other individual components tested were ineffective at inhibiting planktonic cells at the concentrations used.

The earlier study by our group (Bagg *et al.*, 2006) suggested a potential role for TTO in candidosis management, but the anti-biofilm effect remained to be examined in the present study, as biofilm resistance is a crucial obstacle to successful control of these infections as described in Section 1.4 (Seneviratne *et al.*, 2008). In the current study, inhibition of mature biofilm metabolism with 1% TTO was excellent; inhibition rates were generally >50% ($SMFC_{50}$ = 1%). In comparison, T4-ol and α-terpineol both inhibited biofilm metabolism by >50% at only 0.25% ($SMFC_{50}$ = 0.25%), *i.e*. the planktonic MIC_{50} concentration of these two components had powerful fungicidal effects on the *in vitro* biofilms. Statistical analysis indicated that there was no difference in biofilm resistance to treatment between strains isolated from different patient groups. 1,8-cineole, which has an oxygen molecule attached to the terpene ring, had a moderate inhibitory effect

($SMFC_{50}$ = 2%). As predicted from the planktonic studies, other components tested were ineffective at inhibiting pre-formed biofilm metabolism ($SMFC_{50}$ > 2%). These results confirm that biofilm resistance is a factor in protecting *C. albicans*, *i.e*. inhibition of strains growing as biofilms required higher concentrations of TTO and many of the derivative compounds, compared to planktonic inhibition. In the case of TTO, biofilms were inhibited at two times the planktonic MIC values. The only derivatives giving significant biofilm inhibition were T4-ol and *α*-terpineol. Both gave excellent inhibition of biofilms at one quarter the SMFC value of TTO. These experiments used 24 h treatments as an initial screen to determine the most potent antifungal components. This long exposure could be used in a denture cleanser (overnight soak) to potentially kill biofilm cells on dentures, or in an oral gel to treat candidosis on mucosal surfaces.

The MIC values for TTO agree well with published data. An earlier study in Glasgow Dental School (Bagg *et al.*, 2006) gave $PMIC_{50}$ values of 0.5% for 301 yeast isolates, with specific $PMIC_{50}$ values of 0.5% for *C. albicans* strains and 0.25% for *C. glabrata* strains. The $PMIC_{90}$ was 1% for *C. albicans*, *C. glabrata* and *C. dubliniensis* and 0.5% or less for other species. *C. albicans* values are the same as were found in the current study. Many other studies have found broadly similar susceptibilities (Banes-Marshall *et al.*, 2001; Hammer *et al.*, 1998; Vazquez *et al.*, 2000). Variation in PMIC values can be partly explained by differences in methods used and also the source and quality of the tea tree oil. Mondella *et al* found $PMIC_{90}$ values of 0.25% for TTO, 0.06% for T4-ol and 4% for 1,8-cineole (Mondello *et al.*, 2006), confirming the potency of T4-ol and weak activity of 1,8-cineole. Previous reports stated T4-ol to be responsible for antimicrobial activity of the oil (Carson *et al.*, 2006; Mondello *et al.*, 2006). The next most effective TTO component against all *Candida* isolates was *α*-terpineol,

with $PMIC_{90}$ of 0.5% for both *C. albicans* and *C. glabrata*. This study validates these findings. Therefore, further investigations focussed on these derivatives.

The *in vitro* effects of these treatments may not be reproduced *in vivo*, as *C. albicans* biofilms in plastic wells differ from natural biofilms. Oral candida biofilms form in a stressed environment to give protection from salivary flow, chewing and the host immune response. This allows the yeast to attach more strongly to the oral surfaces and hence avoid being detached and washed away (Section 1.4) (Mukherjee *et al.*, 2009). The biofilm produces a matrix of exopolymeric substances (EPS) to protect it *in vivo*, and this EPS increases *in vitro* when a developing biofilm is exposed to a continuous media flow (Figure 1.2). All the antifungal experiments in this study were performed on biofilms grown in static conditions (no flow-stress). Therefore, it would be interesting to use a continuous flow model to produce biofilms of a selection of clinical strains, which could then be treated to determine antifungal effects of TTO and components on more natural biofilms, *i.e.* a more complex structure than the simple artificial biofilms used in this project. In addition, the oral environment is also far more complex than any *in vitro* model; the activity of a TTO-based mouthwash can be influenced by many variables in the mouth, including the influence of salivary components (interactions with proteins) which are not tested in these *in vitro* experiments. Future studies could include saliva (sterile artificial saliva can be prepared) within both the planktonic and sessile sensitivity assays to investigate the influence of saliva on the mouthwash ingredients.

The use of 0.2% TTO mouthwashes may not be sufficient to control candidosis; the data in this study suggest that a minimum concentration of 0.5% TTO is necessary to inhibit planktonic *C. albicans* cells (PMIC50 = 0.5%), and possibly higher concentrations would be required to treat biofilm infections. However,

mouthwashes also contain other active ingredients (such as SDS, fluorides and ethanol) which can augment antimicrobial activity or reduce the effective TTO concentration (synergistic effect). The test solutions used in this study were standard laboratory yeast culture broth with only TTO or components added, plus the detergent Tween®80 (0.25%) to improve dispersal of poorly-soluble TTO components. In retrospect, it would have been useful to have a commercial TTO mouthwash, with fully disclosed ingredients, to determine if the other ingredients in the formulation altered the activity of the TTO compared to the laboratory TTO solution used in these experiments. However, the main point of these experiments was to find an individual molecule that could be used independently, making it more attractive for pharmaceutical companies. The TTO proportion on commercially available formulations is not clear and they only stipulate a maximum concentration of T4-ol, rather than a defined concentration. The TTO and components used in this study were HPLC grade, which were high purity. Therefore, comparison with a lower grade product would not have provided a fair comparison. Clinical studies into TTO-based mouthwashes have been performed, but often the proportion of TTO used is not clearly stated (Vazquez & Zawawi, 2002) [Section 1.7.3]. Therefore, determination of a 'safe' concentration for use in oral products is crucial. Another limiting factor is the strong taste of TTO, with 1% being the maximum tolerated concentration.

It is inadvisable to have widespread use of any drug at ineffective levels, as there is the potential for selection of less-sensitive strains. In this investigation, the multiple exposure TTO study did not result in any change in TTO sensitivity after ten exposures to sub-inhibitory concentrations (Section 3.1.2). However, slightly reduced sensitivity to conventional antifungal drugs was seen in 3 out of 8 strains after ten TTO exposures (Table 3.3). This multiple exposure study was too small to make any conclusions, but there is the potential for creating resistance to other

antifungal drugs, if the TTO mouthwash is ineffective (McMahon *et al.*, 2007). Experimental increases in resistance to antibiotics in *E. coli*, *Salmonella* spp. and *Staphylococcus* spp. have been induced by exposure to sub-lethal concentrations of TTO (McMahon *et al.*, 2007; McMahon *et al.*, 2008; Nelson, 2000). However, another small study could not induce TTO resistance in *C. albicans* (Mondello *et al.*, 2003) and evidence for this type of effect on yeast by TTO is lacking. Moreover, no studies to date have demonstrated whether TTO exposure might upregulate candida efflux pump expression. In addition, there is a potential for repeated exposure to TTO to induce 'stress hardening' of the bacteria, where one antimicrobial treatment can increase general resistance to other agents. Any cells that survive exposure to TTO may become more resistant to future antimicrobial challenge, for example by activating efflux pump mechanisms to excrete drugs more quickly. Also, TTO treatment may alter the overall microbial population, by killing sensitive organisms and allowing expansion of resistant strains/species. This can significantly change the ratio of species, which alters the competition between organisms and thus results in selection of a resistant population.

The final experiment in this chapter focussed on short exposures (2 min to 1 h) of biofilms to TTO and the two best antifungal components, T4-ol and *α*-terpineol (Figure 3.7), to determine effective concentrations and times for use in mouthwash or denture cleansers. Two minute exposures to 2% TTO or 1% T4-ol were not very effective (inhibition of 31% for TTO, 18% for T4-ol) against a strong biofilm former, while inhibition after 2 min in 1% *α*-terpineol was more significant (40%). T4-ol (1%) gave strong biofilm inhibition (70%) with 15 min exposure; *i.e.* at least four times the MIC_{50} was required in a short exposure. TTO mouthwashes generally contain around 0.2% TTO. This concentration is below

the *in vitro* planktonic *C. albicans* inhibitory concentration ($PMIC_{50}$ = 0.5%) found in this study.

Several studies have shown efficacy of TTO against azole-resistant *Candida spp*., and this study confirmed this as itraconazole-resistant *Candida* isolates demonstrated no cross-resistance to the 4 effective TTO components (Bagg *et al.*, 2006; Jandourek *et al.*, 1998; Mondello *et al.*, 2003; Vazquez & Zawawi, 2002). With activity against all tested *Candida spp*., and effective use against *C. albicans* biofilms, TTO has been promoted as an alternative treatment for oral care (Jandourek *et al.*, 1998; Vazquez & Zawawi, 2002).

Another advantage to using a broad-spectrum antimicrobial, such as TTO, instead of conventional antifungals is that OPC often involves mixed microbial biofilms containing multiple yeast species, or yeast with bacterial species, particularly *C. albicans* with oral streptococci (Silverman *et al.*, 2010). Specific antifungal drugs such as azoles are inactive against bacteria, whereas TTO targets most microbial species. Therefore, in future studies it would be important to investigate the effectiveness of TTO and its components on mixed *Candida* biofilms and also on biofilms containing both yeast and bacteria.

Chapter 4:
Inhibition of Biofilm Formation by TTO and Derivative Components

4.1 Introduction

Biofilm experiments up to this point in the study have focussed on treatment of pre-formed biofilms with tea tree oil (TTO) and constituent components, followed by measuring their effects on cellular metabolism. This relates to the potential of treating established biofilms on mucosal surfaces or dentures, *i.e*. oral candidosis. The current section investigates the inhibitory (prophylactic) role, *i.e*. early treatments to prevent both *C. albicans* colonisation and the early stages of biofilm formation.

Studies have shown that morphogenesis plays a pivotal role in *C. albicans* biofilm development, in which hyphae are essential elements for providing structural integrity and for multi-layered architecture (Baillie & Douglas, 1999; Ramage *et al.*, 2002d). A gene encoding a major regulator of hyphal development is *EFG1*, which has been shown to be involved in regulation of the morphological transition and is associated with ability to form coherent biofilm structures (Ramage *et al.*, 2002d). Murillo and colleagues (2005) investigated the early stages of biofilm formation and found that after only 30 min there were substantial differences in gene expression between adherent and non-adherent *C. albicans* cells, which impacts antifungal resistance genes, including efflux pumps which are a defined factor in azole resistance (Murillo *et al.*, 2005).

Small molecules such as farnesol and EDTA have been shown to inhibit the yeast to hyphal transitional stage of *C. albicans*, and are able to reduce biofilm development in a concentration-dependent manner (Ramage *et al.*, 2002b; Ramage *et al.*, 2007). Another class of molecules that affect *C. albicans* morphology includes 3-oxo-C12-homoserine lactone (3OC12HSL), a signalling molecule produced by *Pseudomonas aeruginosa*, and dodecanol (Hogan *et al.*, 2004). More recently, Martins and colleagues demonstrated that *C. albicans* and

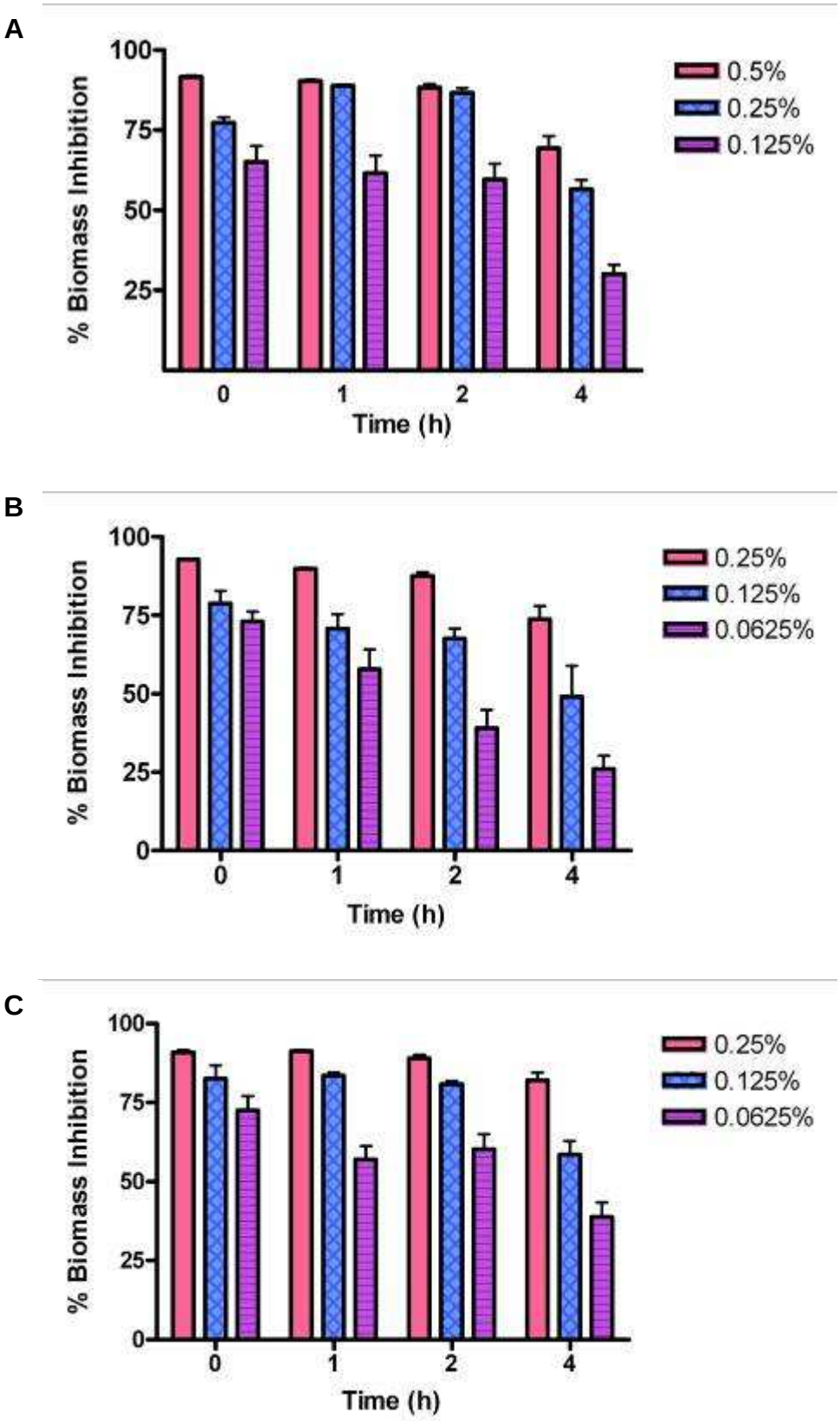
C. dubliniensis planktonic and biofilm cells produce a series of chemical signalling molecules (including isoamyl alcohol, 2-phenylethanol, 1-dodecanol, E-nerolidol, and E,E-farnesol) which all affect morphogenetic transitions, and also that secretion of these alcohols was species, culture mode and growth-time specific (Martins *et al.*, 2007).

Together these studies demonstrate that small molecules can be used to inhibit biofilms through modulation of hyphal formation, which is critical for the basis of its structural stability. This chapter will examine the effects of TTO and the two most effective derivative molecules in the previous chapter on early adherent and immature biofilms to determine their usefulness at preventing *C. albicans* biofilms. It is the hypothesis that lower concentrations of these molecules may inhibit adhesion and *C. albicans* hyphal formation, therefore preventing biofilm formation. This would reduce the need for active biofilm treatment at potentially toxic concentrations.

4.2 Prolonged treatment during early adhesion inhibits biofilm formation

To investigate inhibitory effects of TTO and its two main antifungal components on early stages of adhesion, four *C. albicans* strains (YH3, YH28, YH31 and YH32 from neonates, all forming coherent biofilms *in vitro*) were inoculated into a 96-well plate. At 0, 1, 2 or 4 h after inoculation of the yeast cells into wells, cells were treated with TTO (0.5, 0.25, 0.125%), T4-ol (0.25, 0.125, 0.0625%) or *α*-terpineol (0.25, 0.125, 0.0625%), all in RPMI / 0.25% Tween®80, (quadruplicate treatments for each strain and time-point), then incubated for a 24 h period (with TTO or component still present) at 37°C (Section 2.1.8). Control wells for each strain were treated with RPMI / 0.25% Tween®80 at each time-point. The biofilm was then quantified at 24 h using the crystal violet biomass assay (Section 2.1.7). Biofilm inhibition for each treatment was calculated by comparing to untreated control biofilms without TTO or component (in RPMI / 0.25% Tween®80), as illustrated in Figure 4.1. Figure 4.2 shows all the replicate TTO inhibition data for strain YH3 as an example (part of Figure 4.1 data), showing a statistically significant inhibitory effect of 0.125% TTO when cells treated within 2 h of attachment. Repeat measures ANOVA tests were performed, with Bonferroni's correction, using the General linear model in SPSS to identify significant changes. In addition, morphological effects were assessed microscopically, *i.e*. blocking of hyphae formation as a mechanism of biofilm inhibition (Figure 4.3 and 4.4).

Figure 4.1: Mean inhibition of *C. albicans* biofilm development by A) TTO, B) T4-ol and C) *α*-terpineol. At 0, 1, 2 or 4 h after inoculation of the yeast cells into a 96-well plate (2×10^5 cells/ well), cells were treated with TTO (0.5, 0.25, 0.125%), T4-ol (0.25, 0.125, 0.0625%) or *α*-terpineol (0.25, 0.125, 0.0625) then incubated for a 24 h period at 37^{o}C (mean of 4 wells for each strain, n = 4 strains [YH3, YH28, YH31 and YH32]) The biofilm was then quantified at 24 h using the crystal violet biomass assay, and % inhibition calculated by comparing to untreated controls for each strain at each time point. Each column represents the mean inhibition of 4 strains (4 replicates for each strain), with bars showing standard error of mean. The highest concentration of each is equivalent to the planktonic MIC_{50}, which effectively inhibited biofilm formation at all time points. It was shown that each treatment was most effective if given within 2 h of inoculation, with most significant differences in inhibition observed, relative to time treated, at lower concentrations. Jonckheere-Terpstra non-parametric multiple comparison tests were performed using SPSS software, followed by a one-way ANOVA with Bonferroni's correction, to identify significant changes. Statistical analysis on timing of treatment (hours after inoculation) gave adjusted p values: 0.125% TTO 0 h versus 4 h $p<0.001$, 2 h versus 4 h $p<0.05$, no significance for 0.5% and 0.25% TTO 0 h versus 4 h ($p>0.05$), 0.125% T4-ol 0 h versus 4 h $p<0.005$, no significant difference due to timing with higher concentration ($p>0.05$), and no significant difference for 0.125% *α*-terpineol due to timing (*i.e*. delay of treatment was not significant - treating cells at 0, 1, 2 or 4 h after inoculation all gave similar results).


A
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75
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1
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Figure 4.1

TTO effectively inhibited the ability of *C. albicans* to filament and form mature biofilms at a concentration equivalent to the planktonic MIC_{50} (0.5%), at all pre-treatment times tested (Figure 4.1A). Treatment within 2 h of inoculation gave 90% inhibition, while treatment at 4 h resulted in a 70% mean biomass reduction. At a lower TTO concentration of 0.25%, similar levels of inhibition were demonstrated up to 2 h (80%). Scanning electron microscopy analysis (SEM) demonstrated reduced hyphal growth and filamentous extensions, with a scant covering of the substrate, compared to the no-TTO control in RPMI / 0.25% Tween®80 (Figure 4.4A and B). However, after 4 h adhesion and colonisation, the inhibition was reduced overall to a mean of 56%. 0.125% TTO also produced around 60% inhibition of biofilm formation if present within 2 h of adhesion, *i.e.* at early stages of hyphae elongation. However, at 4 h adhesion this resulted in only a 30% mean inhibition. Figure 4.2 shows replicate data for TTO treatment of strain YH3, while Figure 4.3 shows cells treated with 0.125% TTO at different stages of biofilm formation.

T4-ol effectively inhibited the ability of *C. albicans* to filament and form mature biofilms at a concentration equivalent to the planktonic MIC_{50} (0.25%), at all pre-treatment times tested (Figure 4.1B), giving very similar results to TTO treatment. Treatment within 2 h of inoculation gave around 90% inhibition, while treatment at 4 h resulted in a 74% mean biomass reduction. SEM analysis demonstrated poor overall hyphal growth with limited filamentous extensions, with a scant covering of the substrate (Figure 4.4C). At a lower concentration of 0.125%, the inhibition was time-dependant with respect to adhesion, with inhibitory levels decreasing with increasing adhesion/elongation time, ranging from 80 to 50% mean inhibition from 0 to 4 h adhesion. At 0.0625% the pattern was similar, but exhibiting a range of 75 to 25% mean inhibition for treatment 0 to 4 h after adhesion.

α-terpineol similarly inhibited the ability of *C. albicans* to filament and form mature biofilms at a concentration equivalent to the planktonic MIC_{50} (0.25%), at all pre-treatment times tested (Figure 4.1C). Treatment within 2 h of inoculation gave 90% inhibition, while treatment at 4 h resulted in an 82% mean biomass reduction. SEM analysis demonstrated stunted hyphal extension, with minimal substrate coverage (results not shown). At a lower concentration of 0.125%, the inhibition was consistent up to 2 h adhesion (80%), dropping to 58% after 4h adhesion. At the lowest concentration tested (0.0625%), inhibition was high at 0 h adhesion (72%), dropping to only 39% after 4 h adhesion.

SEM was used to examine the morphology of a 24 h *C. albicans* biofilm, comparing untreated (in RPMI / 0.25% Tween®80) cells with TTO or T4-ol exposed cells. Strain YH28 (neonate throat swab), which forms a coherent *in vitro* biofilm, was inoculated onto Thermanox™ coverslips (Nunc Inc, Thermo Fisher Scientific) at 10^6 cells/ml. Biofilms were allowed to attach to coverslip for 2 h, washed briefly in PBS, then treated with TTO (0.25%) or T4-ol (0.125%). Untreated cells were included as a control. Coverslips were incubated overnight at 37ºC, before treatment solutions were removed and coverslips washed carefully in PBS to detach any loosely adherent cells. Coverslips were processed for SEM and images taken of representative cells (Figure 4.4). Both treatments effectively blocked biofilm formation, with only short hyphae present after 24 h.

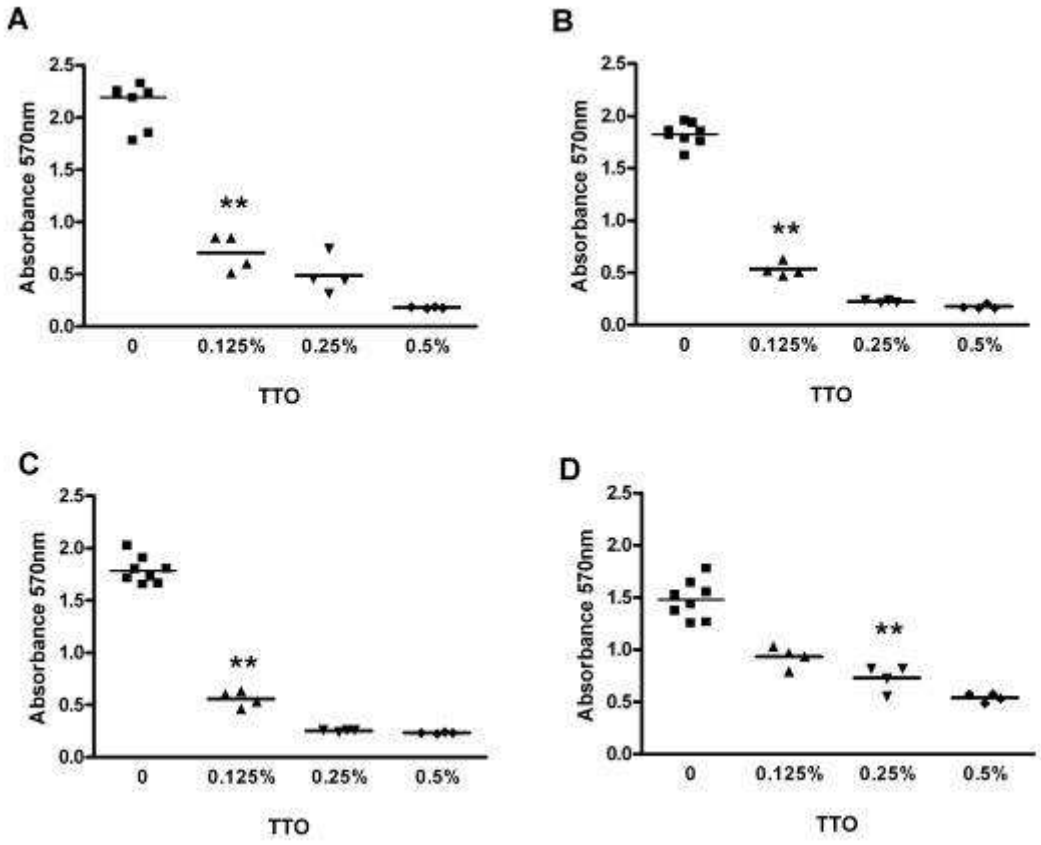


Figure 4.2: Inhibition of biofilm development in *C. albicans* strain YH3 by addition of TTO at A) 0 h, B) 1 h, C) 2 h and D) 4 h. At 0, 1, 2 or 4 h after inoculation of the yeast cells into a 96-well plate (2×10^5 cells / well), cells were treated in quadruplicate with TTO (0.5, 0.25, 0.125%), then incubated for a 24 h period at 37°C. The biofilm was then quantified at 24 h using the crystal violet biomass assay. Mean absorbance after each treatment is shown by horizontal line, and % inhibition calculated by comparing treated values to untreated controls for this strain. Repeat measure ANOVA tests were performed, with Bonferroni's correction, using the General linear model in SPSS® to identify significant changes. The highest concentration is equivalent to the $PMIC_{50}$, which effectively inhibited biofilm formation at all time points. It was shown that 0.125% TTO treatment was most effective if given within 2 h of inoculation (** $p<0.01$), while inhibition at 4 h was not statistically significant ($p>0.05$).

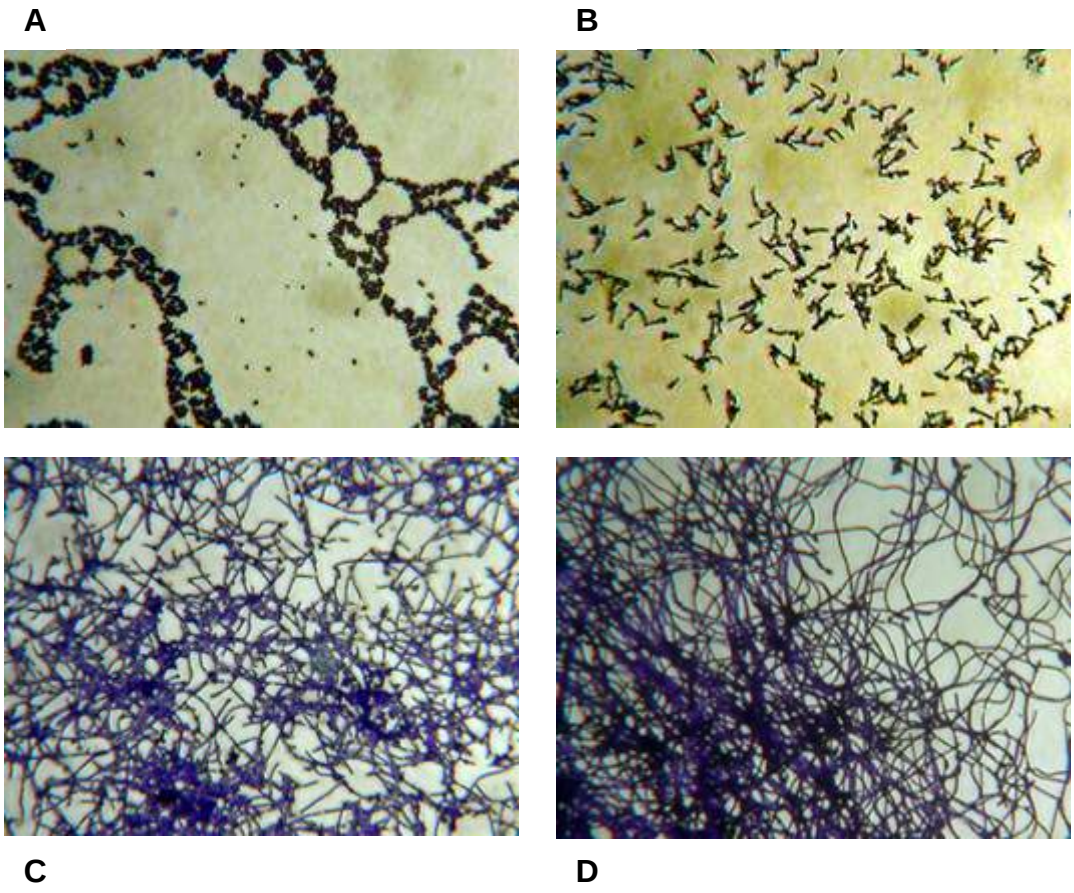


Figure 4.3: *C. albicans* biofilm formation can be blocked by 0.125% TTO. Light microscopy images (x 40) of *C. albicans* biofilm (strain YH3) 24 h after treatment with 0.125% TTO at **A**) 0 h post-inoculation, **B**) 2 h post-inoculation, **C**) 4 h post-inoculation and **D)** untreated control (in RPMI / 0.25% Tween®80). 0.125% TTO completely blocked biofilm formation if present when cells were attaching to surface (**A**), stopping both cell division and morphogenesis of blastospores into hyphae. In (**B**), hyphae were very short, showing early filamentation in first two hours, followed by inhibition when TTO was added. Cells in (**C**) had more elongated hyphae, due to the longer time they had to develop prior to treatment, while the untreated control biofilm showed extensive filamentation (**D**).

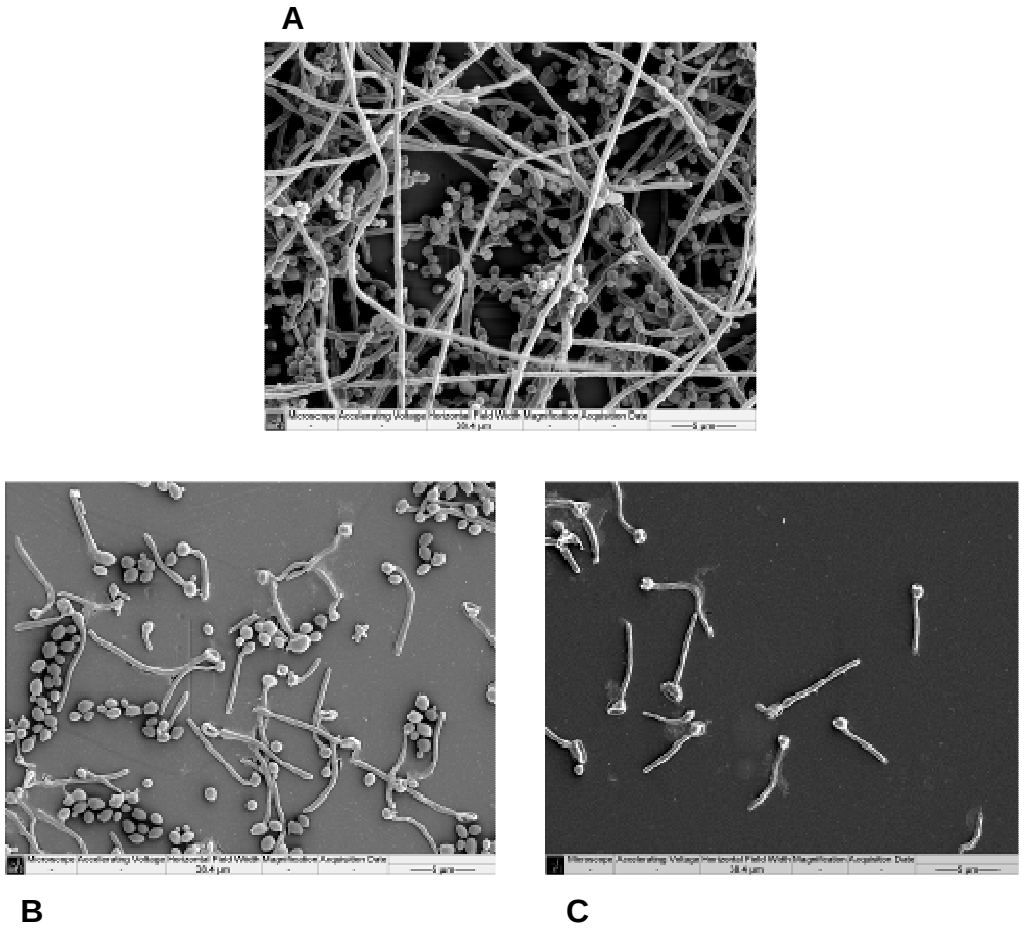


Figure 4.4: Scanning electron micrographs of 24 h *C. albicans* biofilm after various treatments (SEM x1000). *C. albicans* (YH28, neonate throat swab) was plated onto coverslips in a 24-well plate, and treated 2 h after inoculation with **B)** TTO (0.25%) or **C)** T4-ol (0.125%). Untreated cells (**A**, in RPMI / 0.25% Tween®80) were included as a positive control. Cells were fixed on coverslips at 24 h and processed for scanning electron microscopy. Untreated adherent cells developed into mature biofilms consisting of both yeast and hyphae within a complex 3-D architecture (**A**). Adherent cells treated with TTO were only able to form stunted hyphae which were unable to develop into full hyphae and mature biofilms (**B**). Cells treated with T4-ol were also inhibited from hyphal extension, and the cells could only form a scant monolayer (**C**). Due to the inability to form a coherent framework of hyphae, both TTO and T4-ol treated cells were less adherent than untreated cells, and many were removed during the SEM processing procedure.

4.3 Short treatment (1 h) during early adhesion inhibits biofilm formation at high concentrations

These experiments were designed to assess the potential of TTO and constituent components in terms of a short treatment denture cleanser, by assessing how a one hour treatment of adherent cells, impacted on growth and biofilm formation when cells were removed from the active agent. *C. albicans* (YH28 – neonate throat swab) was plated as above, allowed to adhere for only 1 h, then treated in duplicate wells for 1 h with varying concentrations of TTO, T4-ol and *α*-terpineol (control cells were treated with RPMI / 0.25% Tween®80 for 1 h), washed in PBS and then incubated in fresh RPMI for 24 h (Section 2.1.8). Inhibition of treated biofilms were calculated by comparing to the control cells (n=4). Resultant cellular growth was quantified using the crystal violet biomass assay (Figure 4.5).

Treating the 1 h adherent population with TTO at concentrations of 0.125 and 0.25% did not affect subsequent biofilm growth, whereas 0.5% TTO was shown to reduce biofilm formation by 53%. For T4-ol, both 0.625% and 0.125% did not reduce the biomass compared to the untreated control. However, 0.25% was able to reduce biofilm growth by 90%. *α*-terpineol was less effective than T4-ol, with 0.25% giving a mean 53% reduction in biofilm development, T4-ol was more effective than TTO and *α*-terpineol for this strain, but data needs confirmation with a variety of clinical strains. Figure 4.6 shows biofilms in wells treated for 1 h with a range of TTO concentrations, illustrating the inhibitory effect of 0.5% TTO.

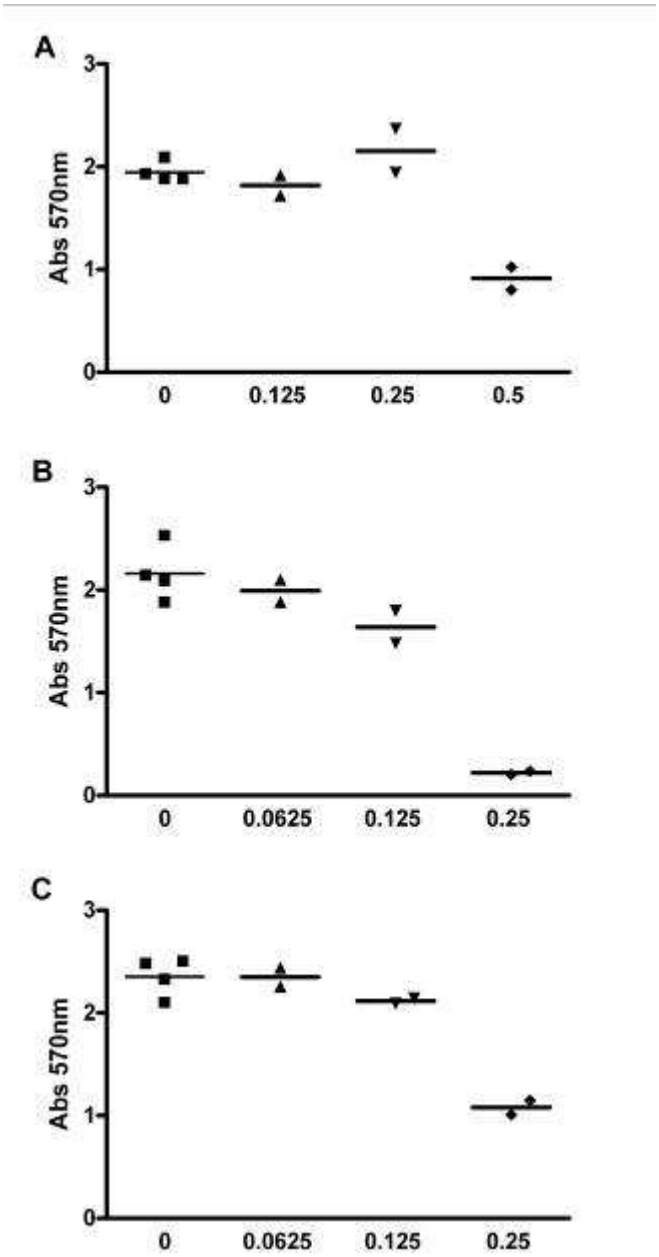


Figure 4.5: Inhibition of *C. albicans* biofilm development by 1 h treatment with A) TTO, B) T4-ol and C) *α*-terpineol. *C. albicans* YH28 was inoculated into a 96-well plate (2×10^5 cells / well), allowed to adhere for 1 h, and then treated in duplicate for 1 h with varying concentrations of TTO, T4-ol and *α*-terpineol or a positive control of RPMI / 0.25% Tween®80. Treatment solutions were removed, cells washed in PBS and then incubated for 24 h in RPMI. T4-ol was more effective than TTO and α-terpineol -reductions in mean biomass of the biofilms (represented by horizontal bar) were as follows: 90% for 0.25% T4-ol, and 53% for 0.5% TTO and 0.25% *α*-terpineol.

0.5% TTO **0.25% TTO** **0.125% TTO** **Control**

Figure 4.6: Crystal violet stained biofilms at 24 h. Cells were treated with TTO for 1 h, washed in PBS, incubated for 24 h in RPMI then stained with crystal violet. A positive control of RPMI / 0.25% Tween®80 was included. Inhibition of biofilm formation by 0.5% TTO is apparent in the left hand well, and marginally at 0.25%.

4.4 Pre-coating of substrate with TTO inhibits biofilm formation

This experiment was designed to assess the effect of coating a substrate with TTO prior to attachment of yeast cells. 96-well plates were coated with serial doubling dilutions of TTO from 2% to 0.015%, in RPMI containing 0.25% Tween®80, and 40 µl added to individual wells, including a media control (RPMI / 0.25% Tween®80). Plates were sealed with adhesive lids, stored overnight at 4ºC to allow adsorption to the plate, then solution removed. Four *C.albicans* strains (DS18, 20, 22, 23 - denture stomatitis isolates) were selected, and plated in replicates at 2 x 10^5 cells / well (duplicates of each strain for each TTO concentration). Plates were sealed with parafilm and incubated overnight at 37^{o}C. Biofilms were examined microscopically to observe morphology, and quantified using the crystal violet assay (Figure 4.7). TTO (1%) consistently inhibited biofilm formation for all 4 strains (** $p<0.01$), while concentrations below 0.5% had little effect.

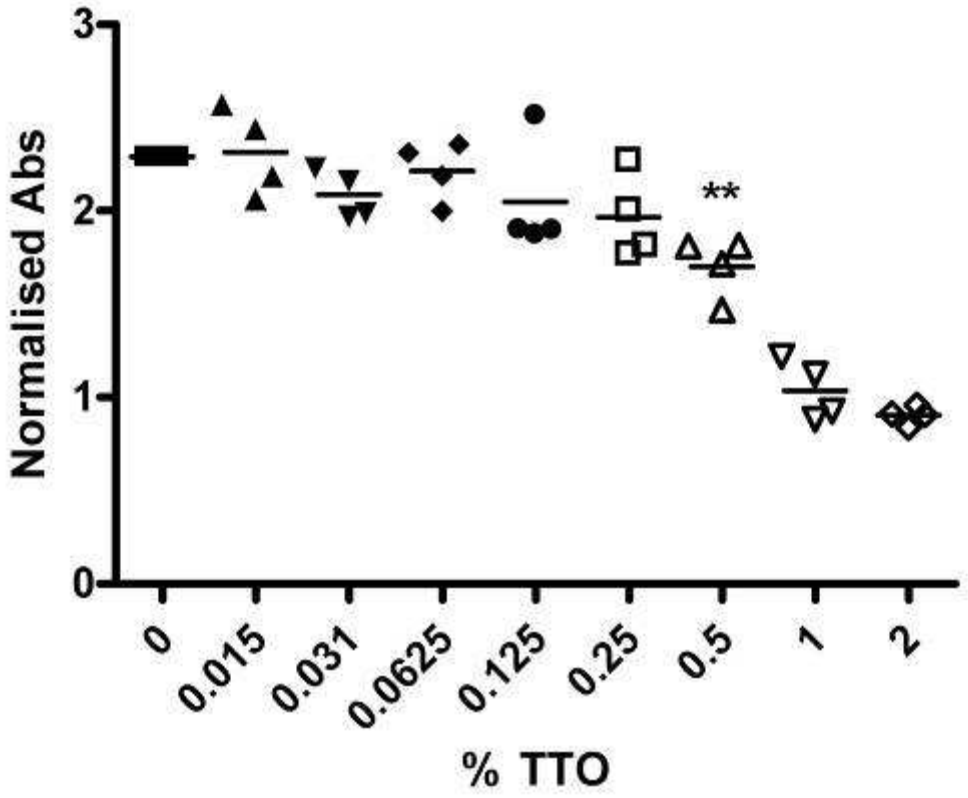


Figure 4.7: Effect of adsorbing (coating) substrate with TTO. Four *C. albicans* strains DS18, DS20, DS22 and DS23 were inoculated in duplicate after overnight treatment of wells with TTO at concentrations from 2 to 0.015%, including a media control. Biofilm formation was measured at 24 h using crystal violet assay. Data were normalised using mean of all control data. Data-points represent mean of each strain, while horizontal bars represent mean biofilm absorbance of all four strains. Statistical analyses were performed using a one-way ANOVA and Dunnett's t-test to compare all treatments with untreated controls. 0.5% TTO was required to reduce biofilm formation significantly, while 1% TTO gave stronger inhibition of all 4 strains tested (** $p<0.01$).

4.4.1 Scanning electron microscopy of pre-treated denture acrylic

An additional experiment was performed to test the potential inhibitory effect of treating denture material with TTO or T4-ol, prior to yeast inoculation. This was also a way to illustrate morphological effects of such pre-treated denture material on *C. albicans* cells with scanning electron microscopy (SEM). Denture acrylic discs (polymethylmethacrylate (PMMA), made in Glasgow Dental Hospital by Dr. Donald Cameron) were soaked in 0.5% TTO or 0.5% T4-ol for 60 min, inoculated with 10^5 *C.albicans* cells (BCO71- denture stomatitis isolate), as described previously, and incubated for 24 h at 37^{o}C. Discs were removed at 24 h, fixed and processed for SEM analysis (Section 2.1.11). Images are shown in Figure 4.8.

0.5% T4-ol inhibited biofilm formation in this strain by reducing the visible number of cells able to attach to surface, or by blocking morphogenesis. Any reduction in hyphae with TTO pre-treatment is marginal. This data is only observational, but demonstrated the physical impact of treatment. Parallel quantitative analysis with defined software would be appropriate with a number of different strains.

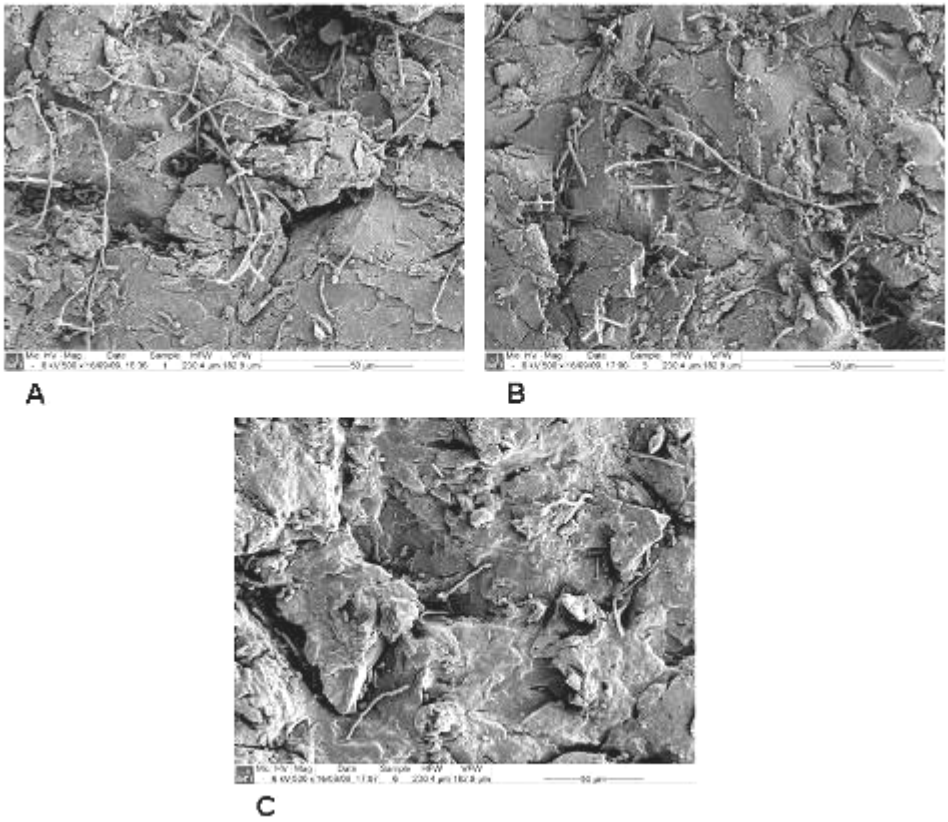


Figure 4.8: Denture acrylic discs pre-treated for 1 h with A) PBS, B) 0.5% TTO or C) 0.5% T4-ol, prior to inoculation with *C. albicans* (BC071) and harvested at 24 h (SEM x1000). Untreated disc at 24 h **(A)** had both extensive hyphae and yeast cells at quite low density. Pre-treatment with 0.5% TTO (**B**) had no obvious effect on hyphal length or number of cells attached to surface, whereas 0.5% T4-ol (**C**) appeared to reduce length of hyphae and attached cell numbers.

4.5 Summary and Discussion

This chapter investigated the potential inhibitory role of TTO and two key derivatives, shown in Chapter 3 to have effective antifungal properties. Intrinsic biofilm resistance results in higher concentrations of TTO being necessary to effectively inhibit planktonic cells to the same extent. Therefore, a treatment used to kill planktonic cells before they attach to oral surfaces and form a biofilm should require lower antifungal concentrations than candidosis treatment.

For 24 h exposures (Figure 4.1), biofilms were successfully inhibited by addition of TTO, T4-ol or *α*-terpineol (all at 0.125%) within 2 h of cell attachment to surface, *i.e.* before morphogenesis has started. SEM images in Figure 4.4 clearly show blocking of hyphal extension with 0.25% TTO and 0.125% T4-ol, which results in biofilm inhibition (illustrated for TTO in Figure 4.2C and 4.3B).

In the shorter 1 h treatment experiments (Figure 4.5), 0.5% TTO or 0.25% T4-ol was required to block biofilm formation. At these concentrations, T4-ol gave stronger inhibition than TTO (90% versus 53%) and 0.25% *α*-terpineol (53% inhibition). When concentrations were below $PMIC_{50}$ levels, removal of the antifungal after 1 h allowed regrowth of the biofilm, i.e. sub PMIC levels did not kill cells in 1 h. This observation requires a larger study with more strains to confirm that T4-ol is more effective than TTO, and also to determine a minimum effective prophylactic T4-ol concentration. These concentrations were shown to be cytotoxic to *in vitro* human oral cells (Chapter 5). These results contrast with the initial experiment (Figure 4.1), where longer exposures were effective at lower doses, and illustrate the time-dependent effect of these antifungals. The nature of the oral cavity demands that an effective antimicrobial agent has to work using a short exposure, or that the compound has to remain on mucosal and tooth surfaces long enough to have an effect, resisting salivary flow. This is

known as substantivity, where an antiseptic such as chlorhexidine can adsorb to a surface and be released gradually, resulting in longer term protection. The final part of this section investigated the potential substantivity of TTO in small pilot studies.

Coating the plastic well surface with 1% TTO prior to *C. albicans* inoculation was very effective in blocking biofilm formation, while 0.5% TTO had some inhibitory effect (Figure 4.7). Clearly some of the TTO adsorbs to the plastic, resulting in a reduction in the ability of *Candida* cells to attach and effectively colonise the surface. This was investigated further by pre-soaking denture material to test the ability of TTO and T4-ol to adsorb to a more clinically relevant material. Coating with 0.5% T4-ol, prior to *C. albicans* inoculation, resulted in reduced biofilm formation as visualised by SEM, whereas 0.5% TTO had no effect (Figure 4.8). This data is not quantitative and requires further work with more than one strain. Quantification would be possible using appropriate software to sample random sections of treated and untreated materials. This could be applied to a denture soak, to reduce biofilm formation on dentures, or a longer-term inhibitory strategy could be the impregnation of denture materials with TTO / T4-ol. The use of TTO to pre-soak polydimethyl siloxane discs has recently been investigated (De Prijck *et al.*, 2010). A 24 h pre-soak with 20% TTO solution produced a 2 $\log_{10}$ reduction in *C. albicans* biofilm biomass, which was comparable with a 0.02% miconazole pre-treatment, while a 2.5% TTO solution gave a $\log_{10}$ reduction. The effectiveness of this strategy *in vivo* is uncertain as the effects of saliva on this anti-biofilm effect are unknown, and also the long-term safety and stability upon denture materials remains to be determined. A recent study has shown that denture cleansers with the active ingredients EDTA, sodium bicarbonate, sodium perborate, hydrogen peroxide and sodium hypochlorite were largely effective against *C. albicans* biofilms, but required additional mechanical disruption to be

entirely effective (Jose *et al.*, 2010). The preliminary studies described in this chapter and Chapter 3 indicate that TTO could potentially be more effective than these denture cleansers, and may have the same applications as chlorhexidine.

Chlorhexidine is considered by dentists as the gold standard of antimicrobial mouthwashes. It has broad-spectrum antimicrobial activity through membrane disruption, is highly active against the planktonic cells present in saliva, which contribute to colonisation and subsequent biofilm formation (Giuliana *et al.*, 1997; Shapiro *et al.*, 2002) and demonstrates substantivity by adsorbing to protein, extracellular material and surfaces, resulting in extended protection from infections. It has been shown to give excellent inhibition of *C. albicans* biofilms when impregnated into polymer materials (Redding *et al*., 2009), or when used in mouthwashes (0.12%) against fungal biofilms (Meiller *et al.*, 2001). It was recently reported that substantivity could be improved by duration of exposure, so it is likely that an increased duration of exposure to greater than 60 sec would improve overall *C. albicans* killing, which is similar to what has been shown within this chapter for the molecules studied (Tomas *et al*., 2010). It has also been shown that chlorhexidine is significantly more effective than the azoles against *C. albicans* biofilms grown in a constant depth film fermenter (Lamfon *et al.*, 2004).

Biofilm formation in *C. albicans* is associated with increases in expression of genes associated with surface adhesion of cells and maturation of the biofilm (Murillo *et al.*, 2005). Azole drug therapy can increase expression of genes associated with drug / toxin efflux (Mateus *et al.*, 2004), and this can be accompanied by a corresponding decrease in expression of biofilm-associated genes, which can prevent biofilm formation (Bruzual *et al.*, 2007). Bruzual *et al.* showed that presence of fluconazole can inhibit biofilm formation, even in

fluconazole-resistant strains. This is similar to the effect of TTO and derivatives described in Section 4.2. This inhibitory effect can also be produced by factors released by bacteria such *as Pseudomonas aeruginosa* (Holcombe *et al.*, 2010). The formation of a biofilm can therefore be influenced by various antifungal factors, and the inhibition seen in this chapter from treating cells with TTO and derivatives during adhesion and early biofilm formation could be explained by such effects on genes involved in this process, which have also been reported elsewhere (Ramage *et al.*, 2002, Hogan *et al.*, 2004, Murillo *et al.*, 2005, Martins *et al*., 2007).

Another strategy for reducing biofilm formation on artificial surfaces such as denture materials is impregnating them with antimicrobials that are released gradually over time (Bach *et al.*, 1994; Solomon & Sherertz, 1987). There have been many studies into inhibiting bacterial colonisation through the incorporation of various antiseptics (chlorhexidine, silver sulphadiazine) and antibiotics into catheters, indwelling medical devices and dentures (Price *et al.*, 1996; Schierholz *et al.*, 1998; Wilson & Wilson, 1993; Wu & Grainger, 2006). Impregnation or coating of polymer materials with antifungals has been less well studied (Pigno *et al.*, 1994), but several recent publications show the potential of such strategies for controlling fungal attachment and hence reducing the problems associated with *C. albicans* biofilm infections (Arai *et al.*, 2009; De Prijck *et al.,* 2010; Redding *et al.*, 2009).

Overall, the data from this chapter indicates that tea tree oil based compounds are inhibitory and this effect has the capacity to be utilised within mouthwash formulations. Further comparative studies, with chlorhexidine for example, are required to fully establish their potential.

Chapter 5:
Cytotoxic Effects of TTO and Derivative Components

5.1 Introduction

The data from previous chapters demonstrate that TTO and its components are effective antifungal compounds. TTO and its components offer a potentially effective prophylactic or therapeutic mouthwash. In this context, human oral epithelial cells would be regularly exposed to these antifungal agents; therefore the possible cytotoxic effects of TTO and its components needed to be determined. In addition, cytotoxicity would influence the reported 'immunomodulatory' effects of TTO. Previous studies have documented cytotoxic effects of TTO on various cell lines after one or more hours exposure (Table 1.3). As an example, a 1 h exposure to 0.03% TTO was toxic to primary fibroblasts and primary epithelial cells (Soderberg *et al.*, 1996), although another study found a 4 h exposure to 0.28% TTO was required to inhibit the HeLa epithelial cell line by 50% (Hayes *et al.*, 1997). It seems that the laboratory-adapted HeLa cell line is less susceptible to damage from TTO than primary cells. *In vivo,* fibroblasts support keratinocyte growth and differentiation. Substances that may impact on epithelial cells may therefore also exert effects on fibroblasts, which are located in close proximity to oral keratinocytes. The aims of these studies include assessment of immunomodulatory effects of TTO and its components therefore it was essential to determine cytotoxic effects so that these could be delineated from immunomodulatory effects. Therefore, *in vitro* toxicity of TTO and its components was investigated, using a 2 min exposure to model a mouthwash, in both oral epithelial and oral fibroblast cells. Chlorhexidine gluconate (CHX), arguably the current 'gold standard' antifungal mouthwash agent, has previously demonstrated marked *in vitro* cytotoxicity, at concentrations below those found in oral products (0.2%), in keratinocyte and fibroblast skin cells (Boyce *et al.*, 1995) and also in gingival fibroblasts (Flemingson *et al.*, 2008; Wilken *et al.*, 2001). Therefore, 0.12% CHX was used throughout these experiments as a relevant positive control, known to have cytotoxic effects.

5.2 Toxicity of TTO and components to periradicular fibroblast cells

Toxicity of TTO and its constituents was initially investigated using primary human periradicular (PRD) fibroblasts (Section 2.2.1), which are readily available and easily cultured. Fibroblasts from one donor were exposed to planktonic MIC_{50} concentrations of TTO and its constituent components (in KSFM / 0.25% Tween®80 (KSFMT) for 2 min, in order to reflect the time of exposure during a mouthwash treatment. All cells were then washed with PBS to remove residual TTO / component. The metabolism of treated cells was measured using the XTT assay and compared to controls (2 min in KSFMT). The reduction in metabolism for each exposure is presented as proportional data in Table 5.1. In addition, several dilutions of TTO and the two most effective antifungal components (terpinen-4-ol [T4-ol] and *α*-terpineol) were used to confirm non-cytotoxic concentrations (Figure 5.1). The data indicates that antifungal $PMIC_{50}$ levels of TTO (0.5%) and T4-ol (0.25%) were largely cytotoxic to the PRD fibroblasts. *α*-terpineol demonstrated no apparent cytotoxicity at its $PMIC_{50}$ (0.25%) concentration. T4-ol was not toxic at 0.125%, half the $PMIC_{50}$. In contrast, TTO remained moderately cytotoxic (approximately 37% inhibition) at half the $PMIC_{50}$ concentration (0.25%). Other components tested were cytotoxic at concentrations below the $PMIC_{50}$ for *C. albicans*.

Table 5.1: Cytotoxicity of TTO and components to PRD fibroblast cells

	Concentration tested (%) *	Mean % Inhibition
TTO	0.5	86
terpinen-4-ol	0.25	87
***α*-terpineol**	0.25	0
1,8-cineole	1	91
terpinolene	1 #	77
***α*-terpinene**	2 #	88
γ-terpinene	2 #	89

* Concentrations represent the planktonic MIC_{50} for *C. albicans* of individual components

Planktonic MIC_{50} values of these compounds were all greater than that tested

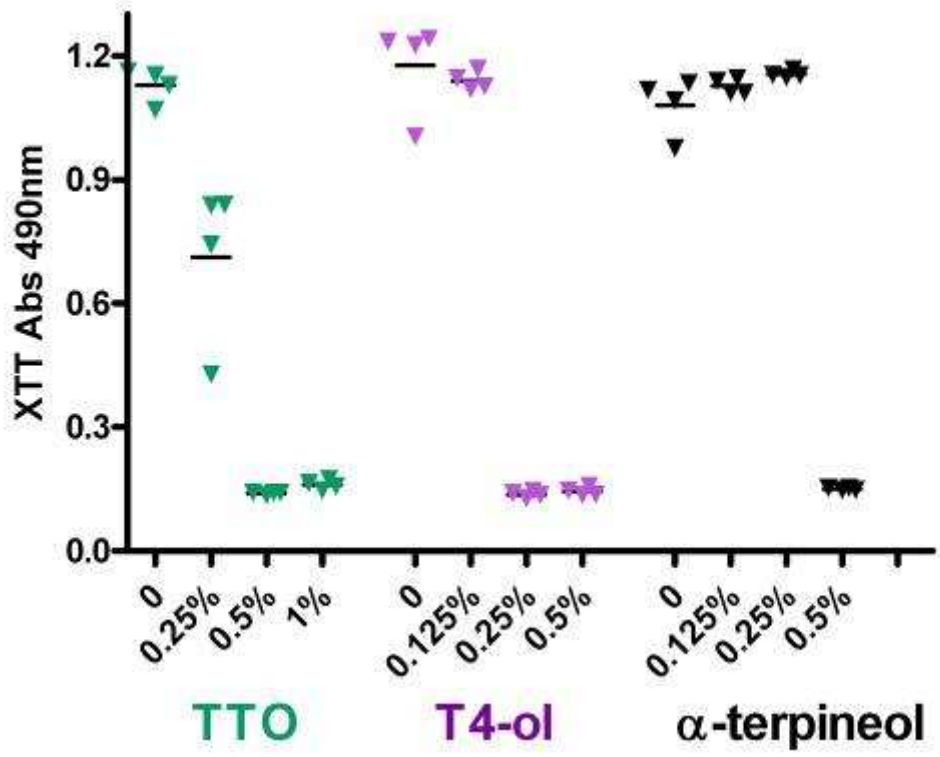


Figure 5.1: Effects of TTO and constituent components on PRD fibroblast metabolism. Fibroblasts were treated in quadruplicate for 2 min then washed gently in PBS. Dilutions were prepared in KSFMT, which was also used to treat controls (0). The metabolic activity of fibroblast cells was then assessed by XTT assay. Horizontal bars represent mean absorbance values. The results indicate that 0.5% of each component inhibited metabolic activity of PRD cells. 0.25% T4-ol was cytotoxic, while 0.125% T4-ol was non-toxic. In contrast, 0.25% *α*-terpineol was not inhibitory and 0.25% TTO demonstrated variable inhibition. The two constituent components of TTO shown here were not inhibitory at 0.125%.

5.3 Toxicity of TTO and terpinen-4-ol to oral epithelial cells

The human oral epithelial cell line OKF6-TERT2 was used to further evaluate toxicological properties of TTO and T4-ol. This cell line was immortalised by forced expression of telomerase, and has been shown to exhibit 'normal' cellular characteristics (Dickson *et al.*, 2000). Cells were grown in a 96-well plate until around 70% confluent and then treated for 2 min with defined concentrations of TTO or T4-ol in KSFMT. Control cells (0) were treated for 2 min with KSFMT. All

cells were washed in Hanks' balanced salt solution (HBSS). It was decided to focus on T4-ol because of its potent antifungal effect (low $SMFC_{50}$), and because of its abundance in TTO. Although *α*-terpineol also has strong antifungal properties, its epithelial cell toxicity was not tested as it is not toxic to fibroblasts at MIC_{50} levels, and is a minor TTO component (around 2.5% content).

These short exposures of TTO and T4-ol were highly toxic at 0.25%, while 0.125% of each had no significant effect (Figure 5.2). 0.12% chlorhexidine digluconate (CHX) in KSFMT was used as a known toxic control to give a comparison with toxicity of TTO and T4-ol. The figure shows that both 0.5% TTO and 0.25% T4-ol have comparable *in vitro* cytotoxic effect to 0.12% CHX. It is not surprising that 0.5% TTO and 0.25% T4-ol give equivalent effects, as T4-ol makes up 40% of the TTO, i.e. 0.5% TTO contains approximately 0.2% T4-ol. While Figure 5.2 illustrates data obtained from the range of concentrations tested in one experiment, it should be noted that multiple immuno-modulatory experiments using 0.125% TTO and 0.125% T4-ol exposures in OKF6-TERT2 cells confirmed this concentration to be non-toxic. In addition, alternative cell viability assays were also performed to confirm the XTT results (data not shown).

To ensure the results were representative of oral epithelial cells, and not a result of any atypical properties of a particular cell line, a further epithelial cell, the squamous cell carcinoma cell line TR146, was tested to confirm toxicity levels. In these studies, similar results were observed (data not shown). 0.25% TTO appeared to be more toxic to OKF6-TERT2 epithelial cells than to PRD fibroblasts.

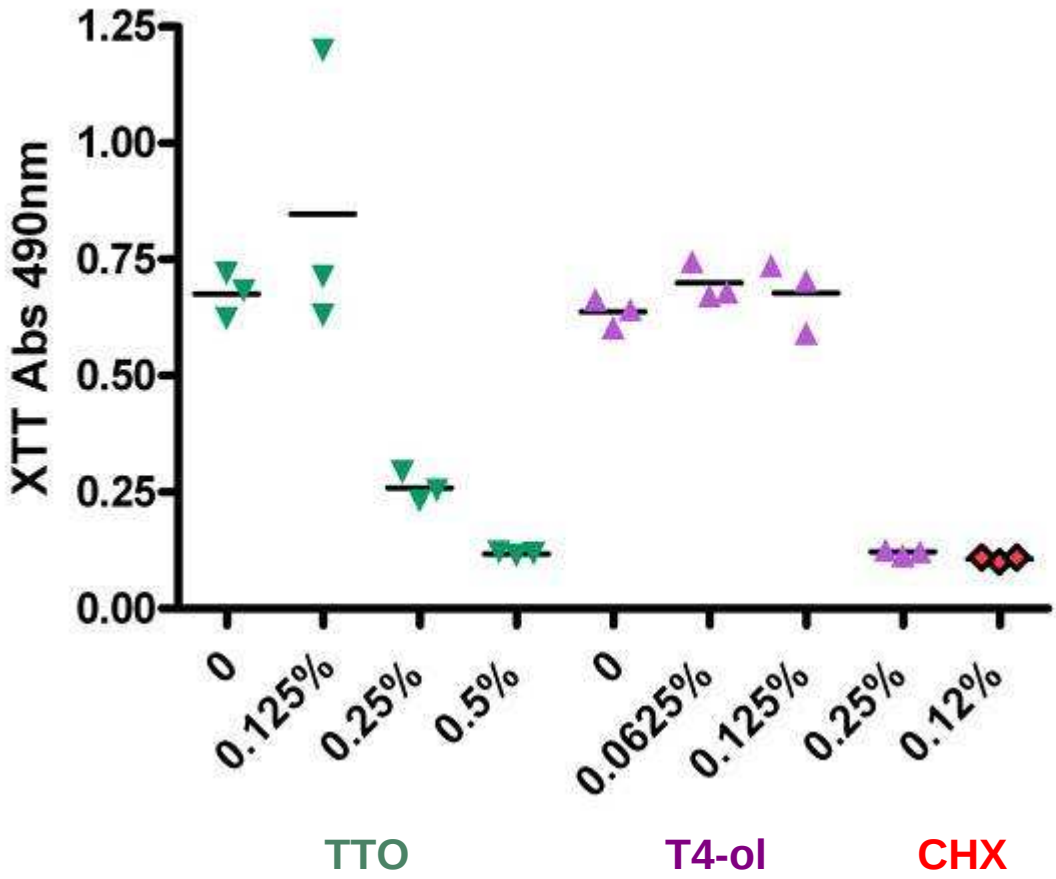
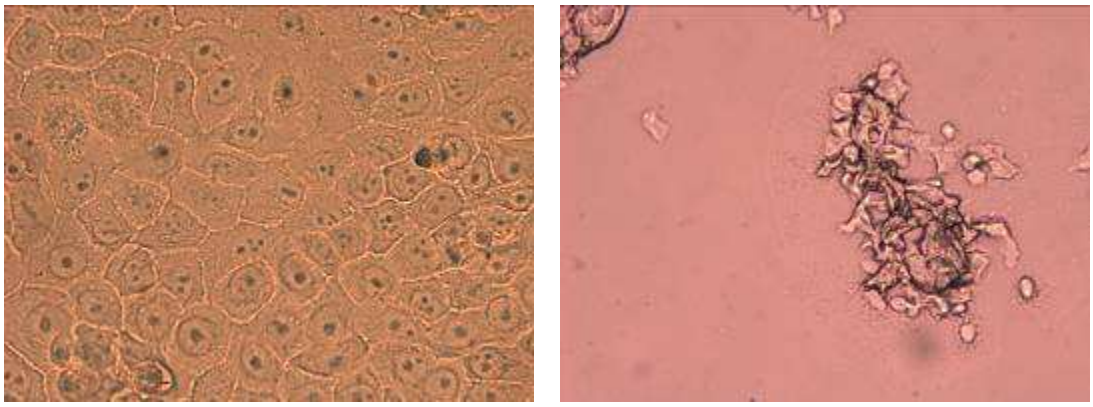


Figure 5.2: Effects of TTO and T4-ol on OKF6-TERT2 cell metabolism. Epithelial cells were treated in triplicate with TTO / T4-ol solutions in KSFMT for 2 min then washed gently in HBSS (controls (0): 2 min in KSFMT, then washed in HBSS). The metabolic activity of these cells was then assessed by XTT assay. Horizontal bars represent mean absorbance values. 0.25% TTO and 0.25% T4-ol both exhibited an inhibitory effect on OKF6-TERT2 cell metabolism. Lower concentrations had no inhibitory effect. The toxic control, 0.12% CHX, was as toxic as 0.25% T4-ol and 0.5% TTO.

Figure 5.3A illustrates confluent untreated OKF6-TERT2 cells, appearing orange from XTT reduction, compared with sparse and damaged cells after 2 min 0.25% TTO treatment (Figure 5.3B). Lack of orange colour indicates inhibition of cell metabolism. Figure 5.4 illustrates a 96-well plate with OKF6-TERT2 cells exposed for 2 min to various concentrations of TTO and T4-ol, prior to 4 h zymosan A incubation. Note that 10 μg/ml zym does not alter XTT colour, further discussed in Chapter 6.



A **B**

Figure 5.3: Morphology of OKF6-TERT2 cells after 2 min in TTO. Monolayer cells were exposed for 2 min to **(A)** KSFMT (control), or **(B)** 0.25% TTO in KSFMT, followed by 3 washes in HBSS and 2 h incubation in 100 µl of 0.25 mg/ml XTT in KSFM (400x magnification). TTO clearly destroys the monolayer.

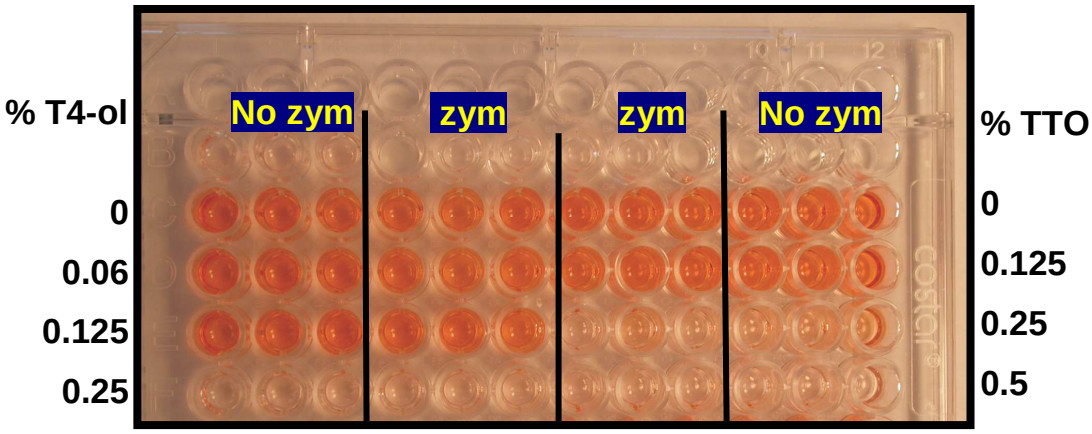


Figure 5.4: OKF6-TERT2 cells exposed to various concentrations of TTO and T4-ol. Monolayer cells were treated in a 96-well plate for 2 min, then washed 3x in HBSS. Cells were then incubated for 4 h in zym (10 µg/ml in KSFM) or KSFM. Cell metabolism was measured using the XTT assay. Lack of orange colour indicates inhibition of cell metabolism by 0.25% TTO and 0.25% T4-ol. 0.125% TTO / T4-ol (2 min exposures) had no effect on overall metabolism of these cells. Central wells show no toxic effect from 10 µg/ml zymosan A.

5.4 Summary and Discussion

The cytotoxic concentrations of TTO and components to oral cells were determined to estimate potential toxicity of TTO. Cells were exposed for 2 minutes to model a typical mouthwash exposure time. Exposure to residual concentrations remaining on mucosa for longer periods may still be significant; however, the current experiments did not address the possibility of TTO binding to, or being taken up by epithelial cells. Initial experiments with fibroblasts indicated that 2 min exposure to antifungal $PMIC_{50}$ levels of TTO or all components used in this study, except *α*-terpineol (toxic at double $PMIC_{50}$), demonstrated cytotoxic effects (Table 5.1). Subsequent experiments using TTO and terpinen-4-ol showed cytotoxicity in OKF6-TERT2 epithelial cells at 0.25%, with 0.125% being non-toxic (Figure 5.2). Variations in experiment design prevent meaningful comparison with data from previous studies (Hayes *et al.*, 1997; Soderberg *et al.*, 1996) using longer exposure times. It is clear that 0.25% TTO / T4-ol rapidly kills OKF6-TERT2 cells, while lower concentrations tested do not. Future work could determine a non-toxic concentration for TTO or components between the two levels tested here (0.25% and 0.125%), *i.e*. 0.2% TTO or component may not be cytotoxic. The variation between studies may be accounted for by several factors including a genuine variation in cytotoxic effect, variations in dose or composition of TTO, and variation in TTO adherence to plastic, which can all result in variable effective concentrations.

It would be interesting to test a much lower concentration of TTO / T4-ol with a longer exposure time (1 h or longer), to determine the susceptibility of oral cells to extended residual exposures. Exposure from mouthrinses is only ever going to be for short periods (minutes), with any residual TTO likely to be diluted and washed away by salivary flow. However, use of a TTO-based gel on oral surfaces would involve longer exposures (hours).

Other mouthwash constituents can influence the toxic properties of TTO and the overall safety of the mouthwash, and the cell monolayer model is different structurally from *in vivo* epithelial cells, which are in a complex multilayered structure, with the outer layer being composed of dead or dying cells. Toxicity of mouthwash components also depends on penetration into the inner epithelial cells. Individual components in TTO have different tissue-penetration potential, *i.e*. how well they can be absorbed, which results in changes in proportion of these constituents reaching inner mucosal layers (Nielsen, 2008).

Tissue-barrier effects, where a drug or topical lotion does not reach certain cells *in vivo*, can be investigated using *in vitro* three-dimensional organotypic models. Three-dimensional models of oral mucosa have been developed using epithelial cells in combination with fibroblasts (Dongari-Bagtzoglou & Kashleva, 2006; Klausner *et al.*, 2007). The stratification and differentiation found in epithelial tissues or organotypic models can act as an antimicrobial barrier which gives protection from bacterial and fungal challenge (Kimball *et al.*, 2006). These models are therefore also potentially useful for studying infections and the subsequent immune response. This study planned to include the use of an organotypic model containing the two monolayer cell types described above, but time did not permit this part of the investigation. Future work with such a cell model would be valuable in understanding the potential effects of any TTO-based oral product.

Extrapolating from *in vitro* toxicity of a mixture such as TTO, with differing bio-availability of components, to *in vivo* toxicity and hence human hazard and risk assessment is not straightforward. Legislative standards, such as ISO 10993-5: 2009 (ISO, 2009), provide a platform for defined *in vitro* assays which may be required for commercialization purposes.

However, the relevance of *in vitro* cytotoxicity testing is challenged by clear *in vitro* cytotoxic effects of CHX, which is widely used clinically. Clinical studies would be required to determine if the reduced cytotoxicity of TTO or its derivatives improved patient acceptance and comfort. These results suggest that TTO and its components demonstrate concentration-dependent cytotoxicity to oral epithelial cells and these observations provide the necessary information to allow investigation of the reported immunomodulatory effects.

Chapter 6:
Immunomodulatory Effects of TTO and Terpinen-4-ol

6.1 Introduction

TTO has been used over many years for treatment of skin infections and insect bites, as it is thought to have anti-inflammatory properties in addition to its antimicrobial effects. However, evidence for immuno-modulation by TTO in the oral cavity is very limited, and previous studies have not always controlled for cytotoxicity.

Inflammation accompanies candidosis, and in some cases is associated with discomfort and / or a burning sensation. Inflammation is a necessary part of the immune defence to control yeast infections and prevent invasion. Reducing inflammation with a bioactive anti-inflammatory agent could be beneficial for short-term control of symptoms, but there are also potential problems with this approach. Several studies have provided a potential link between lower levels of pro-inflammatory cytokines (including IL-1α, IL-6, IL-8 and TNF-α) and susceptibility to oral *C. albicans* infection, suggesting that such cytokines may be involved in immune protection (Dongari-Bagtzoglou & Fidel, 2005). Inflammatory cytokines released from oral epithelial cells have a major role in innate mucosal immunity, via recruitment of neutrophils and macrophages to infected mucosal sites, hence promoting phagocytosis of microbial cells (Schaller *et al*., 2002). Failure of polymorphonuclear leukocyte (PMNL) activation, due to defects in PMNL or cytokines, can lead to candidosis. Patients with defects in neutrophil function or neutropenic patients are more susceptible to oral candidosis (Epstein *et al.*, 2003). Neutrophils have also been shown to reduce *C. albicans* tissue penetration and damage in a 3-D *in vitro* model (Schaller *et al.*, 2004). Anti-inflammatory effects of therapy could hypothetically exacerbate infection problems by decreasing phagocytosis of candida cells and hence reducing control of tissue penetration. Immuno-compromised patients, such as those with HIV infection, are also more susceptible to candidosis (Ellis *et al.*, 1988); it is

clear that many OPC patients have an abnormal immune system. Therefore, there is uncertainty concerning the benefits of attempting to control inflammation in these patients

The role of tea tree oil (TTO) in immuno-modulation was discussed in detail in the introduction (Section 1.8). Previous studies into the effects of TTO on human cells have demonstrated modulation of cytokine and chemokine production, but have not excluded the possibility of toxicity mediating such effects (Brand *et al.*, 2001; Caldefie-Chezet *et al.*, 2006; Hart *et al.*, 2000). Cytotoxic studies in Section 5.3 showed that 2 min exposures to 0.125% TTO and terpinen-4-ol (T4-ol), which can also inhibit biofilm formation, were non-toxic to the OKF6-TERT2 cell line. Therefore, this concentration was chosen to investigate immuno-modulation at the transcriptional and translational level. Zymosan A (zym), a purified fungal cell wall component, was used throughout as a positive control, as an inflammatory agonist to the cells. The experiments in this chapter aimed to test the hypothesis that a TTO-based oral product could be immuno-modulatory, using both zym-stimulated and unstimulated oral epithelial cells.

6.2 Optimising OKF6-TERT2 cell stimulation

To establish an *in vitro* model of an oral epithelial inflammatory response, OKF6-TERT2 cells were challenged with zym, which provided a more efficient and reproducible cell stimulant than live or dead intact candida species. Zym was re-suspended in DMSO, with a maximum final concentration of 1:300 DMSO in cell culture medium. This concentration of DMSO was compared with cell culture medium alone and found to have no impact on mRNA or protein expression (data not shown). Initial experiments were carried out to demonstrate a dose and kinetic response of OKF6-TERT2 cells to zym (Figure 6.1), assessing IL-8 release by ELISA. Briefly, OKF6-TERT2 cells at approximately 70% confluency

were incubated in defined-keratinocyte serum-free medium (KSFM) with zym at 0,10 and 100 μg/ml for 2, 4 and 24 h. Supernatants were harvested at end of incubation and extracellular IL-8 levels determined by ELISA. At 4 h, supernatant from untreated control cells had a mean IL-8 concentration of 553 pg/ml. This increased to 892 pg/ml with 10 μg/ml zym, and 2200 pg/ml with 100 μg /ml zym. These experiments demonstrated that 10 -100 μg/ml zym reproducibly activated IL-8 production in OKF6-TERT2 cells. Therefore, this concentration range was used in further experiments to assess the effect of TTO / T4-ol on this model of an inflammatory response.

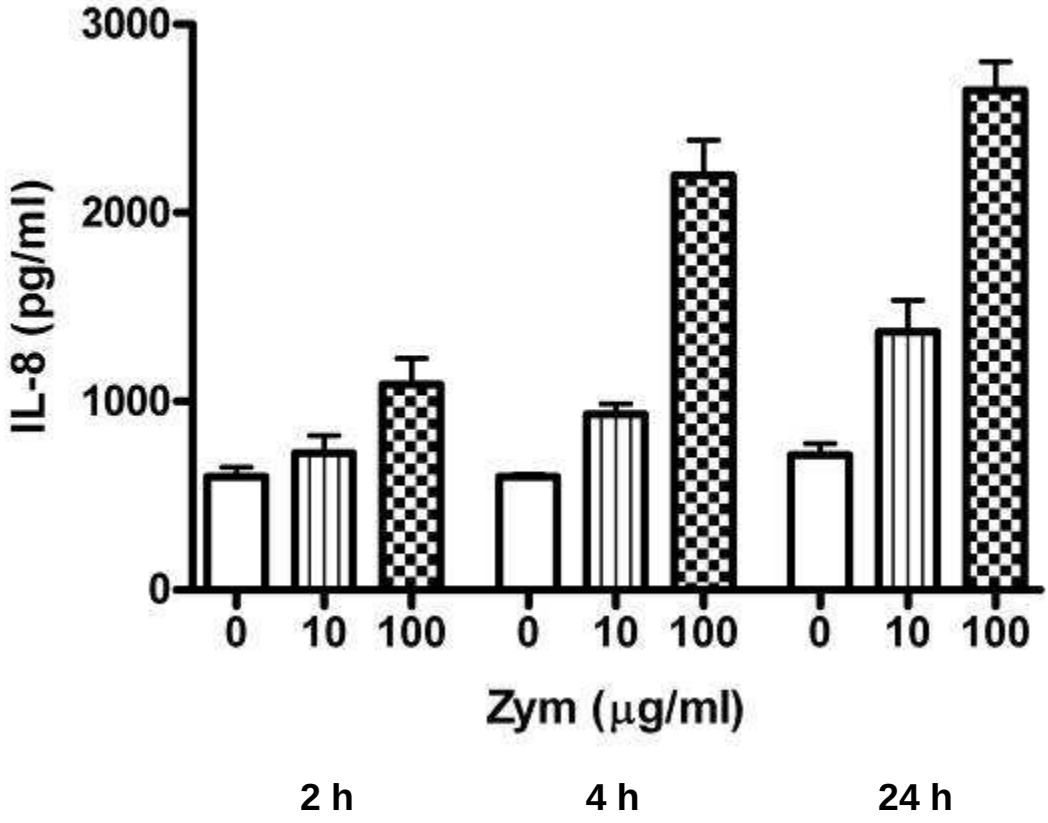


Figure 6.1: IL-8 production by OKF6-TERT2 cells in response to zymosan exposure. Cell supernatants were harvested after 2, 4, and 24 h in zym (or from unstimulated control cells in defined-KSFM) and IL-8 concentration was measured using ELISA. Each column represents 4 replicate treatments in a single experiment, with standard error bars. Zymosan within the range 10 – 100 μg/ml induced IL-8, with longer exposures giving stronger induction. 4 h and 24 h time-points were chosen for all other experiments.

6.3 PCR analysis of cytokine gene expression

6.3.1 Changes in gene expression cannot be detected using standard PCR

OKF6-TERT2 epithelial cells were treated for 2 min with 0.125% T4-ol in defined KSFM with 0.25% Tween®80 (KSFMT), or KSFMT alone, and then incubated in zym (100 μg/ml) or defined-KSFM for 4 h. RNA was harvested using the Trizol® extraction methodology, as described in Section 2.3.1. cDNA was prepared and standard reverse-transcriptase PCR was performed using primers for cytokines

IL-1β, IL-6, IL-8, and the housekeeping gene GAPDH. Similar product levels were seen in each sample visualised on an agarose gel (Figure 6.2), *i.e*. no difference seen in expression, even in the presence of zym.

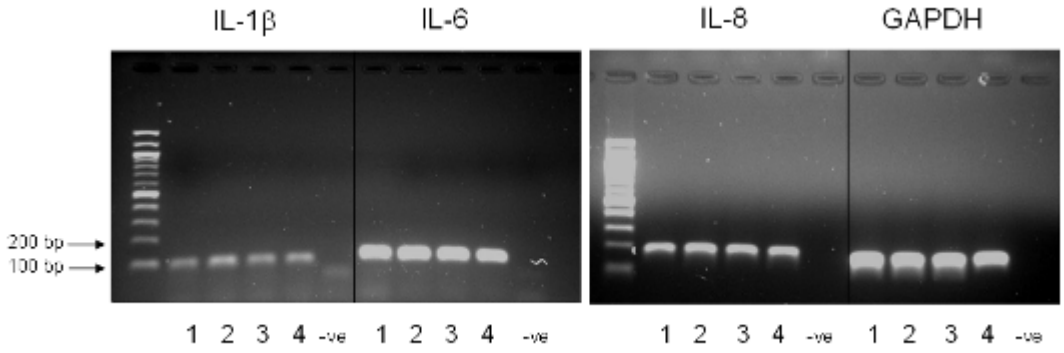


Figure 6.2: Qualitative analysis of cytokine gene expression from treated OKF6-TERT cells. Cells were treated for 2 min with either defined-KSFMT (**1**, **2**) or 0.125% T4-ol (**3**, **4**), washed and then incubated for 4 h in defined-KSFMT (**1**, **3**) or 100 μg/ml zym (**2**, **4**). The final column is the negative (-ve) water control. Products were run on a 1.8% agarose gel and stained with ethidium bromide. Based on visual analysis, the cells from each treatment have equivalent levels of each cytokine after 35 cycles. Similar GAPDH product intensity shows that DNA is at similar levels for each sample, *i.e*. RNA harvested from an equivalent number of cells for each sample.

6.3.2 Quantitative PCR analysis of cytokines from OKF6-TERT2 cells after a short exposure to TTO or T4-ol revealed no differential expression

OKF6-TERT2 cells were treated with TTO, T4-ol (both 0.125% solutions in defined-KSFMT) or defined-KSFMT (control) for 2 min, and then incubated for 4 or 24 h in 50 μg/ml zym or media control (defined-KSFM) [Section 2.4.3]. Treatments were repeated in 2 independent experiments. RNA was then harvested using the Trizol® extraction methodology, as described in section 2.3.1. cDNA was prepared and real-time PCR was performed using primers for cytokines IL-6, IL-8, and the housekeeping gene GAPDH. Real-time PCR is the best method for accurate measurement of gene expression as it measures the

DNA product at each cycle, which in turn is relative to amount of mRNA in sample template and allows calculation of changes in gene expression caused by various treatments. Each sample was run in duplicate to provide a mean C_t value (the threshold cycle when fluorescent product is present at a level above background). C_t values were normalised with the housekeeping gene GAPDH (Section 2.3.4). Untreated samples were given a relative expression value of 1, and relative expression of genes in treated cells was calculated from mean C_t values. Data were analysed using a one-way Anova followed by a Dunnett's t-test, comparing each treatment to the appropriate control cells without pre-treatment.

After 4 h treatment with 50 μg/ml zym, there was a substantial induction of IL-6 and IL-8 mRNA, with around a 40-fold increase in both (Fig. 6.3A). TTO and T4-ol pre-treatment of the cells did not induce IL-8 compared to the control, while the elevated mean levels of IL-6 were not statistically significant ($p>0.05$). Following TTO and T4-ol treatment, zym-stimulated cells showed a similar level of IL-8 induction to that of the untreated control. At 24 h, zym-stimulated mRNA induction was less reproducible (Figure 6.3B), but pre-treatment made no significant difference to gene expression of either cytokine at 24 h, compared to both zym-induced and untreated cells ($p>0.05$).

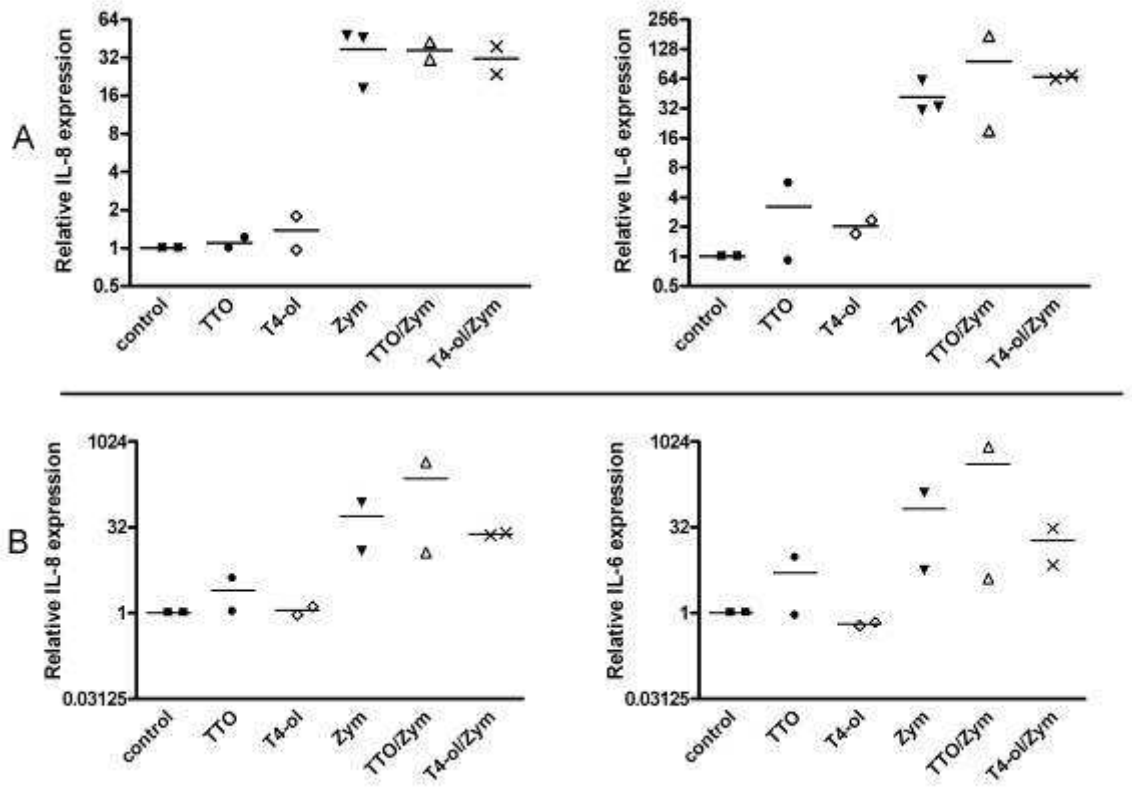


Figure 6.3: TTO and T4-ol pre-treatment produced no significant alteration in IL-6 and IL-8 transcript induction from OKF6-TERT2 cells following stimulation by zym. OKF6-TERT2 cells were treated for 2 min with defined-KSFMT, 0.125% TTO or 0.125% T4-ol, washed and incubated for 4 h (**A**) or 24 h (**B**), in defined KSFMT or 50 μg/ml zym. IL-8 and IL-6 mRNA expression was then assessed by real-time RT-PCR, using GAPDH to assess relative expression. **A**) TTO and T4-ol treatment of the cells did not induce IL-8, while IL-6 expression was increased moderately ($p>0.05$). 50 μg/ml zym produced a 40-fold induction of both IL-6 and IL-8. Following TTO and T4-ol treatment, zym-stimulated cells showed a similar level of IL-8 induction to that of the untreated zym control. IL-6 induction was slightly raised from that of the untreated controls for both TTO and T-4-ol, but not significantly ($p>0.05$). **B**) Pre-treatment with TTO or T4-ol had no significant effect on gene expression of either cytokine at 24 h ($p>0.05$), although induction levels were less reproducible at this time-point, particularly for zym-treated cells. Data shown represent two independent experiments (or three for 4 h zym), the mean of which is indicated by a horizontal bar. Each point represents the mean from PCR duplicates.

6.4 Quantitative protein analysis of cytokines from OKF6-TERT2 cells after a short exposure to TTO or T4-ol revealed no significant effect

The previous experiments suggest that exposure to non-cytotoxic concentrations of TTO or T4-ol does not significantly influence cytokine mRNA expression. To further investigate potential effects of TTO or T4-ol on epithelial cell function, analysis of supernatants was performed to measure changes in extracellular protein concentrations following exposure to various treatments. The following sections present results from the two types of protein assay used in this study.

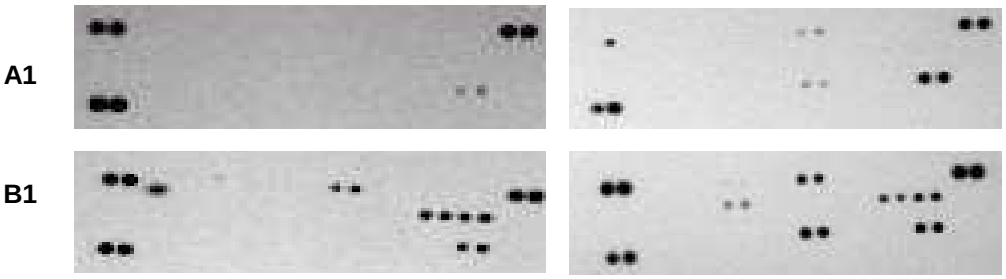
6.4.1 Cytokine Array

The quantitative PCR focussed on IL-6 / IL-8 gene expression. Levels of proteins released by cells are more biologically relevant than levels of gene expression. In addition, the inflammatory response is a very complex process involving many interacting molecules. It is therefore critically important to have an indication of changes in this immuno-modulatory mixture. Frozen supernatants from epithelial cell experiments described in section 6.3 were analysed using the cytokine array procedure (Section 2.4.1). This is a very useful way to simultaneously look at the relative levels of different cytokines within a sample, avoiding multiple PCR or ELISA assays.

6.4.2 Effects of zymosan A on extracellular cytokine levels

OKF6-TERT2 cells were grown in defined-KSFM overnight in 12-well dishes (Section 2.2.6), then treated for 4 or 24 h with 0 or 50 µg/ml zym in defined-KSFM. Supernatants were harvested and frozen at -80^{0}C. 1 ml of each supernatant was incubated with a cytokine array membrane as described in Section 2.4.1. Processed membranes were exposed to X-ray film and the resultant images analysed using Quantity One software (Biorad). This allowed comparison of 36 cytokines in zym-treated versus untreated cells (Figure 6.4),

showing results from two independent experiments. Few cytokines were detectable in untreated cells, with only serpin peptidase inhibitor clade E (serpin E1) giving a signal in both experiments, while 2 additional proteins were detected in experiment 2: macrophage migration inhibitory factor (MIF) and the growth-regulated oncogene CXCL1 (GRO-α). Four-hour zymosan treatment resulted in increases in several cytokines, most notably the pro-inflammatory interleukins IL-6 and IL-8. Interestingly, after 4 h zym-stimulation only four of the 36 proteins were detected in experiment 1, while seven were detected in the second experiment, *i.e.* most proteins being assayed for were below detectable levels. The array kit manufacturer claims that sensitivity of this assay is equivalent to Western blotting and similar to ELISA assays, although sensitivity may vary for the different array proteins. ELISA experiments indicated that 4 h untreated cell supernatants had mean IL-8 concentration of approximately 400 pg/ml (Figure 6.8). The weak IL-8 array signal from this supernatant indicates the sensitivity of the array is close to 400 pg/ml, as signal was not always present in untreated cell supernatant.



+ve	C5a	CD40	G-CFS	GM-CSF	GROα	I-309	sICAM-1	IFNγ	+ve
	IL-1α	IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-8	
	IL-10	IL-12p70	IL-13	IL-16	IL-17	IL-17E	IL-23	IL-27	
	Il-32α	IP-10	I-TAC	MCP-1	MIF	MIP-1α	MIP-1β	Serpin E1	
+ve	RANTES	SDF-1	TNF-α	sTREM-1	-ve				

Figure 6.4: Cytokine profiles of OKF6-TERT2 cell supernatants after 4 h treatment with A) control - no zym, and B) 50 µg/ml zym (1 and 2 are independent experiments). In **A1** only serpin E1 gave a signal, while in **A2** MIF, GRO α and serpin E1 were present. 4 h zym treatment (**B1**/**B2**) produced increases in several cytokines, most notably the pro-inflammatory interleukins IL-6 and IL-8. IL-1 receptor antagonist (IL-1 ra) and colony stimulating factor 3 (G-CSF) were also detected in **B2**.

6.4.3 Effects of TTO and T4-ol on extracellular cytokine levels

OKF6-TERT2 cells were plated in a 12-well cell culture dish, grown overnight, then treated for 2 min with 400 µl TTO, T4-ol (both 0.125% solutions in defined-KSFMT) or control media (defined-KSFMT), before incubation in defined-KSFM with or without zym for 4 and 24 h, as described in Section 2.2.6. Supernatants were harvested and frozen at -80^{o}C for later analysis using a proteome profiler array (R & D Systems) to detect a range of cytokines (Section 2.4.1). Images of array membranes are shown in Figures 6.5 (4 h) and 6.6 (24 h). Density was analysed for each pair of spots using Quantity One software (Biorad), and mean induction from control cells calculated for IL-6 and IL-8, as shown in Figure 6.7.

In the first 4 h array experiment (Figure 6.5A), five proteins were detected in control cell supernatant (i), namely serpin E1, MIF, GRO-α, with weak signals for IL-6 and IL-8. ELISA data from these experiments indicated that IL-8 concentration in untreated control cells was approximately 400 pg/ml (Figure 6.8), so the weak IL-8 signal described above suggests that this concentration is close to the limit of sensitivity for the array. TTO pre-treatment produced slight increases in IL-6, IL-8 and MIF, with little difference in GRO-α at 4 h (Figure 6.5A ii). Zym-induced cell supernatant (Figure 6.5A iv) had much higher levels of IL-6 and IL-8, increased MIF and GRO-α, and also signals for G-CSF (colony stimulating factor-3), IL-1ra, IL-1α and IL-13. TTO pre-treated cells with zym induction (Figure 6.5A iii) gave very similar array profile to zym treated panel. In experiment 2 (Figure 6.5B), only serpin E1 was detected in control cell supernatant (Figure 6.5B i). T4-ol pre-treatment produced a slight increase in serpin E1, with GRO-α also detected, but no IL-6 or IL-8 was detected at 4 h (Figure 6.5B ii), in contrast to TTO result above. Zym-induced cell supernatant (Figure 6.5B iv) had high levels of IL-6 and IL-8, increased serpin E1 and GRO-α, but no other proteins were detected at 4 h. T4-ol pre-treated cells with zym induction (Figure 6.5B iii) gave similar array profile to zym-treated panel (Figure 6.5B iv), but with increased IL-8, GRO-α, and detectable levels of MIF and G-CSF.

At 24 h (Figure 6.6), only serpin E1 and GRO-α gave a signal in untreated control cells (6.6A i). T4-ol pre-treatment had little effect (Figure 6.5A ii), while TTO pre-treatment (Figure 6.6A iii) increased IL-6, IL-8 (Figures 6.7C, 6.7D) and MIF to detectable levels. Zym treatment (Figure 6.6B i) resulted in high levels of pro-inflammatory IL-6 and IL-8 (Figures 6.7C, 6.7D) increases in serpin E1 and GRO-α, and also detectable levels of MIF and G-CSF. T4-ol treatment prior to 24 h zym incubation (Figures 6.6B ii, 6.7C) produced reductions in all proteins detected in zym panel (Figure 6.6B i), with MIF and G-CSF no longer detectable.

In contrast, TTO pre-treated cells (Figures 6.6B iii, 6.7C, 6.7D) showed possible increases in all the zym-induced proteins seen in Figure 6.6B i, particularly IL-6, IL-8, MIF and G-CSF. The changes described here are clearly seen in the original X-ray films, although some weak signals are not clear in the scanned images shown in the figures.

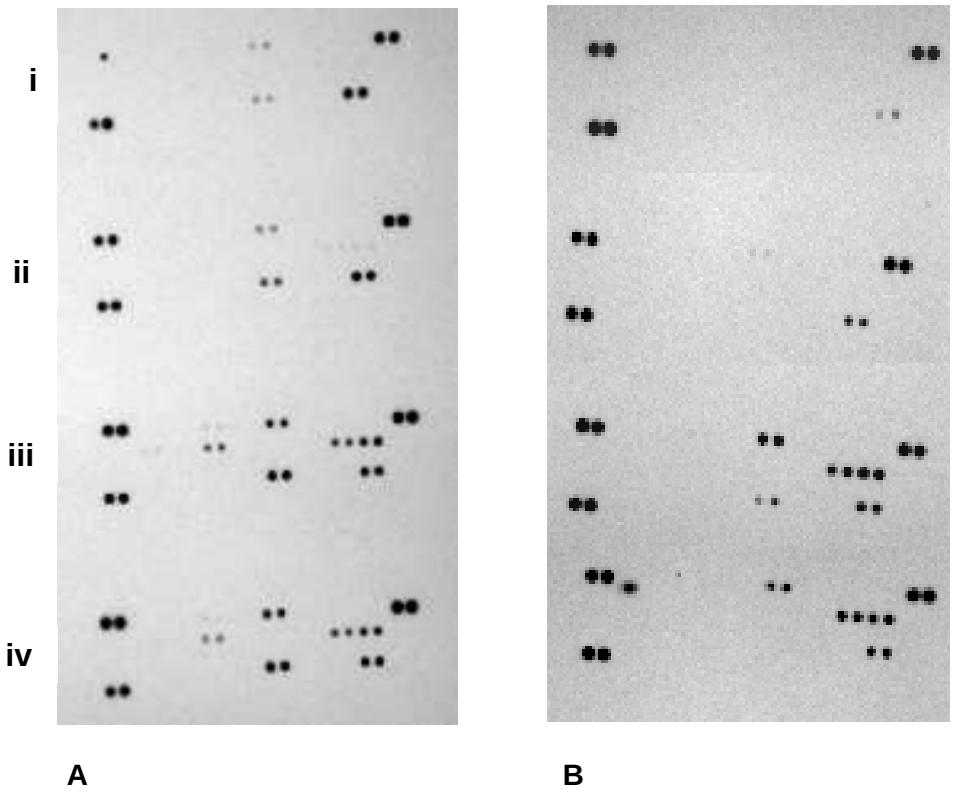


Figure 6.5: Digitised images from X-ray exposure of Proteome profiler™ arrays (4 h). OKF6-TERT2 cells were treated as described below and supernatants harvested for membrane array analysis:
6.5A i) Control cells, **ii**) 2 min TTO, **iii**) 2 min TTO + 4 h zym, **iv**) 4 h zym
6.5B i) Control cells, **ii**) 2 min T4-ol, **iii**) 2 min T4-ol + 4 h zym, **iv**) 4 h zym
Control cells: no pre-treatment or zym. 0.125% TTO/T4-ol used in pre-treatment. Zym used at 50 μg/ml in defined-KSFM. Experiments on 2 panels used independent cell preparations, as reflected in different control array profiles (**i**). TTO induced IL-6 and IL-8 (**A ii**), but much less than zym (**A iv**) which also produced detectable levels of several other proteins. TTO had no visible effect on zymosan induction profile. T4-ol pre-treatment increased serpin E1 and GRO-α (**B ii**), and also increased zym induction of IL-8, GRO-α, MIF and G-CSF (**B iii**). The strong signals at 3 corners are positive controls.

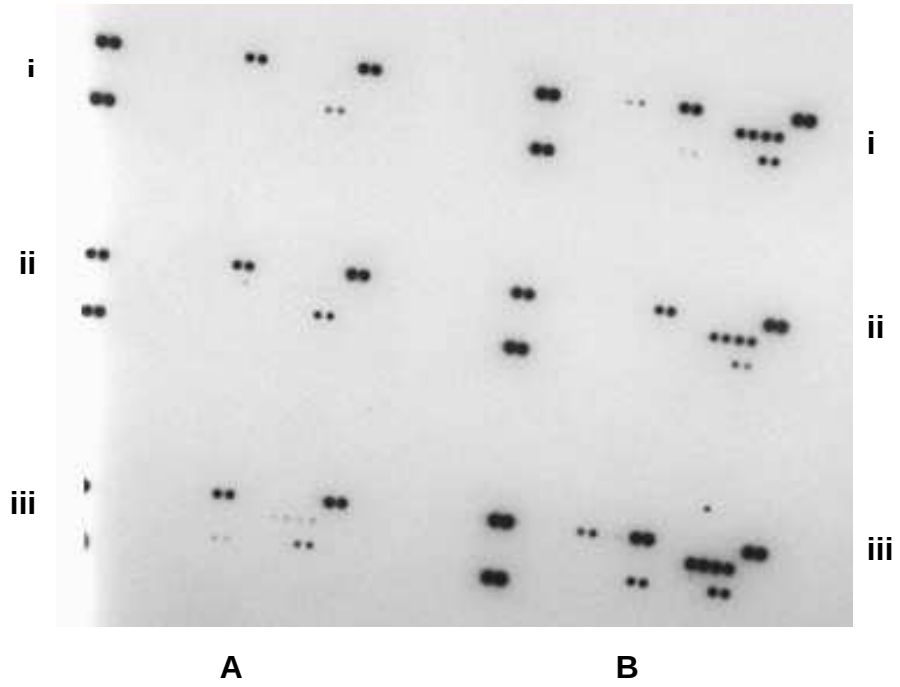


Figure 6.6: Digitised images from X-ray exposure of Proteome profiler™ arrays (24 h).

Cytokine profiles of OKF6-TERT2 cell supernatants after **24 h** treatment with :

A) no zym and **B**) 50 µg/ml zym (both in defined-KSFM), following 2 min pre-treatment with (**i**) control, only KSFMT, (**ii**) T4-ol, (**iii**) TTO (both 0.125% in defined-KSFMT).

Serpin E1 and GRO-α gave a signal in untreated control cells (**A i**), T4-ol pre-treatment had little effect (**A ii**) in contrast to TTO treatment (**A iii**), which increased IL-6, IL-8 and MIF to detectable levels. 24 h zym treatment (**B i**) produced increases in both proteins seen in **A i**, but also resulted in high levels of IL-6 and IL-8, as well as smaller amounts of MIF and G-CSF. T4-ol treatment prior to 24 h zym incubation (**B ii**) produced some reduction in all proteins detected in **B i**, with MIF and G-CSF no longer detectable. In contrast, TTO pre-treated cells (**B iii**) had possible increases in all the proteins seen in **B i**, particularly IL-6, IL-8, MIF and G-CSF.

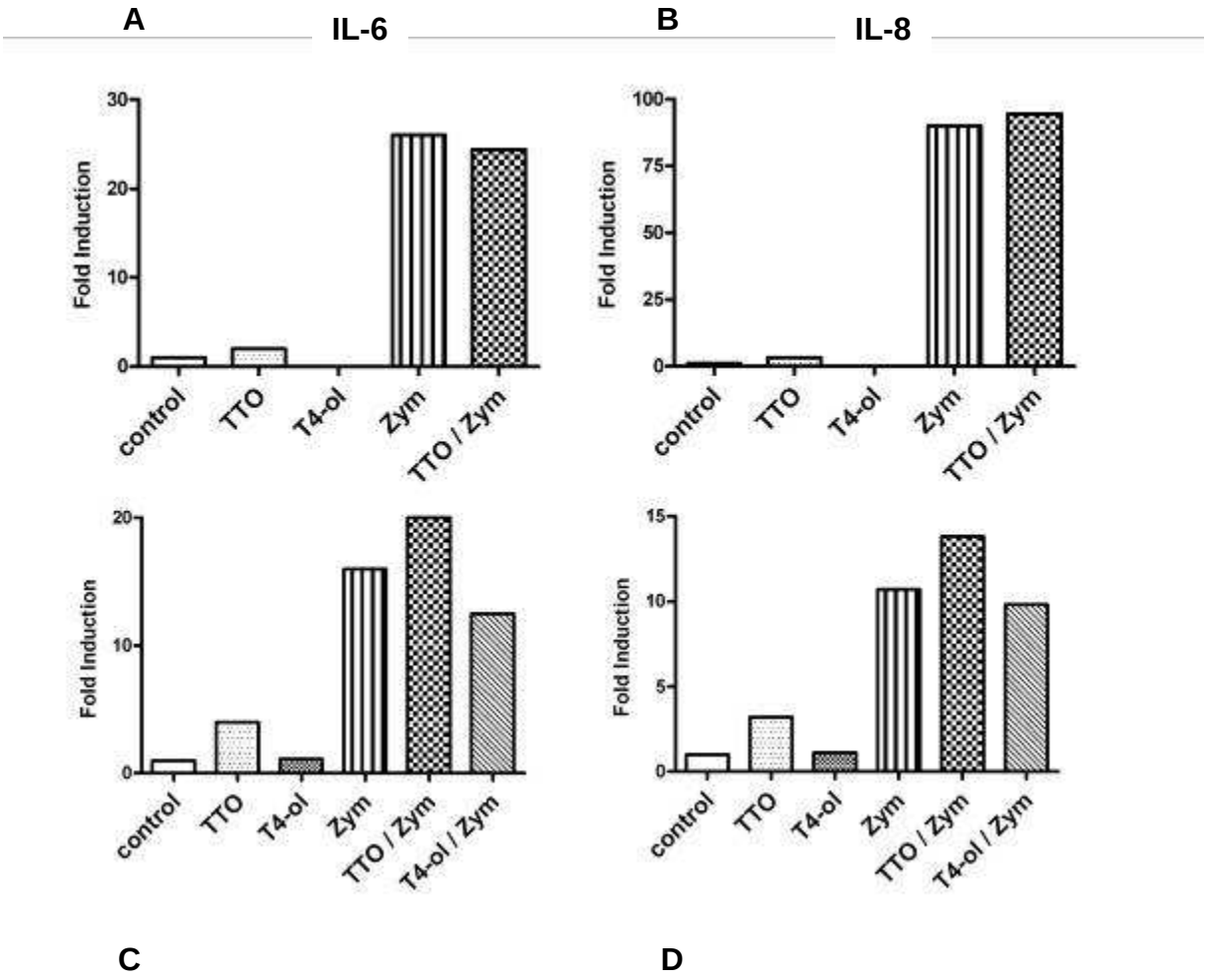


Figure 6.7: Change in spot density of A) IL-6, B) IL-8 (both 4 h), C) IL-6 and D) IL-8 (both 24 h), after 2 min pre-treatment with 0.125 % TTO / T4-ol, followed by 0 or 50 µg/ml zym.

At **4 h** (**A** and **B**), zym produced a marked increase in both cytokines (IL-6: 26 fold, IL-8: 90 fold), while TTO alone produced small increases (IL-6: 2 fold, IL-8: 3.3 fold). TTO pre-treatment had little effect on zym induction. IL-6 and IL-8 in T4-ol pre-treated zym-treated cell supernatant was similar to zym control, but neither cytokine was detected in 'no zym' control, so induction could not be quantified.

At **24 h** (**C** and **D**), zym produced a strong increase in both cytokines (IL-6: 16 fold, IL-8: 11 fold), TTO also produced an increase in both (IL-6: 4 fold, IL-8: 3.2 fold), while T4-ol had little effect. TTO pre-treatment seemed to slightly increase zym induction of IL-6 and IL-8, while the effect of zym on both cytokines was slightly reduced by T4-ol pre-treatment.

6.4.4 ELISA: IL-8 level in supernatant not significantly affected by TTO

An ELISA assay was performed to more accurately quantify changes in extracellular IL-8 protein seen in array experiments on treated cells. To establish a dose response, OKF6-TERT2 cells were grown overnight, treated for 2 min with TTO, T4-ol (both 0.125% solutions in defined-KSFMT) or defined-KSFMT for controls, then incubated in 0, 10 or 50 μg/ml zym in defined-KSFM for 4 h before harvesting supernatants (Sections 2.2.6 and 2.4.2), which were frozen at -80ºC then analysed by IL-8 ELISA (Section 2.4.2). The sensitivity of the IL-8 ELISA assay was found to be approximately 60 pg/ml. The supernatants from cells treated with 0 and 50 μg/ml zym were the same as those used in the cytokine arrays described in the previous section. These experiments measured IL-8 produced by cells and released into supernatant within 4 hours of treatments. Figure 6.8 shows combined data from 3 independent experiments.

Mean concentrations of IL-8 in supernatants from both TTO and T4-ol pre-treated cells were 400 pg/ml, not significantly different from untreated cells. TTO pre-treatment had no effect on IL-8 production by cells exposed to 10 μg/ml zym, while T4-ol pre-treatment resulted in a reduction from 877 to 662 pg/ml. TTO pre-treatment had a small inhibitory effect on IL-8 production from cells exposed to 50 μg/ml zym, while T4-ol pre-treatment resulted in a reduction from 1621 to 1095 pg/ml. This suppression of zym-induced IL-8 protein levels by T4-ol pretreatment was not significant, due to excessive experimental variation, *i.e*. T4-ol had strong inhibitory effect on IL-8 production in one experiment, but this effect was much reduced in the other experiments (Unpaired t-tests were performed on individual experiments at each zym dose, comparing control with TTO or T4-ol. One experiment gave significant p values for control versus T4-ol [no zym: $p=0.0122$, 10 zym: $p=0.0037$, 50 zym: $p<0.0001$], but no difference was seen in other experiments, or for TTO treatment). However, the trend indicates a

possible T4-ol inhibition of IL-8, an important inflammatory cytokine, which merits further investigation**.**

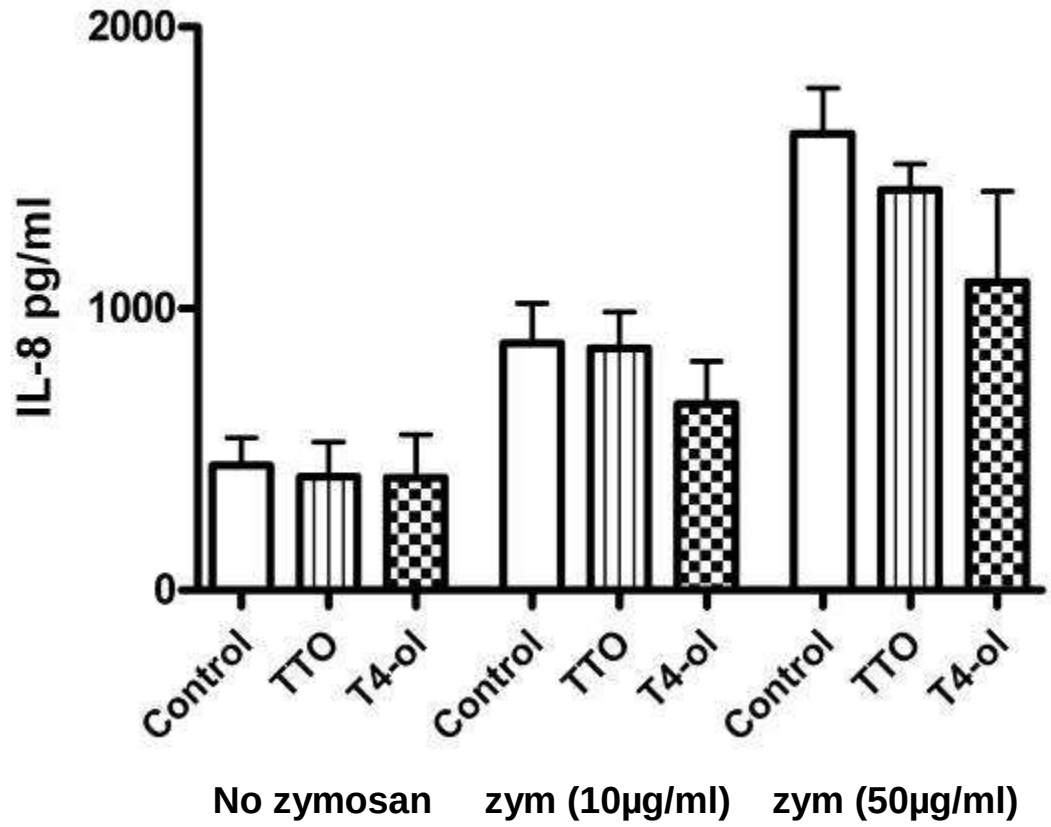


Figure 6.8: Mean zymosan dose response of OKF6-TERT2 cells pre-treated for 2 min with defined-KSFMT, 0.125% TTO or 0.125% T4-ol. Each experiment was performed on quadruplicate wells, on 3 independent cell preparations (n=3). Columns represent mean IL-8 concentration from 3 experiments, with error bars showing standard error of mean. 4 h zym treatment increased IL-8 in the supernatant in a dose-dependent manner at the concentrations tested. Pre-treatment of cells with TTO or T4-ol at 0.125% had no significant effect on IL-8 levels in the supernatant at all zym doses shown ($p>0.05$). Statistical analysis using one-way ANOVA was performed to compare all treatments to controls, followed by Bonferroni's correction for multiple comparisons, to compare all columns, to identify significantly different treatments.

6.5 Summary and Discussion

The data presented in this chapter suggest minimal, if any, immuno-modulatory effects of TTO or T4-ol on oral epithelial cells stimulated with zymosan, and highlight the importance of controlling for cytotoxicity when investigating immuno-modulatory effects. As with any *in vitro* study there are a number of caveats with this conclusion. There were some variability problems between experiments for TTO-treated cells, with one experiment showing no change while the repeat experiment gave a 4-fold induction in IL-6 expression at 4 h. Reproducibility was good for T4-ol treated cells, but less reliable for TTO treatments at both 4 and 24 h. Any cell lines may show variation in response, therefore experiments were repeated independently. Experimental variability in zymosan induction could be due to differences in the health of the cells at the time of exposure. In addition, the TTO and T4-ol are poorly soluble and the complex mixture of molecules in TTO could lead to unequal final concentrations arising in different experiments, although this was minimised by thoroughly vortex mixing all dilutions immediately before use. Small inaccuracies in treatment solutions combined with slight changes in cell health may therefore result in significant changes in cell responses to treatment. Adequate repeats of independent experiments were carried out to provide a good estimation of the effects of TTO and T4-ol.

Zymosan stimulation of an epithelial cell line provides an *in vitro* model system of candidal infection. However, it is possible that this model is too crude to detect subtle changes in cell function, thereby masking any immuno-modulatory effects of TTO or T4-ol. More complex, 3-dimensional tissue culture models are commercially available, which combine fibroblasts and epithelial cells in a collagen matrix (Dongari-Bagtzoglou & Kashleva, 2006; Klausner *et al.*, 2007). Although these are histologically reminiscent of oral mucosa, there is limited evidence documenting *in vitro* functional differences between these models and

cell mono-layers. However, it is possible that such a system may reveal more subtle effects of TTO / T4-ol. In addition, zymosan stimulation mimics only part of the challenge of a candidal biofilm, and an ideal *in vitro* model would arguably combine a more complex host cell structure with a more realistic pathogen challenge. These experiments were, however, outwith the scope of the current studies.

Although there were no statistically significant differences following TTO or T4-ol exposure, there was a small reduction in cytokine production following T4-ol exposure. It could be speculated that this may be due to membrane / lipid raft disruption, which has previously been described as an antimicrobial mechanism of TTO (Hammer *et al.*, 2004). However, further investigation would be required to validate this suggestion.

Finally, the *in vivo* immune response of oral cells to TTO exposure is also influenced by the immuno-competence of the OPC patient. Many OPC infections are in immuno-compromised individuals, including newborns with an immature immune system, AIDS / cancer patients with damaged immune responses, and the elderly population. All these individuals will respond to *Candida* infection in different ways, including variable inflammatory responses. Therefore, it is unwise to predict a possible TTO / T4-ol treatment effect from experiments performed on 'normal' cells. Any *in vitro* anti-inflammatory effect would require a careful clinical study to be undertaken to confirm if this had potential benefits for symptom relief in particular patient groups.

Chapter 7:
General Discussion

The three major aims of this study were to examine the beneficial properties of tea tree oil (TTO) and its derivatives, particularly their antifungal effects and potential anti-biofilm properties, to investigate any detrimental effects on human cells (cytotoxicity), and finally to improve understanding of possible immuno-modulatory effects of TTO components (which may or may not be beneficial). In this study it was shown that terpinen-4-ol (T4-ol) was the most effective TTO component against *C. albicans*, both for free-floating planktonic and sessile biofilm cells. Concentrations of 0.25% T4-ol / 1% TTO were toxic to *Candida albicans* biofilms, but were also cytotoxic to epithelial cells. A lower concentration of 0.125% T4-ol was able to inhibit biofilm growth (although was not toxic to pre-formed biofilms). These lower concentrations were found to be non-cytotoxic to epithelial cells and demonstrated a slight, although statistically insignificant trend to down-regulate the pro-inflammatory chemokine, IL-8. Cytotoxicity can be assessed using standard methods, such as ISO 10993-5: 2009 (ISO, 2009), which recommends particular cell lines and quantitative assays, including the XTT assay used in this study. Human oral cells were used in this study to make the results relevant to oral candidosis. One purpose of the cytotoxic studies was to allow selection of TTO and component concentrations that would not kill cells in the immunomodulation studies, using 2 min exposures. The cytotoxicity results are also useful for assessing the antifungal inhibition in relation to potential patient side-effects. Biocompatibility indexes can be calculated for potential antiseptics, using antimicrobial potency and cytotoxic activity, in an attempt to evaluate agents for clinical usefulness (Muller & Kramer, 2008). The relatively long exposure times used in most of the antifungal tests compared with two minute exposures of human cells also has to be considered. Biofilm formation was inhibited using overnight 0.125% TTO / T4-ol exposures; shorter exposures required higher concentrations. A useful additional experiment would be to test the cytotoxicity of the biofilm-inhibiting dose / time by exposing epithelial cells to

0.125% TTO / T4-ol for longer (1 or more hours instead of 2 min). The ISO method would be important when legal authorisation for clinical use was required. Mouthwashes and toothpastes are classed as cosmetics (EC1223/2009, updated version of EC Cosmetics Directive 76/768/EEC), not medicines, and so do not require as strict safety regulations, or strong evidence of effectiveness as antifungal drugs. This is partly why there are numerous oral products on the market with essential oils, such as TTO, which have inconclusive efficacy. Studies such as this aim to provide scientific evidence to aid better selection of appropriate products with proven active ingredients.

The widespread use of azole anti-mycotics has been a driving force behind the development of resistance, including point mutations in the *ERG* genes and up-regulation of efflux pumps (Niimi *et al.*, 2010; Sanglard & Odds, 2002). Furthermore, azoles have been reported to have little effect against intact biofilms, both phenotypically and genotypically (Kuhn *et al.*, 2002b; Vediyappan *et al.*, 2010). Management of oropharyngeal candidosis (OPC) with commercially available mouthwashes offers an alternative, reducing both the frequency of clinical resistance to azoles and the antimycotic cost burden to health providers. These mouthwashes are highly active against planktonic cells (Balbuena *et al.*, 1998; Jenkins *et al.*, 1994a) that are likely to be present in saliva and contribute to colonisation and subsequent biofilm formation. These active components in the commercially available mouthwashes have broad-spectrum antimicrobial activity through membrane disruption (Hugo & Longworth, 1966; Russell, 1986) which, unlike the azoles and polyenes, is not related to cell membrane ergosterol, resistance and toxicity (White, 1997). Defined, purified components of these mouthwashes were not available to the current study; therefore direct comparisons with TTO and T4-ol could not be made. Moreover, compounds such as chlorhexidine demonstrate substantivity by adsorbing to protein and

extracellular material, which can be improved by duration of exposure (Bonesvoll, *et al*., 1974; Jenkins *et al*., 1994b; Tomas *et al.*, 2009; Tomas *et al*., 2010). This increases the antimicrobial effect of CHX, but potentially can also increase the risk of cytotoxic / irritant effects. It is therefore difficult to compare *in vitro* antifungal activity of TTO with CHX and link this to a clinical effect in the oral environment.

There are however detrimental aspects to over-the-counter (OTC) mouthwashes, such as bad taste, enamel staining, burning sensation and, more controversially, the high alcohol content which has been implicated in oral cancer (McCullough & Farah, 2008; Weaver *et al.*, 1979). As a result, alternatives such as TTO have attracted some attention, as these compounds may be a more desirable alternative to prescription anti-mycotics for the management of OPC.

With regard to alcohol mouthrinses, a recent review found that 8 epidemiological studies had been done in the past 25 years and no link between oral cancer and use of alcohol-containing mouthrinses was found (Lewis & Murray, 2006). Certain mouthrinses have high ethanol content (McCullough & Farah, 2008), particularly some Listerine™ brand products (>20% alcohol), and it could be argued that these should not be used long-term, particularly in smokers and heavy drinkers who have been shown to be at a 50-fold increased risk of developing oral cancer (Castellsague *et al.*, 2004). The data from the McCullough study and other studies over a number of years are open to debate (Conway, 2009; La Vecchia, 2009). The combination of short exposure times with mouthrinse, and the influence of saliva in flushing away any residual alcohol would indicate that the risks involved are probably low (Ciancio, 2009; Smigel, 1991). However, the British Dental Journal advised that dentists should inform patients of the ‘ hypothetical risk for the development of oral cancer from repeated use of alcohol containing mouthwashes ’ (Werner & Seymour, 2009). To counteract

these potential problems, essential oils such as thymol and TTO have been proposed to have anti-cancerous properties. For example, TTO and T4-ol have been shown to induce cell cycle arrest in aggressive tumour cells, by increasing apoptosis and inducing necrotic cell death (Calcabrini *et al.*, 2004; Greay *et al.*, 2010; Medina-Holguin *et al.*, 2008).

OPC results from overgrowth of *Candida* spp. as biofilms on hard and soft tissues, such as oral epithelial surfaces, or artificial denture surfaces. Protection within the biofilm is a major factor in failure of conventional antiseptics found in mouthwashes and denture cleansers (Ramage *et al.*, 2009). It was recently reported by our group that whilst denture cleansers exhibit effective anti-biofilm activity, both in terms of removal and disinfection, residual biofilm retention could lead to regrowth and denture colonization (Jose *et al.*, 2010). The paper concluded that alternative mechanical disruptive methods may be an appropriate mechanism to enhance biofilm removal. However, are other chemotherapeutic approaches available? *In vitro* studies have demonstrated that TTO has broad antimicrobial effects and because of this it may have the potential for controlling overgrowth of detrimental oral bacteria and fungi (Bagg *et al.*, 2006; Hammer *et al.*, 2003b). Mouthwashes and denture-cleansers containing TTO are currently available, with TTO concentration of approximately 0.2% As the purpose of this study was to compare TTO with its derivatives, defined compounds were required. The OTC mouthwashes and denture cleaners contain TTO of undefined composition and a wide variation in other ingredients therefore excluding their use in the current study. Moreover, evidence to support the use of TTO or its derivatives is lacking and an initially reductionist approach was required to elucidate their effects. The complexity of the oils chemical composition and requirement for oil extraction from plants grown in native conditions has limited its large-scale development. Therefore, lack of clinical evidence, safety concerns

and limitations with growth conditions has restricted its widespread use to its native Australia.

As man-made antiseptics and drugs are often toxic, expensive, and can create resistance problems, there is much interest in natural alternatives (Edris, 2007). Plant essential oils are complex mixtures of aromatic chemicals which have been used for centuries in medicine and cooking. However, further research is required to understand the mode of action and safety profiles of TTO before it is fully accepted as drug (SCCP, 2004; SCCP, 2008).

Cell toxicity of TTO and T4-ol was dose-dependent. This has the potential to be minimised, as bioactive molecules derived from natural sources can be synthesised as exogenous molecules or adapted from their current form to improve activity, availability, reduce costs, and reduce side-effects. TTO is a complex mixture of molecules, which limits the potential for modification. However, the data from this study demonstrated that T4-ol was the most potent and abundant molecule of those tested, which would make modification and/or derivatisation an attractive option. Given that T4-ol possesses a hydroxyl group, which is one likely source of its antimicrobial action, then this would provide a starting point for the engineering of a new antimicrobial. In nature it is a ubiquitous molecule found in a wide variety of plants, including oranges, origanum, the New Zealand lemonwood tree, Japanese cedar and black pepper (Camp, 2004). Therefore, presumably T4-ol functions as an ancient innate antimicrobial molecule within the plants defences to protect against microbial infection, thus supporting rationale for its further study and development. T4-ol has been shown to control *C. albicans* vaginal infections in rats (Mondello *et al.*, 2006).

Another natural plant product currently being investigated for denture stomatitis treatment is propolis gel, which is extracted from bee honey (Santos *et al.*, 2008). Propolis is a complex mixture of chemicals including terpenes, and like TTO it has reported antifungal and anti-inflammatory properties (Borrelli *et al.*, 2002; Ota et al., 2001), giving it the potential as an agent against candidosis. Similarly, plant extracts from chewing sticks used in African (More *et al.*, 2008), Muslim (al-Otaibi, 2004), and Indian traditional medicine (Prashant *et al.*, 2007) have recently been investigated for antimicrobial activity against oral micro-organisms. For the African plants, several extracts were shown to be anti-bacterial, but these did not inhibit *C. albicans*. In contrast, clinical studies found that miswak extract could inhibit *C. albicans* (al-Bagieh *et al.*, 1994) and also the periodontal bacterial pathogen *Actinobacillus actinomycetemcomitans* (al-Otaibi, 2004), resulting in plaque and gingival index reduction compared to tooth-brushing. Prashant *et al*. found that extracts from neem tree twigs could inhibit growth of oral streptococci. A recent review of the herbal medicine literature concluded that evidence from controlled clinical studies for the antifungal efficacy of plant extracts is generally weak, although 'the most thoroughly clinically tested is tea tree oil, which holds some promise' (Martin & Ernst, 2004). In fact, the earlier work by our group also suggested a potential role for TTO in candidosis management, but the lack of any clear anti-biofilm work remained a concern (Bagg *et al.*, 2006), as biofilm resistance has been reported to be a crucial obstacle to the successful control of these infections (Seneviratne *et al.*, 2008).

The treatment of biofilms always remains problematic, and this was no different in this study. This study focussed on *C. albicans* isolates, as it is the main OPC pathogen and it forms biofilms more readily than many other *Candida* spp. This is also seen *in vitro*, where *C. glabrata* isolates generally do not form biofilms. Therefore, C. albicans was the most appropriate species to use for investigating effects of TTO and components on yeast biofilms. Although effective biofilm

activity was demonstrated by T4-ol and TTO, toxicity was associated with these concentrations, as has been reported elsewhere (Hammer *et al.*, 2006; Soderberg *et al.*, 1996). Therefore, an arm of this study was devised to evaluate what the impact of T4-ol and TTO was upon inhibition of *C. albicans* growth, morphogenesis and biofilm formation. Given that morphogenesis is pivotal to *C. albicans* biofilm formation (Ramage *et al.*, 2002b), preventing hyphal development would potentially help in the management of oral candidosis. In line with other TTO studies (D'Auria *et al.*, 2001; Hammer *et al.*, 2000), T4-ol at these non-toxic concentrations was effective at inhibiting morphogenesis and biofilm formation. The implications of this are that T4-ol has the potential to be incorporated within oral formulations of mouthwashes and toothpastes that would act as a preventative management strategy. However, it may be the case that the TTO mixture is a more effective treatment due to the different properties of the molecules in the mixture. In addition, development of microbial resistance to this mixture may be less likely than for an individual derivative.

Little is known about how the T4-ol molecule interacts with salivary proteins and whether substantivity is relevant, as is the case with chlorhexidine (Tomas *et al.*, 2009). It was shown that the molecule acted in a time-dependant manner, therefore, it may be that T4-ol may be more appropriate in relation to denture cleansers that have a longer contact time with the denture and retained microorganisms. Moreover, it would be useful to investigate whether T4-ol could be incorporated within denture lining materials, PMMA [poly methyl methacrylate acrylic resin] or other dental materials, as has been previously investigated with other antimicrobial agents (Amin *et al.*, 2009; Chandra *et al.*, 2005; Douglas & Walker, 1973; Tallury *et al.*, 2007; Wilson & Wilson, 1993). Preliminary experiments in this study showed that a 1 hour treatment of denture material with 0.5% T4-ol could inhibit *C. albicans* morphogenesis and hence block biofilm formation. The widespread use of this compound as an oral chemotherapeutic is

of less concern than for azoles. This study showed that repeat TTO exposure, whilst in a limited time-frame (10 exposures), did not alter the sensitivity of *C. albicans* to TTO. Furthermore, in this limited study the sensitivity towards azoles and other antifungal agents was not altered over the same time-frame. Induced *in vitro* resistance to TTO has been reported in *Staphylococcus aureus* (Nelson, 2000), and reductions in TTO sensitivity and antibiotic sensitivities were also detected in staphylococci after repeated exposure to sub-lethal concentrations of TTO (McMahon *et al.*, 2008). Antibiotic sensitivities reverted to normal when cells were sub-cultured in the absence of TTO, but TTO sensitivity remained lower. The stress of the TTO treatment may slow down cell growth and metabolism, causing reduced activity of the antimicrobial agent, or upregulate efflux pumps to remove any drug molecules from the bacterial cells more rapidly. Hence, it is inadvisable to have widespread use of TTO or any other drug at ineffective levels, as there is the potential for selection of less-sensitive strains (McMahon *et al.*, 2007). In contrast, it was demonstrated that induction of TTO resistance in *C. albicans* could not be achieved (Mondello *et al.*, 2003). Further detailed studies using T4-ol opposed to TTO would have to be performed, but the preliminary evidence is positive.

Combinations of chlorhexidine (CHX) with essential oils have been investigated as alternative mouthwashes (Filoche *et al.*, 2005). Bacterial biofilm inhibition was achieved using lower concentrations of chlorhexidine when cinnamon oil, Leptospermum morrisonii oil, manuka oil and Listerine®, were included in the treatment. This paper suggested that CHX had a bacteriostatic effect on *Streptococcus mutans* biofilms at the concentrations used. Chlorhexidine is thought to have bacteriostatic activity *in vivo* (Beighton *et al.*, 1991*)*; therefore use of a CHX / essential oil mixture may improve the bacteriocidal activity. Use of lower CHX concentrations could help in reducing side-effects such as staining

and tissue irritation, while also preventing the potential selection and persistence of bacteria with reduced antibiotic sensitivities (Russell, 2002; Russell, 2004). These lower concentrations may also provide opportunity for immunomodulation, as these non-toxic concentrations are likely to induce cellular responses without killing the oral cells. However, the evidence in this study for the use of TTO-based oral products in control of inflammation is inconclusive, partly due to reproducibility problems. The effect of TTO, T4-ol or other components on epithelial cells may well be significantly anti-inflammatory, if these cells respond in a similar way to other *in vitro* cells (Brand *et al.*, 2001; Caldefie-Chezet *et al.*, 2006; Hart *et al.*, 2000). TTO may reduce inflammation caused by invasive strains of *C. albicans*, but it remains to be seen whether this is a positive outcome, as the pathogen may continue to spread when unchecked by innate immune factors and cause more serious problems. These studies were performed in cell culture conditions, so the translation of the data is limited. To improve this the effects of TTO on cytokine production could be studied in organotypic cell models, which are more representative of the oral mucosa (Dongari-Bagtzoglou & Kashleva, 2006).

Convincing evidence for the immunomodulation from TTO, or its constituents, may strengthen the case for its use in many conditions, including eczema, burns and periodontal disease. It could well be the case that the complex combination of chemicals in the TTO affects multiple immuno-modulatory processes, and by only concentrating on a single molecule, like T4-ol, subtle effects may be missed. Further work is required on TTO and its components to determine their effects on immune responses and the overall clinical result of TTO-based interventions. Nevertheless, T4-ol does offer potential as a bioactive molecule that merits further study.

Conclusion

Candida albicans is the predominant yeast associated with oropharyngeal candidosis (OPC), characterised by formation of multispecies biofilms on the oral epithelium or artificial denture surfaces. Current antifungal treatments have limited success due to various resistance mechanisms and patient compliance issues, with recurring infections common. Therefore, alternative methods for suppressing or eradicating biofilms are desirable. The aims of this study were to evaluate the efficacy of tea tree oil (TTO) and key derivatives against biofilms formed by a diverse panel of clinical *C. albicans* isolates, and to assess their effects on a clinically relevant oral cell line, both in terms of their toxicological and immunomodulatory properties. TTO components have powerful activity against *C. albicans* biofilms, with terpinen-4-ol (T4-ol) and *α*-terpineol exhibiting greater anti-biofilm activity than TTO. TTO and T4-ol were significantly less toxic than chlorhexidine at therapeutic levels. TTO did not exhibit any clear immunomodulatory effect at non-toxic levels, whereas T4-ol showed possible anti-inflammatory effects. The use of a single TTO component has advantages over the complete oil in terms of product safety and consistency. Therefore, T4-ol could be developed for use in both prophylactic oral hygiene products, such as mouthrinses and denture cleansers, and as a novel treatment for established OPC infections, as it has potent antifungal activity and is minimally toxic. Preclinical and clinical trials of mouthwashes or denture cleansers, containing the range of T4-ol concentrations (0.125 - 0.5%) investigated in these studies would be required to validate the clinical use of such a product. Future work is required to determine if T4-ol has an anti-inflammatory effect in the oral environment, both *in vitro* and *in vivo*, and if so, whether this is beneficial to the patient. It is also important to investigate the potential for development of resistance to T4-ol after multiple exposures. Finally, more robust clinical studies are required to provide evidence for the use of TTO-based oral products.

Reference list

Abu-Elteen, K. H. & Abu-Alteen, R. M. (1998). The prevalence of *Candida albicans* populations in the mouths of complete denture wearers. *New Microbiol* **21**, 41-48.

Adams, D. & Addy, M. (1994). Mouthrinses. *Adv Dent Res* **8**, 291-301.

Akins, R. A. (2005). An update on antifungal targets and mechanisms of resistance in *Candida albicans***.** *Med Mycol* **43,** 285-318

Akpan, A. & Morgan, R. (2002). Oral candidiasis. *Postgrad Med J* **78**, 455-459.

al-Bagieh, N. H., Idowu, A. & Salako, N. O. (1994). Effect of aqueous extract of miswak on the in vitro growth of *Candida albicans*. *Microbios* **80**, 107-113.

Al-Otaibi, M. (2004). The miswak (chewing stick) and oral health. Studies on oral hygiene practices of urban Saudi Arabians. *Swed Dent J Suppl* **167**, 2-75.

Al-Otaibi, M. & Angmar-Mansson, B. (2004). Oral hygiene habits and oral health awareness among urban Saudi Arabians. *Oral Health Prev Dent* **2**, 389-396.

Al-Tannir, M. A. & Goodman, H. S. (1994). A review of chlorhexidine and its use in special populations. *Spec Care Dentist* **14**, 116-122.

Altman, D. G. (1991). *Practical Statistics for Medical Research*. London: Chapman and Hall.

Altman, P. M. (1988). Australian tea tree oil. *Aust J Pharm* **69**, 276-278.

Amin, W. M., Al-Ali, M. H., Salim, N. A. & Al-Tarawneh, S. K. (2009). A new form of intraoral delivery of antifungal drugs for the treatment of denture-induced oral candidosis. *Eur J Dent* **3**, 257-266.

Anonymous (1933a). Ti-trol oil. *Br Med J* **ii**, 927.

Anonymous (1933b). An Australian antiseptic oil. *Br Med J* **i**, 966.

Arai, T., Ueda, T., Sugiyama, T. & Sakurai, K. (2009). Inhibiting microbial adhesion to denture base acrylic resin by titanium dioxide coating. *J Oral Rehabil* **36**, 902-908.

Ayad, F., Berta, R., Petrone, M., De Vizio, W. & Volpe, A. (1995). Effect on plaque removal and gingivitis of a triclosan-copolymer pre-brush rinse: a six-month clinical study in Canada. *J Can Dent Assoc* **61**, 53-56, 59-61.

Bach, A., Bohrer, H., Motsch, J., Martin, E., Geiss, H. K. & Sonntag, H. G. (1994). Prevention of bacterial colonization of intravenous catheters by antiseptic impregnation of polyurethane polymers. *J Antimicrob Chemother* **33**, 969-978.

Bachmann, S. P., VandeWalle, K., Ramage, G., Patterson, T. F., Wickes, B. L., Graybill, J. R. & Lopez-Ribot, J. L. (2002). *In vitro* activity of caspofungin against *Candida albicans* biofilms. *Antimicrob Agents Chemother* **46**, 3591-3596.

Bagg, J., Jackson, M. S., Petrina Sweeney, M., Ramage, G. & Davies, A. N. (2006). Susceptibility to *Melaleuca alternifolia* (tea tree) oil of yeasts isolated from the mouths of patients with advanced cancer. *Oral Oncol* **42**, 487-492.

Bagg, J., Sweeney, M. P., Lewis, M. A., Jackson, M. S., Coleman, D., Al, M. A., Baxter, W., McEndrick, S. & McHugh, S. (2003). High prevalence of non-albicans yeasts and detection of anti-fungal resistance in the oral flora of patients with advanced cancer. *Palliat Med* **17**, 477-481.

Bagg, J., Sweeney, M. P., Davies, A. N., Jackson, M. S. & Brailsford, S. (2005). Voriconazole susceptibility of yeasts isolated from the mouths of patients with advanced cancer. *J Med Microbiol* **54**, 959-964.

Baillie, G. S. & Douglas, L. J. (1999). Role of dimorphism in the development of *Candida albicans* biofilms. *J Med Microbiol* **48**, 671-679**.**

Baillie, G. S. & Douglas, L. J. (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J Antimicrob Chemother* **46**, 397-403.

Balbuena, L., Stambaugh, K. I., Ramirez, S. G. & Yeager, C. (1998). Effects of topical oral antiseptic rinses on bacterial counts of saliva in healthy human subjects. *Otolaryngol Head Neck Surg* **118**, 625-629.

Banes-Marshall, L., Cawley, P. & Phillips, C. A. (2001). *In vitro* activity of *Melaleuca alternifolia* (tea tree) oil against bacterial and *Candida spp*. isolates from clinical specimens. *British Journal of Biomedical Science* **58**, 139-145.

Basson, N. J., Quick, A. N. & Thomas, C. J. (1992). Household products as sanitising agents in denture cleansing. *J Dent Assoc S Afr* **47**, 437-439.

Beighton, D., Decker, J. & Homer, K. A. (1991). Effects of chlorhexidine on proteolytic and glycosidic enzyme activities of dental plaque bacteria. *J Clin Periodontol* **18**, 85-89.

Bonesvoll, P., Lokken, P., Rolla, G. & Paus, P. N. (1974). Retention of chlorhexidine in the human oral cavity after mouth rinses. *Arch Oral Biol* **19**, 209-212.

Borrelli, F., Maffia, P., Pinto, L., Ianaro, A., Russo, A., Capasso, F. & Ialenti, A. (2002). Phytochemical compounds involved in the anti-inflammatory effect of propolis extract. *Fitoterapia* **73 Suppl 1**, S53-63.

Boyce, S. T., Warden, G. D. & Holder, I. A. (1995). Cytotoxicity testing of topical antimicrobial agents on human keratinocytes and fibroblasts for cultured skin grafts. *J Burn Care Rehabil* **16**, 97-103.

Bradshaw, D. J., Marsh, P. D., Hodgson, R. J. & Visser, J. M. (2002). Effects of glucose and fluoride on competition and metabolism within *in vitro* dental bacterial communities and biofilms. *Caries Res* **36**, 81-86.

Brand, C., Ferrante, A., Prager, R. H., Riley, T. V., Carson, C. F., Finlay-Jones, J. J. & Hart, P. H. (2001). The water-soluble components of the essential oil of *Melaleuca alternifolia* (tea tree oil) suppress the production of superoxide by human monocytes, but not neutrophils, activated in vitro. *Inflamm Res* **50**, 213-219.

Brand, C., Grimbaldeston, M. A., Gamble, J. R., Drew, J., Finlay-Jones, J. J. & Hart, P. H. (2002a). Tea tree oil reduces the swelling associated with the efferent phase of a contact hypersensitivity response. *Inflamm Res* **51**, 236-244.

Brand, C., Townley, S. L., Finlay-Jones, J. J. & Hart, P. H. (2002b). Tea tree oil reduces histamine-induced oedema in murine ears. *Inflamm Res* **51**, 283-289.

Brophy, J. J., Davies, N. W., Southwell, I. A., Stiff, I. A. & Williams, L. R. (1989). Gas chromatographic quality control for oil of *Melaleuca* terpinen-4-ol type (Australian tea tree). *J Agric Food Chem* **37**, 1330-1335.

Brown, G. D. **(2010)**. How fungi have shaped our understanding of mammalian immunology. *Cell Host Microbe* **7**, 9-11.

Bruzual, I., Riggle, P., Hadley, S. & Kumamoto, C. A. (2007). Biofilm formation by fluconazole-resistant *Candida albicans* strains is inhibited by fluconazole. *J Antimicrob Chemother* **59**, 441-450.

Budtz-Jorgensen, E. (1990a). Etiology, pathogenesis, therapy and prophylaxis of oral yeast infections. *Acta Odontol Scand* **48**, 61-69.

Budtz-Jorgensen, E. (1990b). *Oral Candidosis: Candida-associated denture stomatitis and angular cheilitis*, pp156-183.. London.

Budtz-Jorgensen, E. & Kelstrup, J. (1977). Enzymes as denture cleansers. *Scand J Dent Res* **85**, 209-215.

Buergers, R., Rosentritt, M., Schneider-Brachert, W., Behr, M., Handel, G. & Hahnel, S. (2008). Efficacy of denture disinfection methods in controlling *Candida albicans* colonization in vitro. *Acta Odontol Scand* **66**, 174-180.

Caelli, M., Porteous, J., Carson, C. F., Heller, R. & Riley, T. V. (2000). Tea tree oil as an alternative topical decolonization agent for methicillin-resistant *Staphylococcus aureus*. *J Hosp Infect* **46**, 236-237.

Calcabrini, A., Stringaro, A., Toccacieli, L. & other authors (2004). Terpinen-4-ol, the main component of *Melaleuca alternifolia* (Tea tree) oil inhibits the *in vitro* growth of human melanoma cells. *Journal of Investigative Dermatology* **122**, 349-360.

Caldefie-Chezet, F., Fusillier, C., Jarde, T., Laroye, H., Damez, M., Vasson, M. P. & Guillot, J. (2006). Potential anti-inflammatory effects of *Melaleuca alternifolia* essential oil on human peripheral blood leukocytes. *Phytother Res* **20**, 364-370.

Calderone, R. A. & Fonzi, W. A. (2001). Virulence factors of *Candida albicans*. *Trends Microbiol* **9**, 327-335.

Calnan, C. D. (1962). Contact dermatitis from drugs. *Proc R Soc Med* **55**, 39-42.

Camp, R. D. (2004). The Wizard of Oz, or the intriguing tale of the tea tree. *J Invest Dermatol* **123**, xviii-xix.

Campanha, N. H., Pavarina, A. C., Brunetti, I. L., Vergani, C. E., Machado, A. L. & Spolidorio, D. M. (2007). *Candida albicans* inactivation and cell membrane integrity damage by microwave irradiation. *Mycoses* **50**, 140-147.

Cannon, R. D., Holmes, A. R., Mason, A. B. & Monk, B. C. (1995). Oral Candida: clearance, colonization, or candidiasis? *J Dent Res* **74**, 1152-1161.

Cannon, R. D., Lamping, E., Holmes, A. R., Niimi, K., Tanabe, K., Niimi, M. & Monk, B. C. (2007). *Candida albicans* drug resistance - another way to cope with stress. *Microbiology* **153**, 3211-3217.

Cannon, R. D., Lamping, E., Holmes, A. R. & other authors (2009). Efflux-mediated antifungal drug resistance. *Clin Microbiol Rev* **22**, 291-321.

Carson, C. F., Hammer, K. A. & 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. & Riley, T. V. (2006). *Melaleuca alternifolia* (Tea Tree) oil: a review of antimicrobial and other medicinal properties. *Clin Microbiol Rev* **19**, 50-62.

Carson, C. F., Mee, B. J. & Riley, T. V. (2002). Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob Agents Chemother* **46**, 1914-1920.

Carson, C. F. & Riley, T. V. (1995). Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. *J Appl Bacteriol* **78**, 264-269.

Castellsague, X., Quintana, M. J., Martinez, M. C. & other authors (2004). The role of type of tobacco and type of alcoholic beverage in oral carcinogenesis. *Int J Cancer* **108**, 741-749.

Catalan, A., Pacheco, J. G., Martinez, A. & Mondaca, M. A. (2008). *In vitro* and *in vivo* activity of *Melaleuca alternifolia* mixed with tissue conditioner on *Candida albicans*. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **105**, 327-332.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T. & Ghannoum, M. A. (2001a). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* **183**, 5385-5394.

Chandra, J., Mukherjee, P. K., Leidich, S. D., Faddoul, F. F., Hoyer, L. L., Douglas, L. J. & Ghannoum, M. A. (2001b). Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. *J Dent Res* **80**, 903-908.

Chandra, J., Patel, J. D., Li, J., Zhou, G., Mukherjee, P. K., McCormick, T. S., Anderson, J. M. & Ghannoum, M. A. (2005). Modification of surface properties of biomaterials influences the ability of *Candida albicans* to form biofilms. *Appl Environ Microbiol* **71**, 8795-8801.

Charles, C. H., Pan, P. C., Sturdivant, L. & Vincent, J. W. (2000a). *In vivo* antimicrobial activity of an essential oil-containing mouthrinse on interproximal plaque bacteria. *J Clin Dent* **11**, 94-97.

Charles, C. H., Vincent, J. W., Borycheski, L., Amatnieks, Y., Sarina, M., Qaqish, J. & Proskin, H. M. (2000b). Effect of an essential oil-containing dentifrice on dental plaque microbial composition. *Am J Dent* **13**, 26C-30C.

Choo, A., Delac, D. M. & Messer, L. B. (2001). Oral hygiene measures and promotion: review and considerations. *Aust Dent J* **46**, 166-173.

Ciancio, S. (2003). Essential Oils in Oral Health Management: a review. *J Clin Periodontol* **30 (supplement 5)**, 1-24.

Ciancio, S. G. (2009). Alcohol-containing mouthwashes. *Aust Dent J* **54**, 179-182; author reply 182-173.

CLSI, Clinical Laboratory Standards Institute (2008). Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard, 3rd ed. In *CLSI document M27-A3*.

Coco, B. J., Bagg, J., Cross, L. J., Jose, A., Cross, J. G. & Ramage, G. (2008). Mixed *Candida albicans* and *Candida glabrata* populations associated with the pathogenesis of denture stomatitis. *Oral Microbiol Immunol* **23**, 377-383.

Coenye, T. & Nelis, H. J. (2010). *In vitro* and *in vivo* model systems to study microbial biofilm formation. *J Microbiol Methods* **83**, 89-105.

Conway, D. (2009). Oral health, mouthwashes and cancer - what is the story? *Evid Based Dent* **10**, 6-7.

Coulthwaite, L. & Verran, J. (2007). Potential pathogenic aspects of denture plaque. *Br J Biomed Sci* **64**, 180-189.

Cox, S. D., Gustafson, J. E., Mann, C. M., Markham, J. L., Liew, Y. C., Hartland, R. P., Bell, H. C., Warmington, J. R. & Wyllie, S. G. (1998). Tea tree oil causes K^+ leakage and inhibits respiration in *Escherichia coli*. *Lett Appl Microbiol* **26**, 355-358.

Cox, S. D., Mann, C. M., Markham, J. L., Bell, H. C., Gustafson, J. E., Warmington, J. R. & Wyllie, S. G. (2000). The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J Appl Microbiol* **88**, 170-175.

Cox, S. D., Mann, C. M., Markham, J. L., Gustafson, J. E., Warmington, J. R. & Wyllie, S. G. (2001). Determining the antimicrobial actions of tea tree oil. *Molecules* **6**, 87-91.

Cross, D. L. & Short, L. J. (2008). Angular cheilitis occurring during orthodontic treatment: a case series. *J Orthod* **35**, 229-233.

Crump, J. A. & Collignon, P. J. (2000). Intravascular catheter-associated infections. *Eur J Clin Microbiol Infect Dis* **19**, 1-8.

D'Auria, F. D., Laino, L., Strippoli, V., Tecca, M., Salvatore, G., Battinelli, L. & Mazzanti, G. (2001). *In vitro* activity of tea tree oil against *Candida albicans* mycelial conversion and other pathogenic fungi. *Journal of Chemotherapy* **13**, 377-383.

Davies, A. N., Brailsford, S., Broadley, K. & Beighton, D. (2002). Oral yeast carriage in patients with advanced cancer. *Oral Microbiol Immunol* **17**, 79-84.

Davies, A. N., Brailsford, S. R. & Beighton, D. (2006). Oral candidosis in patients with advanced cancer. *Oral Oncol* **42**, 698-702.

Davies, A. N., Brailsford, S. R., Beighton, D., Shorthose, K. & Stevens, V. C. (2008). Oral candidosis in community-based patients with advanced cancer. *J Pain Symptom Manage* **35**, 508-514.

Davies, R. M. (2007). The clinical efficacy of triclosan/copolymer and other common therapeutic approaches to periodontal health. *Clin Microbiol Infect* **13 Suppl 4**, 25-29.

de Brugerolle de, F., Picarles, V., Chibout, S. & other authors (1999). Predictivity of an *in vitro* model for acute and chronic skin irritation (SkinEthic) applied to the testing of topical vehicles. *Cell Biol Toxicol* **15**, 121-135.

de Groot, A. C. & Weyland, J. W. (1992). Systemic contact dermatitis from tea tree oil. *Contact Dermatitis* **27**, 279-280.

Denning, D. W. (2003). Echinocandin antifungal drugs. *Lancet* **362**, 1142-1151.

De Prijck, K., De Smet, N., Honraet, K., Christiaen, S., Coenye, T., Schacht, E. & Nelis, H. J. (2010). Inhibition of *Candida albicans* biofilm formation by antimycotics released from modified polydimethyl siloxane. *Mycopathologia* **169**, 167-174

Dickson, M. A., Hahn, W. C., Ino, Y., Ronfard, V., Wu, J. Y., Weinberg, R. A., Louis, D. N., Li, F. P. & Rheinwald, J. G. (2000). Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol* **20**, 1436-1447.

Dixon, D. L., Breeding, L. C. & Faler, T. A. (1999). Microwave disinfection of denture base materials colonized with *Candida albicans*. *J Prosthet Dent* **81**, 207-214.

Dongari-Bagtzoglou, A. & Fidel, P. L., Jr. (2005). The host cytokine responses and protective immunity in oropharyngeal candidiasis. *J Dent Res* **84**, 966-977.

Dongari-Bagtzoglou, A. & Kashleva, H. (2003). *Candida albicans* triggers interleukin-8 secretion by oral epithelial cells. *Microb Pathog* **34**, 169-177.

Dongari-Bagtzoglou, A. & Kashleva, H. (2006). Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. *Nat Protoc* **1**, 2012-2018.

Donlan, R. M. (2008). Biofilms on central venous catheters: is eradication possible? *Curr Top Microbiol Immunol* **322**, 133-161.

Donlan, R. M. & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**, 167-193.

Donlan, R. M., Murga, R., Bell, M., Toscano, C. M., Carr, J. H., Novicki, T. J., Zuckerman, C., Corey, L. C. & Miller, J. M. (2001). Protocol for detection of biofilms on needleless connectors attached to central venous catheters. *J Clin Microbiol* **39**, 750-753.

Douglas, L. J. (2002). Medical importance of biofilms in Candida infections. *Rev Iberoam Micol* **19**, 139-143.

Douglas, L. J. (2003). *Candida* biofilms and their role in infection. *Trends Microbiol* **11**, 30-36.

Douglas, W. H. & Walker, D. M. (1973). Nystatin in denture liners - an alternative treatment of denture stomatitis. *Br Dent J* **135**, 55-59.

Dranginis, A. M., Rauceo, J. M., Coronado, J. E. & Lipke, P. N. (2007). A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. *Microbiol Mol Biol Rev* **71**, 282-294.

Dreizen, S. (1984). Oral candidiasis. *Am J Med* **77**, 28-33.

Ebo, D. G., Stevens, W. J., Bridts, C. H. & Matthieu, L. (1998). Contact allergic dermatitis and life-threatening anaphylaxis to chlorhexidine. *J Allergy Clin Immunol* **101**, 128-129.

Edris, A. E. (2007). Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytother Res* **21**, 308-323.

Ellepola, A. N. & Samaranayake, L. P. (2001). Adjunctive use of chlorhexidine in oral candidoses: a review. *Oral Dis* **7**, 11-17.

Ellis, M., Gupta, S., Galant, S., Hakim, S., VandeVen, C., Toy, C. & Cairo, M. S. (1988). Impaired neutrophil function in patients with AIDS or AIDS-related complex: a comprehensive evaluation. *J Infect Dis* **158**, 1268-1276.

Epstein, J. B. (1990). Antifungal therapy in oropharyngeal mycotic infections. *Oral Surg Oral Med Oral Pathol* **69**, 32-41.

Epstein, J. B. & Polsky, B. (1998). Oropharyngeal candidiasis: a review of its clinical spectrum and current therapies. *Clin Ther* **20**, 40-57.

Epstein, J. B., Hancock, P. J. & Nantel, S. (2003). Oral candidiasis in hematopoietic cell transplantation patients: an outcome-based analysis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **96**, 154-163.

Eren, K., Ozmeric, N. & Sardas, S. (2002). Monitoring of buccal epithelial cells by alkaline comet assay (single cell gel electrophoresis technique) in cytogenetic evaluation of chlorhexidine. *Clin Oral Investig* **6**, 150-154.

Erlandsen, S. L., Kristich, C. J., Dunny, G. M. & Wells, C. L. (2004). High-resolution visualization of the microbial glycocalyx with low-voltage scanning electron microscopy: dependence on cationic dyes. *J Histochem Cytochem* **52**, 1427-1435.

Evans, R. J. (1992). Acute anaphylaxis due to topical chlorhexidine acetate. *BMJ* **304**, 686.

Filoche, S. K., Soma, K. & Sissons, C. H. (2005). Antimicrobial effects of essential oils in combination with chlorhexidine digluconate. *Oral Microbiol Immunol* **20**, 221-225.

Fine, D. H., Furgang, D., Barnett, M. L., Drew, C., Steinberg, L., Charles, C. H. & Vincent, J. W. (2000). Effect of an essential oil - containing antiseptic mouthrinse on plaque and salivary *Streptococcus mutans* levels. *J Clin Periodontol,* **27**, 157-61.

Fine, D. H., Markowitz, K., Furgang, D., Goldsmith, D., Charles, C. H., Lisante, T.A. & Lynch, M. C. (2007a). Effect of an essential oil-containing antimicrobial mouthrinse on specific plaque bacteria *in vivo*. *J Clin Periodontol,* **34**, 652-657.

Fine, D. H., Markowitz, K., Furgang, D., Goldsmith, D., Ricci-Nittel, D., Charles, C. H., Peng, P. & Lynch, M. C. (2007b). Effect of rinsing with an essential oil-containing mouthrinse on subgingival periodontopathogens. *J Periodontol,* **78**,1935-1942.

Finlay, I. & Davies, A. (2005). Fungal infections*. In Oral care in advanced disease*, pp 55-71. Oxford: Oxford University Press.

Fischman, S. L. (1994). A clinician's perspective on antimicrobial mouthrinses. *J Am Dent Assoc* **125 Suppl 2**, 20S-22S.

Flemingson, Emmadi, P., Ambalavanan, N., Ramakrishnan, T. & Vijayalakshmi, R. (2008). Effect of three commercial mouth rinses on cultured human gingival fibroblast: an in vitro study. *Indian J Dent Res* **19**, 29-35.

Fraser, V. J., Jones, M., Dunkel, J., Storfer, S., Medoff, G. & Dunagan, W. C. (1992). Candidemia in a tertiary care hospital: epidemiology, risk factors, and predictors of mortality. *Clin Infect Dis* **15**, 414-421.

Freitas, M. D., Blanco-Carrion, A., Gandara-Vila, P., Antunez-Lopez, J., Garcia-Garcia, A. & Gandara Rey, J. M. (2006). Clinicopathologic aspects of oral leukoplakia in smokers and nonsmokers. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **102**, 199-203.

Garcia-Sanchez, S., Aubert, S., Iraqui, I., Janbon, G., Ghigo, J. M. & d'Enfert, C. (2004). *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot Cell* **3**, 536-545.

Ghannoum, M. A., Jurevic, R. J., Mukherjee, P. K., Cui, F., Sikaroodi, M., Naqvi, A. & Gillevet, P. M. (2010). Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog* **6**, e1000713.

Gilbert, P., Das, J. & Foley, I. (1997). Biofilm susceptibility to antimicrobials. *Adv Dent Res,* **11**, 160-167.

Gilbert, P., McBain, A. & Sreenivasan, P. (2007). Common therapeutic approaches for the control of oral biofilms: microbiological safety and efficacy. *Clin Microbiol Infect* **13 Suppl 4**, 17-24.

Giordani, C., Molinari, A., Toccacieli, L., Calcabrini, A., Stringaro, A., Chistolini, P., Arancia, G. & Diociaiuti, M. (2006). Interaction of tea tree oil with model and cellular membranes. *J Med Chem* **49**, 4581-4588.

Giuliana, G., Pizzo, G., Milici, M. E., Musotto, G. C. & Giangreco, R. (1997). *In vitro* antifungal properties of mouthrinses containing antimicrobial agents. *J Periodontol* **68**, 729-733.

Golab, M. & Skwarlo-Sonta, K. (2007). Mechanisms involved in the anti-inflammatory action of inhaled tea tree oil in mice. *Exp Biol Med (Maywood)* **232**, 420-426.

Greay, S. J., Ireland, D. J., Kissick, H. T., Levy, A., Beilharz, M. W., Riley, T. V. & Carson, C. F. (2010). Induction of necrosis and cell cycle arrest in murine cancer cell lines by *Melaleuca alternifolia* (tea tree) oil and terpinen-4-ol. *Cancer Chemother Pharmacol* **65**, 877-888.

Green, C. B., Cheng, G., Chandra, J., Mukherjee, P., Ghannoum, M. A. & Hoyer, L. L. (2004). RT-PCR detection of *Candida albicans* ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. *Microbiology* **150**, 267-275.

Guggenheimer, J. & Moore, P. A. (2003). Xerostomia: etiology, recognition and treatment. *J Am Dent Assoc* **134**, 61-69; quiz 118-119.

Hammer, K. A., Carson, C. F. & Riley, T. V. (1998). *In-vitro* activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against Candida spp. *J Antimicrob Chemother* **42**, 591-595.

Hammer, K. A., Carson, C. F. & Riley, T. V. (2000). *Melaleuca alternifolia* (tea tree) oil inhibits germ tube formation by *Candida albicans*. *Med Mycol* **38**, 355-362.

Hammer, K. A., Carson, C. F. & Riley, T. V. (2003a). Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. *J Appl Microbiol* **95**, 853-860.

Hammer, K. A., Carson, C. F. & Riley, T. V. (2004). Antifungal effects of *Melaleuca alternifolia* (tea tree) oil and its components on *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*. *J Antimicrob Chemother* **53**, 1081-1085.

Hammer, K. A., Carson, C. F., Riley, T. V. & Nielsen, J. B. (2006). A review of the toxicity of *Melaleuca alternifolia* (tea tree) oil. *Food Chem Toxicol* **44**, 616-625.

Hammer, K. A., Carson, C. F., Dunstan, J. A., Hale, J., Lehmann, H., Robinson, C. J., Prescott, S. L. & Riley, T. V. (2008) Antimicrobial and anti-inflammatory activity of five *Taxandria fragrans* oils *in vitro*. *Microbiol Immunol,* **52**, 522-30.

Hammer, K. A., Dry, L., Johnson, M., Michalak, E. M., Carson, C. F. & Riley, T. V. (2003b). Susceptibility of oral bacteria to *Melaleuca alternifolia* (tea tree) oil *in vitro*. *Oral Microbiol Immunol* **18**, 389-392.

Hart, P. H., Brand, C., Carson, C. F., Riley, T. V., Prager, R. H. & Finlay-Jones, J. J. (2000). Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. *Inflamm Res* **49**, 619-626.

Hausen, B. M., Reichling, J. & Harkenthal, M. (1999). Degradation products of monoterpenes are the sensitizing agents in tea tree oil. *Am J Contact Dermat* **10**, 68-77.

Hawser, S. P. & Douglas, L. J. (1994). Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infect Immun* **62**, 915-921.

Hawser, S. P. & Douglas, L. J. (1995). Resistance of *Candida albicans* biofilms to antifungal agents in vitro. *Antimicrob Agents Chemother* **39**, 2128-2131.

Hayes, A. J., Leach, D. N. & Markham, J. L. (1997). *In vitro* cytotoxicity of Australian tea tree oil using human cell lines. *J Essent Oil Res* **9**, 572-582.

Hayes, A. J. & Markovic, B. (2003). Toxicity of Australian essential oil *Backhousia citriodora* (lemon myrtle). Part 2. Absorption and histopathology following application to human skin. *Food Chem Toxicol* **41**, 1409-1416.

Hiom, S. J., Furr, J. R., Russell, A. D. & Dickinson, J. R. (1992). Effects of chlorhexidine diacetate on *Candida albicans*, *C. glabrata* and *Saccharomyces cerevisiae*. *J Appl Bacteriol* **72**, 335-340.

Hogan, D. A., Vik, A. & Kolter, R. (2004). A Pseudomonas aeruginosa quorum-sensing molecule influences *Candida albicans* morphology. *Mol Microbiol* **54**, 1212-1223.

Holcombe, L. J., McAlester, G., Munro, C. A. & other authors (2010). *Pseudomonas aeruginosa* secreted factors impair biofilm development in *Candida albicans*. *Microbiology* **156**, 1476-1486.

Homann, N., Jousimies-Somer, H., Jokelainen, K., Heine, R. & Salaspuro, M. (1997). High acetaldehyde levels in saliva after ethanol consumption: methodological aspects and pathogenetic implications. *Carcinogenesis* **18**, 1739-1743.

Homann, N., Tillonen, J., Rintamaki, H., Salaspuro, M., Lindqvist, C. & Meurman, J. H. (2001). Poor dental status increases acetaldehyde production from ethanol in saliva: a possible link to increased oral cancer risk among heavy drinkers. *Oral Oncol* **37**, 153-158.

Huber, A. R. & Weiss, S. J. (1989). Disruption of the subendothelial basement membrane during neutrophil diapedesis in an *in vitro* construct of a blood vessel wall. *J Clin Invest* **83**, 1122-1136.

Hugo, W. B. & Longworth, A. R. (1966). The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of *Escherichia coli* and *Staphylococcus aureus*. *J Pharm Pharmacol* **18**, 569-578.

ISO, International Organization for Standardization (2004). ISO/FDIS 4730 Final draft, International Standard Oil of Melaleuca, terpinen-4-ol type (Tea Tree oil).

ISO, International Organization for Standardization (2009). ISO 10993-5 Biological evaluation of medical devices-Part 5: Tests for *in vitro* cytotoxicity.

Jackson, R. J. (1997). Metal salts, essential oils and phenols - old or new? *Periodontol 2000* **15**, 63-73.

Jagger, D. C. & Harrison, A. (1995). Denture cleansing - the best approach. *Br Dent J* **178**, 413-417.

Jandourek, A., Vaishampayan, J. K. & Vazquez, J. A. (1998). Efficacy of melaleuca oral solution for the treatment of fluconazole refractory oral candidiasis in AIDS patients. *AIDS* **12**, 1033-1037.

Jenkins, S., Addy, M. & Newcombe, R. G. (1994a). A comparison of cetylpyridinium chloride, triclosan and chlorhexidine mouthrinse formulations for effects on plaque regrowth. *J Clin Periodontol* **21**, 441-444.

Jenkins, S., Addy, M., Wade, W. & Newcombe, R. G. (1994b). The magnitude and duration of the effects of some mouthrinse products on salivary bacterial counts. *J Clin Periodontol* **21**, 397-401.

Johansen, C., Falholt, P. & Gram, L. (1997). Enzymatic removal and disinfection of bacterial biofilms. *Appl Environ Microbiol* **63**, 3724-3728.

Jose, A., Coco, B. J., Milligan, S., Young, B., Lappin, D. F., Bagg, J., Murray, C. & Ramage, G. (2010). Reducing the Incidence of Denture Stomatitis: Are Denture Cleansers Sufficient? *J Prosthodont*. **19,** 252-257.

Kauffman, C. A. & Carver, P. L. (2008). Update on echi.nocandin antifungals. *Semin Respir Crit Care Med* **29**, 211-219.

Keating, G. & Figgitt, D. (2003). Caspofungin: a review of its use in oesophageal candidiasis, invasive candidiasis and invasive aspergillosis. *Drugs* **63**, 2235-2263.

Kennedy, H. F., Shankland, G. S., Bagg, J., Chalmers, E. A., Gibson, B. E. & Williams, C. L. (2006). Fluconazole and itraconazole susceptibilities of *Candida spp*. isolated from oropharyngeal specimens and blood cultures of paediatric haematology/oncology patients. *Mycoses* **49**, 457-462.

Khalil, Z., Pearce, A. L., Satkunanathan, N., Storer, E., Finlay-Jones, J. J. & Hart, P. H. (2004). Regulation of wheal and flare by tea tree oil: complementary human and rodent studies. *J Invest Dermatol* **123**, 683-690.

Kimball, J. R., Nittayananta, W., Klausner, M., Chung, W. O. & Dale, B. A. (2006). Antimicrobial barrier of an *in vitro* oral epithelial model. *Arch Oral Biol* **51**, 775-783.

Kitahara, M., Ishiguro, F., Takayama, K., Isowa, K. & Nagai, T. (1993). Evaluation of skin damage of cyclic monoterpenes, percutaneous absorption enhancers, by using culture human skin cells. *Biol Pharm Bull* **16**, 912-916.

Klausner, M., Ayehunie, S., Breyfogle, B. A., Wertz, P. W., Bacca, L. & Kubilus, J. (2007). Organotypic human oral tissue models for toxicological studies. *Toxicol In Vitro* **21**, 938-949.

Koh, K. J., Pearce, A. L., Marshman, G., Finlay-Jones, J. J. & Hart, P. H. (2002). Tea tree oil reduces histamine-induced skin inflammation. *Br J Dermatol* **147**, 1212-1217.

Kojic, E. M. & Darouiche, R. O. (2004). *Candida* infections of medical devices. *Clin Microbiol Rev* **17**, 255-267.

Krautheim, A. B., Jermann, T. H. & Bircher, A. J. (2004). Chlorhexidine anaphylaxis: case report and review of the literature. *Contact Dermatitis* **50**, 113-116.

Kuhn, D. M., Chandra, J., Mukherjee, P. K. & Ghannoum, M. A. (2002a). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun* **70**, 878-888.

Kuhn, D. M., George, T., Chandra, J., Mukherjee, P. K. & Ghannoum, M. A. (2002b). Antifungal susceptibility of Candida biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob Agents Chemother* **46**, 1773-1780.

Kurkivuori, J., Salaspuro, V., Kaihovaara, P., Kari, K., Rautemaa, R., Gronroos, L., Meurman, J. H. & Salaspuro, M. (2007). Acetaldehyde production from ethanol by oral streptococci. *Oral Oncol* **43**, 181-186.

La Vecchia, C. (2009). Mouthwash and oral cancer risk: an update. *Oral Oncol* **45**, 198-200.

Lachenmeier, D. W., Gumbel-Mako, S., Sohnius, E. M., Keck-Wilhelm, A., Kratz, E. & Mildau, G. (2009). Salivary acetaldehyde increase due to alcohol-containing mouthwash use: a risk factor for oral cancer. *Int J Cancer* **125**, 730-735.

Lamfon, H., Porter, S. R., McCullough, M. & Pratten, J. (2004). Susceptibility of *Candida albicans* biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study. *J Antimicrob Chemother* **53**, 383-385.

Laudenbach, J. M. & Epstein, J. B. (2009). Treatment strategies for oropharyngeal candidiasis. *Expert Opin Pharmacother* **10**, 1413-1421.

Lee, H. M., Yuk, J. M., Shin, D. M. & other authors (2009). Apurinic/apyrimidinic endonuclease 1 is a key modulator of keratinocyte inflammatory responses. *J Immunol* **183**, 6839-6848.

Lennon, M. A. & Davies, R. M. (1975). A short-term evaluation of a chlorhexidine gel on plaque deposits and gingival status. *Pharmacol Ther Dent* **2**, 13-19.

Lewis, M. A. O. & Murray, S. (2006). Safety of alcohol-containing mouthwashes. *Dental Health* **45(1)**.

Lewis, M. A., Samaranyake, L. P. & Lamey, P. J. (1991). Diagnosis and treatment of oral candidosis. *J Oral Maxillofac Surg* **49**, 996-1002.

Li, L., Redding, S. & Dongari-Bagtzoglou, A. (2007). *Candida glabrata*: an emerging oral opportunistic pathogen. *J Dent Res* **86**, 204-215.

Lie, T. & Enersen, M. (1986). Effects of chlorhexidine gel in a group of maintenance-care patients with poor oral hygiene. *J Periodontol* **57**, 364-369.

Lilly, E. A., Shetty, K. V., Leigh, J. E., Cheeks, C. & Fidel, P. L., Jr. (2005). Oral epithelial cell antifungal activity: approaches to evaluate a broad range of clinical conditions. *Med Mycol* **43**, 517-523.

Lo, H. J., Kohler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. & Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939-949.

Lodder, J. (1970). *The Yeasts: A Taxonomic Study*, 2nd edition.

Loughlin, R., Gilmore, B. F., McCarron, P. A. & Tunney, M. M. (2008). Comparison of the cidal activity of tea tree oil and terpinen-4-ol against clinical bacterial skin isolates and human fibroblast cells. *Lett Appl Microbiol* **46**, 428-433.

Low, T. (1990). *Bush medicine*. North Ryde, NSW, Australia: Harper Collins Publishers.

Lundstrom, I. M., Anneroth, G. B. & Holmberg, K. (1984). *Candida* in patients with oral lichen planus. *Int J Oral Surg* **13**, 226-238.

MacFarlane, T. W. & Helnarska, S. J. (1976). The microbiology of angular cheilitis. *Br Dent J* **140**, 403-406.

Marquis, R. E., Clock, S. A. & Mota-Meira, M. (2003). Fluoride and organic weak acids as modulators of microbial physiology. *FEMS Microbiol Rev,* **26**, 493-510.

Martin, K. W. & Ernst, E. (2004). Herbal medicines for treatment of fungal infections: a systematic review of controlled clinical trials. *Mycoses* **47**, 87-92.

Martins, M., Henriques, M., Azeredo, J., Rocha, S. M., Coimbra, M. A. & Oliveira, R. (2007). Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signalling molecules produced by planktonic and biofilm cells. *Eukaryot Cell* **6**, 2429-2436.

Mateus, C., Crow, S. A., Jr. & Ahearn, D. G. (2004). Adherence of *Candida albicans* to silicone induces immediate enhanced tolerance to fluconazole. *Antimicrob Agents Chemother* **48**, 3358-3366.

Maynard, J. H., Jenkins, S. M., Moran, J., Addy, M., Newcombe, R. G. & Wade, W. G. (1993). A 6-month home usage trial of a 1% chlorhexidine toothpaste (II). Effects on the oral microflora. *J Clin Periodontol* **20**, 207-211.

McBain, A. J., Bartolo, R. G., Catrenich, C. E., Charbonneau, D., Ledder, R. G. & Gilbert, P. (2003). Effects of a chlorhexidine gluconate-containing mouthwash on the vitality and antimicrobial susceptibility of *in vitro* oral bacterial ecosystems. *Appl Environ Microbiol* **69**, 4770-4776.

McCoy, L. C., Wehler, C. J., Rich, S. E., Garcia, R. I., Miller, D. R. & Jones, J. A. (2008). Adverse events associated with chlorhexidine use: results from the Department of Veterans Affairs Dental Diabetes Study. *J Am Dent Assoc* **139**, 178-183.

McCullough, M. J. & Farah, C. S. (2008). The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes. *Aust Dent J* **53**, 302-305.

McMahon, M. A., Blair, I. S., Moore, J. E. & McDowell, D. A. (2007). Habituation to sub-lethal concentrations of tea tree oil (*Melaleuca alternifolia*) is associated with reduced susceptibility to antibiotics in human pathogens. *J Antimicrob Chemother* **59**, 125-127.

McMahon, M. A., Tunney, M. M., Moore, J. E., Blair, I. S., Gilpin, D. F. & McDowell, D. A. (2008). Changes in antibiotic susceptibility in *staphylococci* habituated to sub-lethal concentrations of tea tree oil (*Melaleuca alternifolia*). *Lett Appl Microbiol* **47**, 263-268.

Medina-Holguin, A. L., Holguin, F. O., Micheletto, S., Goehle, S., Simon, J. A. & O'Connell, M. A. (2008). Chemotypic variation of essential oils in the medicinal plant, *Anemopsis californica*. *Phytochemistry* **69**, 919-927.

Meiller, T. F., Kelley, J. I., Jabra-Rizk, M. A., Depaola, L. G., Baqui, A. A. & Falkler, W. A., Jr. (2001). *In vitro* studies of the efficacy of antimicrobials against fungi. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **91**, 663-670.

Meurman, J. H. & Uittamo, J. (2008). Oral micro-organisms in the etiology of cancer. *Acta Odontol Scand* **66**, 321-326.

Migliorati, C. A. & Madrid, C. (2007). The interface between oral and systemic health: the need for more collaboration. *Clin Microbiol Infect* **13 Suppl 4**, 11-16.

Mikus, J., Harkenthal, M., Steverding, D. & Reichling, J. (2000). *In vitro* effect of essential oils and isolated mono- and sesquiterpenes on *Leishmania major* and *Trypanosoma brucei*. *Planta Med* **66**, 366-368.

Mondello, F., De Bernardis, F., Girolamo, A., Salvatore, G. & Cassone, A. (2003). *In vitro* and *in vivo* activity of tea tree oil against azole-susceptible and -resistant human pathogenic yeasts. *J Antimicrob Chemother* **51**, 1223-1229.

Mondello, F., De Bernardis, F., Girolamo, A., Cassone, A. & Salvatore, G. (2006). *In vivo* activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic Candida species. *BMC Infect Dis* **6**, 158.

More, G., Tshikalange, T. E., Lall, N., Botha, F. & Meyer, J. J. (2008). Antimicrobial activity of medicinal plants against oral microorganisms. *J Ethnopharmacol* **119**, 473-477.

Moretti, M. D. L., Peana, A. T. & Satta, M. (1997). A study on anti-inflammatory and peripheral analgesic action of *Salvia sclarea* oil and its main components. *J Essent Oil Res* **9**, 199-204.

Mowat, E., Butcher, J., Lang, S., Williams, C. & Ramage, G. (2007). Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *J Med Microbiol* **56**, 1205-1212.

Mozelsio, N. B., Harris, K. E., McGrath, K. G. & Grammer, L. C. (2003). Immediate systemic hypersensitivity reaction associated with topical application of Australian tea tree oil. *Allergy Asthma Proc* **24**, 73-75.

Mukherjee, P. K., Chandra, J., Kuhn, D. M. & Ghannoum, M. A. (2003). Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun* **71**, 4333-4340.

Mukherjee, P. K., Chand, D. V., Chandra, J., Anderson, J. M. & Ghannoum, M. A. (2009). Shear stress modulates the thickness and architecture of *Candida albicans* biofilms in a phase-dependent manner. *Mycoses* **52**, 440-446.

Mukherjee, P. K., Zhou, G., Munyon, R. & Ghannoum, M. A. (2005). *Candida* biofilm: a well-designed protected environment. *Med Mycol* **43**, 191-208.

Muller, G. & Kramer, A. (2008). Biocompatibility index of antiseptic agents by parallel assessment of antimicrobial activity and cellular cytotoxicity. *J Antimicrob Chemother* **61**, 1281-1287.

Murillo, L. A., Newport, G., Lan, C. Y., Habelitz, S., Dungan, J. & Agabian, N. M. (2005). Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. *Eukaryot Cell* **4**, 1562-1573.

Naglik, J. R., Moyes, D., Makwana, J. & other authors (2008). Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology* **154**, 3266-3280.

Nelson, R. R. (2000). Selection of resistance to the essential oil of *Melaleuca alternifolia* in *Staphylococcus aureus*. *J Antimicrob Chemother* **45**, 549-550.

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

Netea, M. G., Van Der Graaf, C. A., Vonk, A. G., Verschueren, I., Van Der Meer, J. W. & Kullberg, B. J. (2002). The role of toll-like receptor (TLR) 2 and TLR4 in the host defence against disseminated candidiasis. *J Infect Dis* **185**, 1483-1489.

Nett, J. E., Lepak, A. J., Marchillo, K. & Andes, D. R. (2009). Time course global gene expression analysis of an *in vivo Candida* biofilm. *J Infect Dis* **200**, 307-313.

Nielsen, J. B. (2008). What you see may not always be what you get - bioavailability and extrapolation from *in vitro* tests. *Toxicol In Vitro* **22**, 1038-1042.

Nieminen, M. T., Uittamo, J., Salaspuro, M. & Rautemaa, R. (2009). Acetaldehyde production from ethanol and glucose by non-*Candida albicans* yeasts in vitro. *Oral Oncol* **45**, e245-e248.

Niimi, M., Firth, N. A. & Cannon, R. D. (2010). Antifungal drug resistance of oral fungi. *Odontology* **98**, 15-25.

Nikawa, H., Hamada, T., Yamashiro, H. & Kumagai, H. (1999). A review of *in vitro* and *in vivo* methods to evaluate the efficacy of denture cleansers. *Int J Prosthodont* **12**, 153-159.

Nikawa, H., Samaranayake, L. P., Tenovuo, J. & Hamada, T. (1994). The effect of antifungal agents on the *in vitro* susceptibility of *Candida albicans* to apo-lactoferrin. *Arch Oral Biol* **39**, 921-923.

Nobile, C. J. & Mitchell, A. P. (2006). Genetics and genomics of *Candida albicans* biofilm formation. *Cell Microbiol* **8**, 1382-1391.

Nobile, C. J., Schneider, H. A., Nett, J. E., Sheppard, D. C., Filler, S. G., Andes, D. R. & Mitchell, A. P. (2008). Complementary Adhesin Function in *C. albicans* Biofilm Formation. *Curr Biol* **18**, 1017-1024.

Odds, F. C. (1988). *Candida and candidiasis*, 2nd edn. London: Bailliere Tindall.

Odds, F. C., Hanson, M. F., Davidson, A. D., Jacobsen, M. D., Wright, P., Whyte, J. A., Gow, N. A. & Jones, B. L. (2007). One year prospective survey of *Candida* bloodstream infections in Scotland. *J Med Microbiol* **56**, 1066-1075.

Odds, F. C., Van Nuffel, L. & Dams, G. (1998). Prevalence of *Candida dubliniensis* isolates in a yeast stock collection. *J Clin Microbiol* **36**, 2869-2873.

Ohman, S. C., Dahlen, G., Moller, A. & Ohman, A. (1986). Angular cheilitis: a clinical and microbial study. *J Oral Pathol* **15**, 213-217.

Ohman, S. C. & Jontell, M. (1988). Treatment of angular cheilitis. The significance of microbial analysis, antimicrobial treatment, and interfering factors. *Acta Odontol Scand* **46**, 267-272.

Oliveira, L. V., Mesquita, M. F., Henriques, G. E. & Consani, R. L. (2007). The effect of brushing on surface roughness of denture lining materials. *J Prosthodont* **16**, 179-184.

Olsen, C. H. (2003). Review of the use of statistics in infection and immunity. *Infect Immun* **71**, 6689-6692.

Osorio, R. M., Hefti, A., Vertucci, F. J. & Shawley, A. L. (1998). Cytotoxicity of endodontic materials. *J Endod* **24**, 91-96.

Ota, C., Unterkircher, C., Fantinato, V. & Shimizu, M. T. (2001). Antifungal activity of propolis on different species of *Candida*. *Mycoses* **44**, 375-378.

Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L. & Aderem, A. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci USA* **97**, 13766-13771.

Papadopoulos, C. J., Carson, C. F., Hammer, K. A. & Riley, T. V. (2006). Susceptibility of pseudomonads to *Melaleuca alternifolia* (tea tree) oil and components. *J Antimicrob Chemother* **58**, 449-451.

Pappas, P. G., Kauffman, C. A., Andes, D. & other authors (2009). Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* **48**, 503-535.

Patel, V. K. & Venkatakrishna-Bhatt, H. (1988). Folklore therapeutic indigenous plants in periodontal disorders in India (review, experimental and clinical approach). *Int J Clin Pharmacol Ther Toxicol* **26**, 176-184.

Pearce, A. L., Finlay-Jones, J. J. & Hart, P. H. (2005). Reduction of nickel-induced contact hypersensitivity reactions by topical tea tree oil in humans. *Inflamm Res* **54**, 22-30.

Penfold, A. R. & Grant, R. (1925). The germicidal values of some Australian essential oils and their pure constituents, together with those for some essential oil isolates, and synthetics. Part III. *J R Soc New South Wales* **59**, 346-349.

Peterson, D. E. (1992). Oral candidiasis. *Clin Geriatr Med* **8**, 513-527.

Pierce, C. G., Uppuluri, P., Tristan, A. R., Wormley, F. L., Jr., Mowat, E., Ramage, G. & Lopez-Ribot, J. L. (2008). A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat Protoc* **3**, 1494-1500.

Pigno, M. A., Goldschmidt, M. C. & Lemon, J. C. (1994). The efficacy of antifungal agents incorporated into a facial prosthetic silicone elastomer. *J Prosthet Dent* **71**, 295-300.

Polyzois, G. L., Zissis, A. J. & Yannikakis, S. A. (1995). The effect of glutaraldehyde and microwave disinfection on some properties of acrylic denture resin. *Int J Prosthodont* **8**, 150-154.

Pongprayoon, U., Soontornsaratune, P., Jarikasem, S., Sematong, T., Wasuwat, S. & Claeson, P. (1997). Topical anti-inflammatory activity of the major lipophilic constituents of the rhizome of *Zingiber cassumunar*. Part 1: The essential oil. *Phytomedicine* **3**, 319-322.

Prashant, G. M., Chandu, G. N., Murulikrishna, K. S. & Shafiulla, M. D. (2007). The effect of mango and neem extract on four organisms causing dental caries: *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus mitis*, and *Streptococcus sanguis*: an in vitro study. *Indian J Dent Res* **18**, 148-151.

Price, J. S., Tencer, A. F., Arm, D. M. & Bohach, G. A. (1996). Controlled release of antibiotics from coated orthopedic implants. *J Biomed Mater Res* **30**, 281-286.

Radfar, L., Shea, Y., Fischer, S. H., Sankar, V., Leakan, R. A., Baum, B. J. & Pillemer, S. R. (2003). Fungal load and candidiasis in Sjogren's syndrome. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **96**, 283-287.

Radford, J. R., Beighton, D., Nugent, Z. & Jackson, R. J. (1997). Effect of use of 0.05% cetylpyridinium chloride mouthwash on normal oral flora. *J Dent* **25**, 35-40.

Ramage, G., Bachmann, S., Patterson, T. F., Wickes, B. L. & Lopez-Ribot, J. L. (2002a). Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J Antimicrob Chemother* **49**, 973-980.

Ramage, G., Ghannoum, M. & López-Ribot, J. L. (2006a). Fungal biofilms: agents of disease and drug resistance. In *Molecular Principles of Fungal Pathogenesis*. Edited by J. Heitman: ASM Press.

Ramage, G. & Lopez-Ribot, J. L. (2005). Techniques for antifungal susceptibility testing of *Candida albicans* biofilms. *Methods Mol Med* **118**, 71-79.

Ramage, G., Martinez, J. P. & Lopez-Ribot, J. L. (2006b). *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res* **6**, 979-986.

Ramage, G., Mowat, E., Jones, B., Williams, C. & Lopez-Ribot, J. (2009). Our current understanding of fungal biofilms. *Crit Rev Microbiol* **35**, 340-355.

Ramage, G., Saville, S. P., Wickes, B. L. & Lopez-Ribot, J. L. (2002b). Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol* **68**, 5459-5463.

Ramage, G., Saville, S. P., Thomas, D. P. & Lopez-Ribot, J. L. (2005). *Candida* biofilms: an update. *Eukaryot Cell* **4**, 633-638.

Ramage, G., Tomsett, K., Wickes, B. L., Lopez-Ribot, J. L. & Redding, S. W. (2004). Denture stomatitis: a role for *Candida* biofilms. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **98**, 53-59.

Ramage, G., VandeWalle, K., Wickes, B. L. & Lopez-Ribot, J. L. (2001a). Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* **45**, 2475-2479.

Ramage, G., VandeWalle, K., Wickes, B. L. & Lopez-Ribot, J. L. (2001b). Characteristics of biofilm formation by *Candida albicans*. *Rev Iberoam Micol* **18**, 163-170.

Ramage, G., VandeWalle, K., Bachmann, S. P., Wickes, B. L. & Lopez-Ribot, J. L. (2002c). *In vitro* pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. *Antimicrob Agents Chemother* **46**, 3634-3636.

Ramage, G., VandeWalle, K., Lopez-Ribot, J. L. & Wickes, B. L. (2002d). The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol Lett* **214**, 95-100.

Ramage, G., Wickes, B. L. & Lopez-Ribot, J. L. (2001c). Biofilms of *Candida albicans* and their associated resistance to antifungal agents. *Am Clin Lab* **20**, 42-44.

Ramage, G., Wickes, B. L. & Lopez-Ribot, J. L. (2007). Inhibition of *Candida albicans* biofilm formation using divalent cation chelators (EDTA). Mycopathologia **164**, 301-306.

Raman, A., Weir, U. & Bloomfield, S. F. (1995). Antimicrobial effects of tea-tree oil and its major components on *Staphylococcus aureus*, *Staph. epidermidis* and *Propionibacterium acnes*. *Lett Appl Microbiol* **21**, 242-245.

Ramberg, P., Furuichi, Y., Sherl, D., Volpe, A. R., Nabi, N., Gaffar, A. & Lindhe, J. (1995). The effect of triclosan on developing gingivitis. *J Clin Periodontol* **22**, 442-448.

Rautemaa, R., Hietanen, J., Niissalo, S., Pirinen, S. & Perheentupa, J. (2007). Oral and oesophageal squamous cell carcinoma - a complication or component of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, APS-I). *Oral Oncol* **43**, 607-613.

Redding, S., Bhatt, B., Rawls, H. R., Siegel, G., Scott, K. & Lopez-Ribot, J. (2009). Inhibition of *Candida albicans* biofilm formation on denture material. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **107**, 669-672.

Reichling, J., Fitzi, J., Hellmann, K., Wegener, T., Bucher, S. & Saller, R. (2004). Topical tea tree oil effective in canine localised pruritic dermatitis - a multi-centre randomised double-blind controlled clinical trial in the veterinary practice. *Dtsch Tierarztl Wochenschr* **111**, 408-414.

Rodu, B., Carpenter, J. T. & Jones, M. R. (1988). The pathogenesis and clinical significance of cytologically detectable oral *Candida* in acute leukemia. *Cancer* **62**, 2042-2046.

Roeder, A., Kirschning, C. J., Rupec, R. A., Schaller, M. & Korting, H. C. (2004). Toll-like receptors and innate antifungal responses. *Trends Microbiol* **12**, 44-49.

Rossie, K. & Guggenheimer, J. (1997). Oral candidiasis: clinical manifestations, diagnosis, and treatment. *Pract Periodontics Aesthet Dent* 9, 635-641; quiz 642.

Rupniak, H. T., Rowlatt, C., Lane, E. B., Steele, J. G., Trejdosiewicz, L. K., Laskiewicz, B., Povey, S. & Hill, B. T. (1985). Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. *J Natl Cancer Inst* 75, 621-635.

Russell, A. D. (1986). Chlorhexidine: antibacterial action and bacterial resistance. *Infection* **14**, 212-215.

Russell, A. D. (2002). Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. *J Appl Microbiol* **92 Suppl**, 121S-135S.

Russell, A. D. (2004). Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. *J Hosp Infect* **57**, 97-104.

Russell, A. D. & Day, M. J. (1993). Antibacterial activity of chlorhexidine. *J Hosp Infect* **25**, 229-238.

Russell, M. (1999). *Toxicology of tea tree oil. In: Tea tree: The Genus Melaleuca.* Amsterdam: Harwood Academic Publishers.

Rutherford, T., Nixon, R., Tam, M. & Tate, B. (2007). Allergy to tea tree oil: retrospective review of 41 cases with positive patch tests over 4.5 years. *Australas J Dermatol* **48**, 83-87.

Samaranayake, L. P., Keung Leung, W. & Jin, L. (2009). Oral mucosal fungal infections. *Periodontol 2000* **49**, 39-59.

Sanglard, D. & Odds, F. C. (2002). Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis* **2**, 73-85.

Sanita, P. V., Vergani, C. E., Giampaolo, E. T., Pavarina, A. C. & Machado, A. L. (2009). Growth of *Candida* species on complete dentures: effect of microwave disinfection. *Mycoses* **52**, 154-160.

Santos, V. R., Gomes, R. T., de Mesquita, R. A., de Moura, M. D., Franca, E. C., de Aguiar, E. G., Naves, M. D., Abreu, J. A. & Abreu, S. R. (2008). Efficacy of Brazilian propolis gel for the management of denture stomatitis: a pilot study. *Phytother Res* **22**, 1544-1547.

Saxton, C. A. (1986). The effects of a dentifrice containing zinc citrate and 2,4,4' trichloro-2'-hydroxydiphenyl ether. *J Periodontol* **57**, 555-561.

SCCP (2004). Scientific Committee on Consumer Products, *Opinion on tea tree oil*.

SCCP (2008). Scientific Committee on Consumer Products, *Opinion on tea tree oil*.

Schaller, M., Boeld, U., Oberbauer, S., Hamm, G., Hube, B. & Korting, H. C. (2004). Polymorphonuclear leukocytes (PMNs) induce protective Th1-type cytokine epithelial responses in an in vitro model of oral candidosis. *Microbiology* **150**, 2807-2813.

Schaller, M., Mailhammer, R., Grassl, G., Sander, C. A., Hube, B. & Korting, H. C. (2002). Infection of human oral epithelia with *Candida* species induces cytokine expression correlated to the degree of virulence. *J Invest Dermatol* **118**, 652-657.

Scheinfeld, N. (2007). A review of the new antifungals: posaconazole, micafungin, and anidulafungin. *J Drugs Dermatol* **6**, 1249-1251.

Schierholz, J. M., Lucas, L. J., Rump, A. & Pulverer, G. (1998). Efficacy of silver-coated medical devices. *J Hosp Infect* **40**, 257-262.

Schiott, C. R., Briner, W. W., Kirkland, J. J. & Loe, H. (1976a). Two years oral use of chlorhexidine in man. III. Changes in sensitivity of the salivary flora. *J Periodontal Res* **11**, 153-157.

Schiott, C. R., Briner, W. W. & Loe, H. (1976b). Two year oral use of chlorhexidine in man. II. The effect on the salivary bacterial flora. *J Periodontal Res* **11**, 145-152.

Schnitzler, P., Schon, K. & Reichling, J. (2001). Antiviral activity of Australian tea tree oil and eucalyptus oil against herpes simplex virus in cell culture. *Pharmazie* **56**, 343-347.

Schwartz, I. S., Young, J. M. & Berrong, J. M. (1988). The effect of Listerine antiseptic on denture microbial flora and denture stomatitis. *Int J Prosthodont* **1**, 153-158.

Scully, C., el-Kabir, M. & Samaranayake, L. P. (1994). *Candida* and oral candidosis: a review. *Crit Rev Oral Biol Med* **5**, 125-157.

Seneviratne, C. J., Jin, L. & Samaranayake, L. P. (2008). Biofilm lifestyle of Candida: a mini review. *Oral Dis* **14**, 582-590.

Seo, R. S., Vergani, C. E., Pavarina, A. C., Compagnoni, M. A. & Machado, A. L. (2007). Influence of microwave disinfection on the dimensional stability of intact and relined acrylic resin denture bases. *J Prosthet Dent* **98**, 216-223.

Shakespeare, V., Shakespeare, P. G. & Evans, B. T. (1988). Effects of proprietary oral rinses containing chlorhexidine, hexetidine and benzydamine on the proliferation of human buccal epithelial cells in culture. *Arch Oral Biol* **33**, 881-885.

Sikkema, J., de Bont, J. A. & Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev* **59**, 201-222.

Silverman, R. J., Nobbs, A. H., Vickerman, M. M., Barbour, M. E. & Jenkinson, H. F. (2010). Interaction of *Candida albicans* Cell-Wall Als3 Protein with *Streptococcus gordonii* SspB Adhesin Promotes Development of Mixed Species Communities. *Infect Immun* **78** 4644-4652.

Sims, C. R., Ostrosky-Zeichner, L. & Rex, J. H. (2005). Invasive candidiasis in immunocompromised hospitalized patients. *Arch Med Res* **36**, 660-671.

Smigel, K. (1991). High-alcohol mouthwashes are under scrutiny. *J Natl Cancer Inst* **83**, 751.

Soderberg, T. A., Johansson, A. & Gref, R. (1996). Toxic effects of some conifer resin acids and tea tree oil on human epithelial and fibroblast cells. *Toxicology* **107**, 99-109.

Solomon, D. D. & Sherertz, R. J. (1987). Antibiotic releasing polymers. *J Control Release*, 343-352.

Soukoulis, S. & Hirsch, R. (2004). The effects of a tea tree oil-containing gel on plaque and chronic gingivitis. *Aust Dent J* **49**, 78-83.

Soysa, N. S. & Ellepola, A. N. (2005). The impact of cigarette/tobacco smoking on oral candidosis: an overview. *Oral Dis* **11**, 268-273.

Soysa, N. S., Samaranayake, L. P. & Ellepola, A. N. (2004). Cytotoxic drugs, radiotherapy and oral candidiasis. *Oral Oncol* **40**, 971-978.

Soysa, N. S., Samaranayake, L. P. & Ellepola, A. N. (2006). Diabetes mellitus as a contributory factor in oral candidosis. *Diabet Med* **23**, 455-459.

Soysa, N. S., Samaranayake, L. P. & Ellepola, A. N. (2008). Antimicrobials as a contributory factor in oral candidosis - a brief overview. *Oral Dis* **14**, 138-143.

Spreghini, E., Maida, C. M., Tomassetti, S., Orlando, F., Giannini, D., Milici, M. E., Scalise, G. & Barchiesi, F. (2008). Posaconazole against *Candida glabrata* isolates with various susceptibilities to fluconazole. *Antimicrob Agents Chemother* **52**, 1929-1933.

Sreenivasan, P. & Gaffar, A. (2002). Antiplaque biocides and bacterial resistance: a review. *J Clin Periodontol* **29**, 965-974.

Sugiura, M., Hayakawa, R., Kato, Y., Sugiura, K. & Hashimoto, R. (2000). Results of patch testing with lavender oil in Japan. *Contact Dermatitis* **43**, 157-160.

Sweeney, M. P., Bagg, J., Baxter, W. P. & Aitchison, T. C. (1998). Oral disease in terminally ill cancer patients with xerostomia. *Oral Oncol* **34**, 123-126.

Tallury, P., Alimohammadi, N. & Kalachandra, S. (2007). Poly (ethylene-co-vinyl acetate) copolymer matrix for delivery of chlorhexidine and acyclovir drugs for use in the oral environment: effect of drug combination, copolymer composition and coating on the drug release rate. *Dent Mater* **23**, 404-409.

Ten Cate, J. M. & Featherstone, J. D. (1991). Mechanistic aspects of the interactions between fluoride and dental enamel. *Crit Rev Oral Biol Med,* **2**, 283-296.

Terzi, V., Morcia, C., Faccioli, P., Vale, G., Tacconi, G. & Malnati, M. (2007). *In vitro* antifungal activity of the tea tree (*Melaleuca alternifolia*) essential oil and its major components against plant pathogens. *Lett Appl Microbiol* **44**, 613-618.

Thein, Z. M., Seneviratne, C. J., Samaranayake, Y. H. & Samaranayake, L. P. (2009). Community lifestyle of *Candida* in mixed biofilms: a mini review. *Mycoses* **52**, 467-475.

Thomas, L., Maillard, J. Y., Lambert, R. J. & Russell, A. D. (2000). Development of resistance to chlorhexidine diacetate in *Pseudomonas aeruginosa* and the effect of a "residual" concentration. *J Hosp Infect* **46**, 297-303.

Timpanaro, R., Garozzo, A., Bisignano, B., Stivala, A., Furneri, P. M., Tempera, G. & Castro, A. (2007). Inhibitory effect of *Melaleuca alternifolia* (tea tree oil) on influenza A/PR/8 virus replication. *International Journal of Antimicrobial Agents* **29**, S202-S203.

Tomas, I., Cousido, M. C., Garcia-Caballero, L., Rubido, S., Limeres, J. & Diz, P. (2010). Substantivity of a single chlorhexidine mouthwash on salivary flora: Influence of intrinsic and extrinsic factors. *J Dent* **38**, 541-546

Tomas, I., Garcia-Caballero, L., Cousido, M. C., Limeres, J., Alvarez, M. & Diz, P. (2009). Evaluation of chlorhexidine substantivity on salivary flora by epifluorescence microscopy. *Oral Dis* **15**, 428-433.

Turner, M. D. & Ship, J. A. (2007). Dry mouth and its effects on the oral health of elderly people. *J Am Dent Assoc* **138 Suppl**, 15S-20S.

Uittamo, J., Siikala, E., Kaihovaara, P., Salaspuro, M. & Rautemaa, R. (2009). Chronic candidosis and oral cancer in APECED-patients: production of carcinogenic acetaldehyde from glucose and ethanol by *Candida albicans*. *Int J Cancer* **124**, 754-756.

van der Bijl, P. & Arendorf, T. M. (1993). Itraconazole and fluconazole in oropharyngeal candidiasis. *Ann Dent* **52**, 12-16.

van der Waal, I., Schepman, K. P., van der Meij, E. H. & Smeele, L. E. (1997). Oral leukoplakia: a clinicopathological review. *Oral Oncol* **33**, 291-301.

Vazquez, J. A. (2007). Role of posaconazole in the management of oropharyngeal and esophageal candidiasis. *Ther Clin Risk Manag* **3**, 533-542.

Vazquez, J. A., Arganoza, M. T., Boikov, D., Akins, R. A. & Vaishampayan, J. K. (2000). *In vitro* susceptibilities of *Candida* and *Aspergillus* species to *Melaleuca alternafolia* (tea tree) oil. *Rev Iberoam Micol* **17**, 60-63.

Vazquez, J. A. & Zawawi, A. A. (2002). Efficacy of alcohol-based and alcohol-free melaleuca oral solution for the treatment of fluconazole-refractory oropharyngeal candidiasis in patients with AIDS. *HIV Clin Trials* **3**, 379-385.

Vediyappan, G., Rossignol, T. & d'Enfert, C. (2010). Interaction of *Candida albicans* biofilms with antifungals: transcriptional response and binding of antifungals to beta-glucans. *Antimicrob Agents Chemother* **54**, 2096-2111.

Veien, N. K., Rosner, K. & Skovgaard, G. L. (2004). Is tea tree oil an important contact allergen? *Contact Dermatitis* **50**, 378-379.

Villar, C. C. & Dongari-Bagtzoglou, A. (2008). Immune defence mechanisms and immunoenhancement strategies in oropharyngeal candidiasis. *Expert Rev Mol Med* **10**, e29.

Villar, C. C., Kashleva, H. & Dongari-Bagtzoglou, A. (2004). Role of *Candida albicans* polymorphism in interactions with oral epithelial cells. *Oral Microbiol Immunol* **19**, 262-269.

Villar, C. C., Kashleva, H., Mitchell, A. P. & Dongari-Bagtzoglou, A. (2005). Invasive phenotype of *Candida albicans* affects the host proinflammatory response to infection. *Infect Immun* **73**, 4588-4595.

Volpe, A. R., Petrone, M. E., De Vizio, W., Davies, R. M. & Proskin, H. M. (1996). A review of plaque, gingivitis, calculus and caries clinical efficacy studies with a fluoride dentifrice containing triclosan and PVM/MA copolymer. *J Clin Dent* **7 Suppl**, S1-S14.

Watson, W. A., Litovitz, T. L., Klein-Schwartz, W., Rodgers, G. C., Jr., Youniss, J., Reid, N., Rouse, W. G., Rembert, R. S. & Borys, D. (2004). 2003 annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. *Am J Emerg Med* **22**, 335-404.

Weaver, A., Fleming, S. M. & Smith, D. B. (1979). Mouthwash and oral cancer: carcinogen or coincidence? *J Oral Surg* **37**, 250-253.

Webb, B. C., Thomas, C. J., Willcox, M. D., Harty, D. W. & Knox, K. W. (1998a). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 3. Treatment of oral candidosis. *Aust Dent J* **43**, 244-249.

Webb, B. C., Thomas, C. J., Willcox, M. D., Harty, D. W. & Knox, K. W. (1998b). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 1. Factors influencing distribution of *Candida* species in the oral cavity. *Aust Dent J* **43**, 45-50.

Webb, B. C., Thomas, C. J., Willcox, M. D., Harty, D. W. & Knox, K. W. (1998c). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 2. Oral diseases caused by *Candida* species. *Aust Dent J* **43**, 160-166.

Werner, C. W. & Seymour, R. A. (2009). Are alcohol-containing mouthwashes safe? *Br Dent J* **207**, E19; discussion 488-489.

White, T. C. (1997). Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. *Antimicrob Agents Chemother* **41**, 1482-1487.

Whiteway, M. & Oberholzer, U. (2004). *Candida* morphogenesis and host-pathogen interactions. *Curr Opin Microbiol* **7**, 350-357.

Wilken, R., Botha, S. J., Grobler, A. & Germishuys, P. J. (2001). *In vitro* cytotoxicity of chlorhexidine gluconate, benzydamine-HCl and povidone iodine mouthrinses on human gingival fibroblasts. *SADJ* **56**, 455-460.

Willcox, M. (2005). An evaluation of tea tree oil as an alternative microbicide. *Nurs Times* **101**, 32-33.

Wilson, S. J. & Wilson, H. J. (1993). The release of chlorhexidine from modified dental acrylic resin. *J Oral Rehabil* **20**, 311-319.

Wray, D. & Bagg, J. (1997). *Pocket reference to oral candidosis*: Science Press.

Wu, C. D., Darout, I. A. & Skaug, N. (2001). Chewing sticks: timeless natural toothbrushes for oral cleansing. *J Periodontal Res* **36**, 275-284.

Wu, P. & Grainger, D. W. (2006). Drug/device combinations for local drug therapies and infection prophylaxis. *Biomaterials* **27**, 2450-2467.

Yeater, K. M., Chandra, J., Cheng, G., Mukherjee, P. K., Zhao, X., Rodriguez-Zas, S. L., Kwast, K. E., Ghannoum, M. A. & Hoyer, L. L. (2007). Temporal analysis of *Candida albicans* gene expression during biofilm development. *Microbiology* **153**, 2373-2385.

Yokoyama, A., Tsutsumi, E., Imazeki, H., Suwa, Y., Nakamura, C. & Yokoyama, T. (2007). Contribution of the alcohol dehydrogenase-1B genotype and oral microorganisms to high salivary acetaldehyde concentrations in Japanese alcoholic men. *Int J Cancer* **121**, 1047-1054.

Young, B., Jose, A., Cameron, D., McCord, F., Murray, C., Bagg, J. & Ramage, G. (2009). Attachment of *Candida albicans* to denture base acrylic resin processed by three different methods. *Int J Prosthodont* **22**, 488-489.

Yumoto, H., Nakae, H., Fujinaka, K., Ebisu, S. & Matsuo, T. (1999). Interleukin-6 (IL-6) and IL-8 are induced in human oral epithelial cells in response to exposure to periodontopathic *Eikenella corrodens*. *Infect Immun* **67**, 384-394.

Appendix

Poster Presentation: Society for General Microbiology, Dublin, September 2008.

Novel tea-tree oil based treatment of *Candida* biofilms.

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Candida albicans is the predominant yeast associated with oropharyngeal candidosis (OPC), which presents clinically as thrush or denture stomatitis. These infections are characterised by multispecies biofilms forming on the oral epithelium or artificial denture surfaces. Current antifungal treatments have limited success due to resistance, with recurring infections common. Therefore, alternative methods for suppressing or eradicating biofilms are desirable. The aim of our study was to evaluate the efficacy of tea tree oil (TTO) and key derivatives against biofilms formed by 100 oral *Candida albicans* isolates. These were formed in microtitre plates, treated with individual components and the fungicidal activity evaluated using a metabolic dye to evaluate killing. It was shown that although generic TTO was effective against planktonic cells, it was poorly active against sessile cells (MFC_{90} >2%). However, terpinene-4-ol and *α*-terpineol exhibited anti-biofilm activity (MFC_{90} = 1%). In addition, lower concentrations (0.25%) of these two agents were able to inhibit filamentous growth. Overall, TTO-based mouthwashes may offer a suitable alternative to conventional azole treatment.

Novel tea-tree oil based treatment of *Candida* biofilms

Steven Milligan, Shauna Culshaw, Jeremy Bagg, Petrina Sweeney, Gordon Ramage
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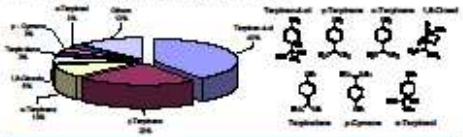
Background

Candida species are part of the normal commensal microflora of the oral cavity. They are frequently isolated from the oral cavity in around 40-80% of the population as asymptomatic carriage. However, with a burgeoning population of immunocompromised individuals, oral candidiasis remains a potential problem (Epstein and Polsky 1998). *C. albicans* can be present in the oral cavity as planktonic (free-floating) cells in the saliva and as biofilms on the soft tissues, hard tissues and dentures. A healthy oral cavity can normally be maintained by regular brushing of teeth and dentures to remove microbial biofilms, which is augmented by the use of antiseptic mouthwashes to control micro-organisms both in plaque and within the saliva. The commercial oral healthcare sector has responded to demand for superior products to manage these superficial infections with the development of new antimicrobial products within toothpastes, mouthwashes and denture cleansers. However, many of these products have side-effects associated with them, such as tooth staining, unpleasant aftertaste, tissue irritation and resistance problems. In addition, current antifungal treatments, such as azoles, are associated with both molecular (efflux pumps) and phenotypic (biofilm) resistance mechanisms.

The use of 'natural' antimicrobials, which are perceived by the general public as being safer, non-toxic and not associated with resistance is an enticing prospect for the commercial oral healthcare sector. Tea tree oil (TTO) is an Australian aboriginal traditional medicine extracted from the leaves of *Melaleuca* species, which is a mixture of over 100 terpene hydrocarbons and has antimicrobial activity against bacteria, fungi, viruses and also protozoa (Carson *et al* 2006). The main derivatives of tea tree and their chemical structures are presented in Figure 1.

Figure 1: Composition and chemical structure of tea tree oil



Aims

The primary objective of this study was to determine whether compounds derived from *Melaleuca alternifolia* (tea-tree) have powerful antifungal properties which could be used as novel mouthwashes and denture-cleansing agents to inhibit and/or kill both planktonic and sessile *C. albicans*.

Methods

Organisms: One hundred *C. albicans* clinical strains isolated from denture stomatitis patients (n = 26), palliative care patients (n = 30), neonatal patients (n = 36) and patients from the ICU (n = 8) were examine in this study. All clinical strains were identified using an API 32C, and maintained on Sabouraud agar plates.

Susceptibility Testing: Tea tree oil and seven derivative compounds (Table 1) were purchased from Sigma at HPLC grade and prepared at stock concentrations in RPMI/Tween (0.05%).
C. albicans planktonic cells were tested using the CLSI M27 broth microdilution methodology, and sessile cells were prepared, tested and quantified using an XTT reduction assay as previously described by Ramage *et al* (2001).

Biofilm Inhibition: *C. albicans* was prepared at a density equivalent to 1 x 10^6 cells/ml in RPMI and added to a flat-bottomed microtitre plate. 4 of the clinical strains were used. Cells were then exposed, at 0, 1, 2 or 4 hrs after plating, to either tea tree oil, terpinen-4-ol or alpha-terpineol, at selected concentrations. Biofilms were quantified using a biomass assay as previously described by Monost *et al* (2007).

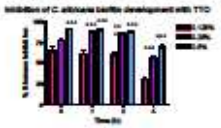
Results

Table 1: *C. albicans* planktonic and sessile susceptibility profiles of TTO derivatives

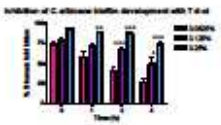
	PLANKTONIC (%)			SESSILE (%)		
	Range	MIC_{50}	MIC_{90}	Range (μg/mL)	$SMIC_{50}$	$SMIC_{90}$
TTO	0.125 - 1.0	0.5	1.0	1.0 - 4	4.0	4.0
Terpinen-4-ol	0.0625 - 0.5	0.25	0.5	0.25 - >16	8.0	0.5
α-Terpineol	0.125 - 0.5	0.5	0.5	0.25 - >16	8.0	0.5
1,8 Cineole	8.0 - >16	>16	>16	>16 - >16	>16	>16
Terpinolene	4.0 - >16	>16	>16	>16 - >16	>16	>16
p-Cymene	2 - >16	>16	>16	4 - >16	>16	>16
α-Terpinene	2 - >16	>16	>16	4 - >16	>16	>16
γ-Terpinene	4 - >16	>16	>16	4 - >16	>16	>16

The data confirms with conventional wisdom, i.e. planktonic cells are more susceptible to antimicrobial challenge than sessile cells. Both terpinen-4-ol and alpha-terpineol were more efficacious than the TTO mixture, against planktonic and sessile cells. The remaining components were less effective than the TTO mixture. Terpinen-4-ol was overall the most effective anti-candidal compound, demonstrating potent anti-biofilm activity.

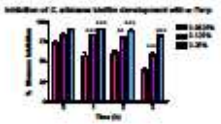
Figure 2: Inhibition of *C. albicans* biofilm development following pretreatment



TTO significantly inhibited the ability of *C. albicans* to filament and form biofilms at the highest concentration (0.5%) at all pre-treatment times ($P<0.001$). However, its effectiveness is time dependent, i.e. *C. albicans* is able to form biofilms if given the opportunity to form hyphae for 4 hours.



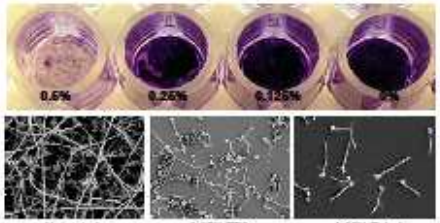
Terpinen-4-ol significantly inhibited the ability of *C. albicans* to filament and form biofilms at 0.25% at 1, 2 and 4 hours ($P<0.01$). At relatively low concentrations (0.125%), biofilms were inhibited by over 50%, compared to the control biomass. Pretreatment with 0.0625% at 0 hours inhibited biofilm formation by 75%.



α-terpineol significantly inhibited the ability of *C. albicans* to filament and form biofilms at 0.25% at all time points examined ($P<0.05$), inhibiting biofilm formation by over 75%. At relatively low concentrations (0.125%), biofilms were inhibited by over 50%, compared to the control biomass. Pretreatment with 0.0625% showed an anti-biofilm effect up to 4 hours.

* = $P<0.05$; ** = $p<0.01$; *** = $p<0.001$

Figure 3: Microscopical analysis of *C. albicans* biofilm inhibition following TTO challenge



TTO and terpinene-4-ol (0.25%) both demonstrate inhibitory properties against *C. albicans* biofilm development. Note the scant biofilms at 0.5% TTO treatment as assessed by crystal violet staining and the stunted filamentous growth and distorted yeast morphology, particularly with terpinen-4-ol pretreatment, following SEM analysis.

Discussion

***Candida albicans* clinical isolates from a range of clinical sources show broadly similar susceptibility profiles to TTO and its derivatives components when examined planktonically. No resistance phenotypes were noted for TTO, whereas some components showed no antifungal activity. Terpinen-4-ol and α-terpineol were able to reduce the metabolic activity of biofilms more effectively than TTO and the other components examined. In addition, pretreatment with these molecules was able to effectively inhibit the growth and development of *C. albicans* biofilms. However, once *C. albicans* have initiated morphological differentiation, then biofilm development is able to progress, although with a reduced overall biomass.**

This study has shown that derivatives of TTO have potential as an alternative to commercially available oral hygiene products for managing superficial oral candidosis and could be used as an adjunct to current therapeutics, such as azole antifungal agents, which are associated with resistance. Studies are ongoing within our laboratory to evaluate these molecule in terms of their cytotoxicity to orally relevant cell lines and their capacity to immuno-modulate.

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References

EPSTEIN, J. B. & POLSKY, B. (1998) Oropharyngeal candidiasis: a review of its clinical spectrum and current therapies. *Clin Ther*, 20, 40-57.

CARSON, C. F., HAMMER, K. A. & RILEY, T. V. (2006) *Melaleuca alternifolia* (Tea Tree) oil: a review of antimicrobial and other medicinal properties. *Clin Microbiol Rev*, 19, 50-62.

RAMAGE, G., VANDE WALLE, K., WICKES, B. L. & LOPEZ-RIBOT, J. L. (2001a) Standardized method for in vitro antifungal susceptibility testing of Candida albicans biofilms. *Antimicrob Agents Chemother*, 45, 2475-9.

MONOST, B., BUTCHER, J., LAND, S., WILLIAMS, C. & RAMAGE, G. (2007) Development of a simple model for studying the effects of antifungal agents on multicellular communities of Aspergillus fumigatus. *J Med Microbiol*, 56, 1205-12.

Oral Presentation: British Society for Dental Research, Glasgow, September 2009.

Candidosis Management: Antifungal and Immuno-modulatory Properties of Tea tree oil. S. G. Milligan, G. Ramage, S. Culshaw, M. P. Sweeney, J. Bagg. University of Glasgow Dental School, Glasgow, Scotland.

Candida albicans is the predominant yeast associated with oropharyngeal candidosis (OPC), which presents clinically as thrush or denture stomatitis. These infections are characterised by multispecies biofilms forming on the oral epithelium or artificial denture surfaces. Current antifungal treatments have limited success due to various resistance mechanisms, with recurring infections common. Therefore, alternative methods for suppressing or eradicating biofilms are desirable.

Objectives: The aims of this study were to evaluate the effectiveness of tea tree oil (TTO) and key derivatives against biofilms formed by a panel of oral *C. albicans* isolates, and to assess the cytotoxic and immuno-modulatory properties of TTO and its derivatives in a clinically relevant oral cell model.

Methods: Biofilms were formed on microtitre plates and treated with various concentrations of individual TTO components. Fungicidal activity was evaluated using the metabolic dye XTT. The most efficacious components were then applied to a human oral epithelial cell line (OKF6-TERT) and the toxicity evaluated using a commercially available assay. Cells were stimulated with zymosan A (yeast cell wall polysaccharide) and treated with TTO components, and cytokine profiles analysed using a commercially available kit. Real-time PCR was also used to measure gene expression changes for selected cytokines.

Results: It was shown that terpinen-4-ol (T4-ol) and *α*-terpineol exhibited greater anti-biofilm activity (MFC_{90} = 0.5%) than TTO (1%). TTO and T4-ol were significantly less toxic than chlorhexidine at therapeutic levels. TTO reduced stimulatory effects of zymosan on pro-inflammatory cytokines.

Conclusions: TTO-derived mouthwashes may offer a suitable alternative to conventional azole treatment, as they exhibit potent antifungal activity, are minimally toxic and can modulate inflammatory mediators.