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Antifungal, cytotoxic, and immunomodulatory properties of tea tree oil and its derivative components: potential role in management of oral candidosis in cancer patients

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Candida albicans forms oral biofilms that cause disease and are difficult to treat with conventional antifungal agents. Tea tree oil (TTO) is a natural compound with reported antimicrobial and immunomodulatory activities. The aims of the study were to evaluate the antifungal efficacy of TTO and key derivatives against *C. albicans* biofilms, to assess the toxicological effects of TTO on a clinically relevant oral cell line, and to investigate its impact on inflammation. TTO and its derivatives were examined against 100 clinical strains of *C. albicans*. Planktonic minimum inhibitory concentrations (MICs) were determined using the CLSI M-27A broth microdilution method. Sessile MICs were determined using an XTT reduction assay. Inhibition, time-kill, and mode of action studies were performed. OKF6-TERT2 epithelial cells were used for cytotoxicity and cytokine expression assays. Planktonic *C. albicans* isolates were susceptible to TTO, terpinen-4-ol (T-4-ol), and α-terpineol, with an MIC_{50} of 0.5, 0.25, and 0.25%, respectively. These three compounds also displayed potent activity against the 69 biofilm-forming strains, of which T-4-ol and α-terpineol displayed rapid kill kinetics. For all three compounds, 1 × MIC_{50} effectively inhibited biofilm growth when *C. albicans* were treated at 0, 1, and 2 h post adhesion. By scanning electron microscopy analysis and PI uptake, TTO and derivative components were shown to be cell membrane active. TTO and T-4-ol were cytotoxic at 1 × MIC_{50}, whereas at 0.5 × MIC_{50} T-4-ol displayed no significant toxicity. Transcript and protein analysis showed a reduction of IL-8 when treated with TTO and T-4-ol. These data provide further *in vitro* evidence that TTO and its derivative components, specifically T-4-ol, exhibit strong antimicrobial properties against fungal biofilms. T-4-ol has safety advantages over the complete essential oil and may be suitable for prophylaxis and treatment of established oropharyngeal candidosis. A clinical trial of T-4-ol is worthy of consideration.

Keywords: tea tree oil, oral candidosis, oral cancer, antifungal agent, terpinen-4-ol

INTRODUCTION

Many cancer therapies have a profound negative impact on oral health, causing serious complications (Dreizen, 1990). These include both non-infectious and infectious side-effects with potential for systemic spread (Wingard, 1990). Oral fungal infections, caused predominantly by *Candida* species, are common in patients suffering from cancer at all stages of the disease, especially those receiving palliative care (Sweeney et al., 1998; Sweeney and Bagg, 2000; Davies et al., 2006). *Candida albicans* is known to form complex biofilms (Ramage et al., 2005; Ganguly and Mitchell, 2011) and other *Candida* spp. also have this ability to varying degrees, though it is strain-dependent (Silva et al., 2009). Such biofilms form on the oral epithelium or on the surfaces of intra-oral prostheses (Williams et al., 2011), and can result in pseudomembranous or erythematous candidosis.

The effectiveness of most antifungal agents is significantly reduced if yeasts are in a biofilm as opposed to the planktonic state (Kuhn and Ghannoum, 2004; Lamfon et al., 2004; d'Enfert, 2006). Furthermore, in recent years there has been growing concern about the increasing prevalence of infections caused by yeasts that are resistant to commonly used antifungal drugs (Bagg et al., 2003), with the emergence of strains of *C. albicans* that are resistant to the azole antifungals (Casalinuovo et al., 2004), in addition to the inherently reduced drug susceptibility of many non-albicans yeasts that can be selected through over-use of antifungal drugs (Davies et al., 2002; Bagg et al., 2003). There is a need to identify new methods of preventing and treating oral candidosis among immunocompromised patients, including those with cancer, both to improve treatment of established infections and to limit further development of drug resistance. Given the extensive evolutionary

interaction that exists between plants and microorganisms it is unlikely that resistance would be cause for concern, nevertheless, clinically we would anticipate natural compounds to augment existing antifungal agents opposed to direct replacement.

One agent which merits consideration is tea tree oil (TTO; Hartford and Zug, 2005; Carson et al., 2006), which has recently reported to have minimal impact on developing resistance (Hammer et al., 2012). TTO is produced as a distillate of leaves of the *Melaleuca alternifolia* shrub, which grows in New South Wales, Australia. It is a complex mixture of essential oils, comprising approximately 100 components, most of which are monoterpenes, sesquiterpenes, and their related alcohols (Carson et al., 2006). TTO has been shown to possess a number of therapeutic properties, including anti-inflammatory activities (Hart et al., 2000; Koh et al., 2002; Pearce et al., 2005) and there is current interest in its possible anti-tumor properties (Bozzuto et al., 2011). However, it is best known for its antimicrobial activity against a wide spectrum of microorganisms, for example *Staphylococcus aureus* (including MRSA; Thompson et al., 2008; Kwiecinski et al., 2009), a range of oral bacteria (Hammer et al., 2003b), and certain viruses, including herpes simplex and influenza viruses (Carson et al., 2001; Garozzo et al., 2011). TTO also has potent activity against many fungi (Hammer et al., 2003a, 2004), including some azole-resistant yeasts (Mondello et al., 2003; Bagg et al., 2006) and there is some evidence for its efficacy in treating fluconazole refractory oral candidosis in AIDS patients. This raises the possibility of using TTO preparations for the prevention and treatment of oral candidal infections. Oral care products are now available containing TTO (Soukoulis and Hirsch, 2004), including an alcohol-free mouthwash. However, hypersensitivity reactions to TTO have been reported (Knight and Hausen, 1994; Mozelsio et al., 2003; Hammer et al., 2006; Rutherford et al., 2007) and the palatability of the agent as an oral preparation is poor. Moreover, given the complex chemical composition of TTO, which results in batch-to-batch variability, then the ability to accurately interpret its clinical utility is limited. Some of the individual components of TTO are believed to have antimicrobial properties (Mondello et al., 2006) and may be more appropriate for development into oral care products with respect to safety and consistency.

This study had three aims. The first was to evaluate the efficacy of TTO and two of its key derivatives [terpinen-4-ol (T-4-ol) and α-terpineol] against biofilms formed by a clinically diverse panel of *C. albicans* isolates. Secondly, the toxicological effects of TTO and derivative components were assessed by means of a clinically relevant oral cell line. Finally, the study aimed to investigate further the previously reported anti-inflammatory effects of TTO.

MATERIALS AND METHODS

STRAINS AND GROWTH CONDITIONS

One-hundred clinical strains of *C. albicans* were used in the course of this study, which were isolated from a wide variety of patient groups, including those with denture stomatitis (Glasgow Dental School, $n = 26$), those receiving palliative care for advanced cancer (Accord Hospice, Paisley, $n = 30$), neonates (Royal Hospital for Sick Children, Glasgow, $n = 36$), and those with bloodstream infections (Glasgow Royal Infirmary, $n = 8$). All strains were maintained routinely on Sabouraud dextrose (SAB) agar (Oxoid, Basingstoke, UK) stored at 4°C, and stored indefinitely on Microbank beads (Prolab Diagnostics) at −80°C.

ANTIFUNGAL COMPOUNDS

Tea tree oil and seven HPLC-grade derivatives of the oil obtained from Sigma-Aldrich (Poole, Dorset) were used in the study. The derivatives included terpinen-4-ol (T-4-ol), α-terpineol, 1-8-cineole, terpinolene, α-terpinene, γ-terpinene, and *p*-cymene. Stock concentrations of each component were prepared in RPMI-1640 AutoMod™ medium (Sigma, UK), containing 0.5% (v/v) Tween® 80 (ICI Americas, Inc.). Each compound was prepared as a 4% v/v solution for testing and serially diluted for both planktonic and sessile susceptibility testing. All procedures were carried out in a laminar flow cabinet (Hera Safe laminar flow cabinet, Kendro, Model K515).

SUSCEPTIBILITY TESTING AND BIOFILM INHIBITION

Antifungal testing to determine planktonic minimum inhibitory concentrations (PMICs) was performed using the CLSI M-27A broth microdilution method (Clinical Laboratory Standards Institute, 2008). For sessile susceptibility testing, *C. albicans* biofilms were formed as previously described on polystyrene, flat-bottomed, 96-well microtiter plates (Corning Incorporated, Corning, NY, USA; Ramage et al., 2001). These were treated with a range of concentrations of each antifungal compound (0.0625–2%) in RPMI-1640 containing 0.5% (v/v) TWEEN 80®. After 24 h challenge the sessile minimum fungicidal concentrations (SMFCs) were determined as a 50% inhibition compared to the untreated control using a XTT reduction assay, as described previously (Ramage et al., 2001). For time-kill studies *C. albicans* biofilms ($n = 3$) were grown for 24 h, then exposed to TTO, T-4-ol, or α-terpineol at a concentration of $2 \times MIC_{90}$ for 2, 5, 15, and 60 min. Metabolic activity was quantified by the XTT assay and biofilm viability assessed as a relative reduction in absorbance in comparison to untreated controls. For inhibition, *C. albicans* isolates ($n = 4$) were plated in 96-well microtiter plates for biofilm growth (1×10^6 cells/ml) then treated at 0, 1, 2, or 4 h after adhesion with TTO, T-4-ol, and α-terpineol at 0.5 and $1 \times MIC_{50}$. The cells were then incubated for a further 24 h at 37°C. The levels of biofilm inhibition were compared to an untreated control using a crystal violet biomass assay, as previously described (Mowat et al., 2007). All experiments outlined above were performed on two independent occasions with a minimum of three clinical isolates in triplicate.

MODE OF ACTION

We investigated whether TTO or T-4-ol interacted with the membrane using a propidium iodide (PI) uptake assay, as previously reported (Sherry et al., 2012). PI is a fluorescent dye that diffuses through the membranes of dead cells and is incorporated within nuclear DNA, therefore increased fluorescence directly correlates with reduced membrane integrity. *C. albicans* (3153A) was grown in an overnight broth as described previously, and standardized to 1×10^7 cells/ml in RPMI-1640 and treated with TTO, T-4-ol, or α-terpineol at a concentration of $2 \times MIC_{50}$ for 10, 20, 30, 40, 50, and 60 min. Following treatment, cells were centrifuged at 10,000 rpm to remove the compounds, washed with

PBS, stained with 20 μM of propidium iodide (PI; Sigma-Aldrich, UK), and incubated at 37°C for 15 min to allow the dye to bind to DNA. One-hundred microliters of each sample were transferred to a black microtiter plate (Corning Incorporated, NY, USA) and fluorescence measured using a microtiter plate reader (FluoStar Omega, BMG Labtech) at excitation and emission wavelengths 535/617 nm, respectively. Raw data were corrected for background fluorescence and the assay was carried out in triplicate. In addition, the physical effect of these compounds was assessed microscopically using scanning electron microscopy (SEM). *C. albicans* was adhered to Thermanox™coverslips (Nunc Inc., Thermo Fisher Scientific) for 2 h within 24 well tissue culture plates then treated with TTO and T-4-ol at 2 × MIC_{50} for 24 h. The cells were fixed using 2% w/v para-formaldehyde, 2% v/v glutaraldehyde, 0.15 M sodium cacodylate, and 0.15% Alcian Blue (pH 7.4), and prepared for SEM as previously described (Erlandsen et al., 2004). The fixed and dried biofilm samples were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope.

ORAL EPITHELIAL CELL LINE

OKF6-TERT2 epithelial cells were used as previously described (Ramage et al., 2012). This is an immortalized human oral keratinocyte cell line provided by the Rheinwald Laboratory (Brigham and Woman's Hospital, Boston). These cells were immortalized by forced expression of telomerase, and resemble primary oral keratinocytes in studies of cytotoxicity or inducible cytokine and beta-defensin expression (Dongari-Bagtzoglou and Kashleva, 2003). The cells were cultured in keratinocyte serum-free medium (KSFM) supplemented with 100 IU penicillin, 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+}). Primary periradicular fibroblasts (PRF, kind gift of Professor Colin Murray, University of Glasgow) were cultured in standard Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU penicillin, and 100 μg/ml streptomycin. Both cell lines were passaged at approximately 90% confluence, using 0.05% Trypsin EDTA. Experiments were used with cells between passages 5 and 10.

TOXICITY STUDIES

TTO and T-4-ol dilutions were prepared in KSFM or DMEM with 0.25% (v/v) Tween® 80 for OKF6 or PRF cells, respectively. To assess cytotoxicity, cells were seeded at 1×10^5 cells per well per ml in KSFM or DMEM in a 24 well culture plate and grown until 90–100% confluent. Medium was then removed and cells exposed to 0.5 and 1 × MIC_{50} of each compound for 2 min, then cells were washed gently (×3) in Hanks balanced salt solution. The viability of the cells was assessed using an XTT assay (0.25 mg/ml XTT and 1 μM of menadione in KSFM or DMEM), in which the cells were incubated in 5% CO_2 at 37°C for 2 h, and the absorbance quantified at 490 nm in an automated microtiter plate reader (Tecan Sunrise, Jencons, UK). Viability was calculated based on unexposed control cells.

CYTOKINE EXPRESSION STUDIES

OKF6 cells were grown in 12 well tissue culture trays were seeded and grown as described above and treated with TTO and T-4-ol at 0.5 × MIC_{50} for 2 min, washed then stimulated with zymosan (50 μg/ml). Media and zymosan controls were also included. Supernatants were collected after 4 and 24 h and RNA extracted using TRIzol® according to the manufacturer's instructions (Invitrogen, Paisley, Scotland). Total RNA was treated with DNase I (Promega, Southampton, UK) at 37°C for 30 min. RNA quality and quantity was assessed using a NanoDrop™spectrophotometer ND-1000 (Labtech International Ltd., Ringmer, East Sussex, UK), prior to cDNA production using Superscript II Reverse Transcriptase (Invitrogen). qPCR was then performed using an MxPro MX3000P Quantitative PCR machine (Stratagene, Amsterdam, Netherlands) with the following primers: IL-8 (F: 5′-CAGAGACAGCAGAGCACACAA-3′; R: 5′-TTAGCACTCCTTGGCAAAAC-3′; 170 bp), and the housekeeping gene GAPDH (F: 5′-GGTGGTGAAGACGCCAGT-3′; R: 5′-CAAGGCTGAGAACGGGAAG-3′). Each duplicate PCR reaction contained the following: 0.5 μl of 1 μg/μl of cDNA, 0.5 μl forward and reverse primer (10 nmol), 0.25 μl ROX reference dye, 12.5 μl SYBR® Green (Invitrogen), and 10.5 μl dH_2O. PCR reaction conditions were as follows: 95°C – 10 min, 40× (94°C – 30 s, 58°C –30 s, 72°C – 30 s) and 72°C – 10 min. Analysis was performed using the Mx3000P software (Stratagene) and gene expression normalized to β-tublin gene according to the $2^{-\Delta\Delta CT}$ method. To assess IL-8 protein levels an ELISA was performed on retained supernatants. ELISA kits (R&D Systems, Abingdon, UK) were used in accordance with the manufacturer's instructions. All assays were optimized and validated prior to use, with standards and samples performed in at least duplicate.

STATISTICAL ANALYSIS

For assessing the statistical significance of observed changes in metabolic activity and biomass of *C. albicans* and IL-8 protein levels in oral keratinocyte culture fluids (data that conformed to a near normal distribution) a one-way ANOVA with Bonferroni multiple comparison post-test was performed. ANOVA and *post hoc* tests were also used on natural log transformations (to normalize) of proportional data for the analysis of IL-8 mRNA abundance in these cells. $p < 0.05$ was considered significant. The analyses were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, CA, USA).

RESULTS

TTO AND DERIVATIVE COMPONENTS ARE EFFECTIVE AGAINST *CANDIDA ALBICANS* BIOFILMS

The planktonic *C. albicans* isolates tested were susceptible to TTO, T-4-ol, and α-terpineol, with an MIC_{50} of 0.5, 0.25, and 0.25%, respectively, and an MIC_{90} of 1.0, 0.5, and 0.5%, respectively (**Table 1**). There was minimal variation in susceptibility as determined by the concentration ranges for the 100 strains tested. These three compounds also displayed potent activity against the 69 strains capable of forming biofilms, with the $SMIC_{50}$ for T-4-ol and α-terpineol showing no change when compared to median effective planktonic concentration ($SMFC_{50} = 0.25\%$). However, the concentration of TTO required to show a similar effect was double that required to inhibit planktonic cells ($SMFC_{50} = 1\%$). All other compounds tested showed relatively poor activity against the isolates tested.

Table 1 | Susceptibility profile of *Candida albicans* to tea tree oil and derivative components.

	Planktonic minimum inhibitory concentrations ($n = 100$)			Sessile minimum inhibitory concentrations ($n = 69$*)		
	MIC_{50} range (%)	MIC_{50} (%)	MIC_{90} (%)	$SMFC_{50}$ range (%)	$SMFC_{50}$ (%)**	Sessile/planktonic MIC_{50} ratio
TTO	0.125–1.0	0.5	1.0	1–4	1	2
T-4-ol	0.0625–0.5	0.25	0.5	<0.25–>1	0.25	1
α-Terpineol	0.125–0.5	0.25	0.5	<0.25–1	0.25	1
1,8-Cineole	0.5–>1	1	>1	<2–>2	2	2
Terpinolene	0.5–>1.0	>1	>1	2–>2	>2	>2
α-Terpinene	2–>2	>2	>2	4–>4	>4	>2
γ-Terpinene	2–>2	>2	>2	4–>4	>4	>2
ρ-Cymene	2–>2	>2	>2	4–>4	>4	>2

**69 of the 100 strains tested were able to form biofilms.*

***Sessile minimum inhibitory concentration at which a 50% reduction in biofilm metabolic activity was achieved.*

The biofilm rate of kill for the three most effective components (TTO, T-4-ol, and α-terpineol) at $2 \times SMIC_{50}$ was performed, where time dependent killing was observed (**Figure 1**). TTO was shown to reduce the viability of the biofilms within 2 min by 44%, but after 5, 15, and 60 min this had declined only slightly to 46, 56, and 65%, respectively. However, both T-4-ol and α-terpineol displayed comparable and more effective killing, reducing the viability rapidly after 2 min by approximately 55%. This anti-biofilm activity remained, reducing biofilm viability by approximately 61, 75, and 94% after 5, 15, and 60 min, respectively. Both T-4-ol and α-terpineol showed significantly better activity than TTO only at 60 min ($p < 0.001$).

Inhibition of biofilm formation was assessed for these three compounds at 0.5 and $1 \times MIC_{50}$ by assessing biofilm biomass. It was shown for all compounds that $1 \times MIC_{50}$ was able to inhibit biofilm growth effectively when treated at 0, 1, and 2 h post adhesion (8.5−12% biomass compared to control). At 4 h post adhesion, by which time cells had began to form hyphae, treatment with TTO, T-4-ol, and α-terpineol further suppressed biofilm proliferation by 71, 74, and 82% that of the control (**Figure 2A**). No significant differences were observed between the treatments at each time point. Treatment with $0.5 \times MIC_{50}$ was overall less effective at inhibiting biofilm growth most notably with TTO, which displayed a time dependent reduction in inhibition, showing a 44% inhibition of cells treated at 4 h (**Figure 2B**). Both T-4-ol and α-terpineol were significantly more effective against 1 and 2 h cells ($p < 0.05$), inhibiting biofilm formation by >80%. However, this inhibition was reduced against the 4 h cells (54 and 58%, respectively).

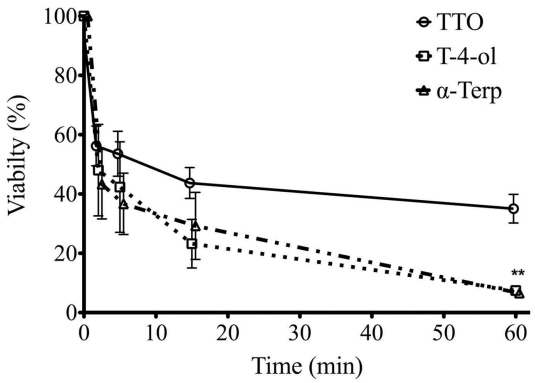

FIGURE 1 | *Candida albicans* biofilm killing by TTO, T-4-ol, and α-terpineol is time dependent. Standardized *C. albicans* (1×10^6 cells/ml) were incubated in flat-bottomed 96-well plates for 24 h, washed in PBS, and treated with $2 \times SMIC_{50}$ at 2, 5, 10, 15, and 60 min. Metabolic activity of treated biofilms was then quantified using the XTT assay by reading at 492 nm in a microtiter plate reader (FluoStar Omega, BMG Labtech). Three isolates were used for each assay, and this was performed on two independent occasions in triplicate. $^{**}p < 0.001$. Error bars represent the ±standard error of the mean.

TTO AND DERIVATIVE COMPONENTS ARE CELL MEMBRANE ACTIVE

Scanning electron microscopy analysis of TTO and T-4-ol treated cells was performed. It was noted that compared to the control (untreated) cells (**Figure 3Ai**), both compounds had ruptured the cells, allowing the cell contents to leak out, giving a punctured appearance (**Figures 3Aii,iii**). The cell damage was shown to be more extensive for T-4-ol treated cells. Given this appearance we hypothesized that cell membrane integrity had been compromised. We therefore undertook PI uptake experiments, as previously reported (Sherry et al., 2012). For TTO, PI uptake was shown to be relatively slow, with maximal fluorescence obtained at 30 min (**Figure 3B**). In comparison, for T-4-ol fluorescence was shown to increase in a time dependent manner up to 40 min, twice that of TTO, after which time this reached a plateau. These data show similar kinetics to the time-kill data presented.

TTO AND TERPINEN-4-ol ARE BIOLOGICALLY ACTIVE AGAINST MAMMALIAN CELLS

The effect of a short exposure (2 min) of TTO and T-4-ol on cellular toxicity was investigated. It was shown that TTO and T-4-ol were toxic to both fibroblast and epithelial cells at $1 \times MIC_{50}$, reducing the viability of PRF to approximately 12% and OKF6

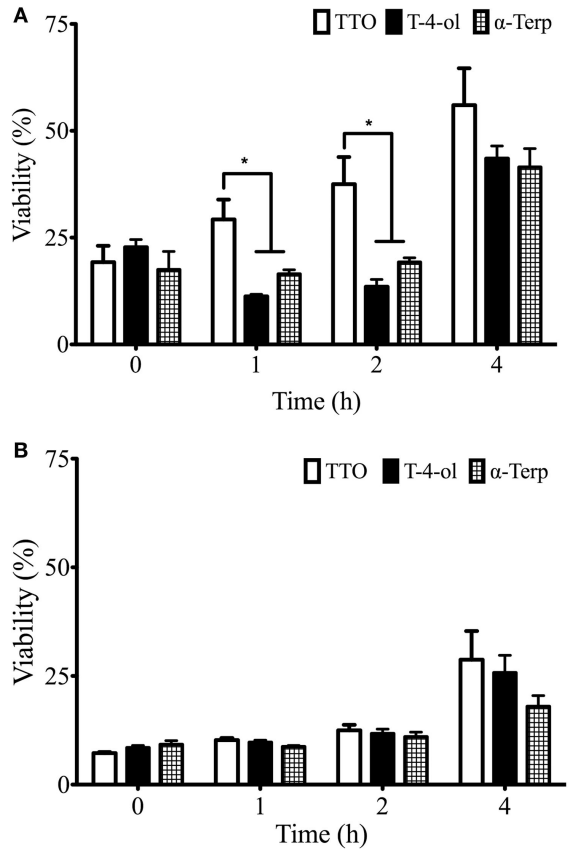

FIGURE 2 | *Candida albicans* biofilm development is inhibited by TTO, T-4-ol, and α-terpineol. Standardized *C. albicans* (1×10^6 cells/ml) were added to a flat-bottomed 96-well plate and treated with **(A)** 0.5 and **(B)** $1 \times SMIC_{50}$ for 0, 1, 2, and 4 h post incubation. Biofilms were subsequently allowed to develop for 24 h. Resultant biofilms were quantified using a crystal violet assay read at 570 nm in a microtiter plate reader (FluoStar Omega, BMG Labtech). Four isolates were used for each assay, and this was performed on two independent occasions in triplicate. $*p < 0.05$. Error bars represent the ±standard error of the mean.

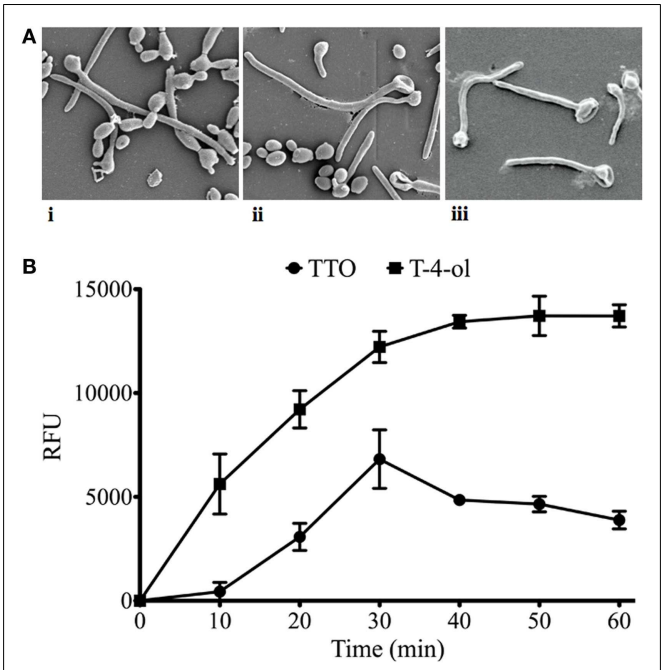

FIGURE 3 | Tea tree oil and T-4-ol are cell membrane active. (A) Adherent cells (3153A) were adhered for 2 h on Thermanox™coverslips and were **(i)** untreated (control), or treated with **(ii)** TTO, or **(iii)** T-4-ol at $2 \times MIC_{50}$ for 24 h. These were then processed and viewed on an SEM. **(B)** *C. albicans* (3153A) planktonic cells (5×10^7 cells/ml) were treated with TTO, T-4-ol, or α-terpineol at a concentration of $2 \times MIC_{50}$ for 10, 20, 30, 40, 50, and 60 min. The cells were washed by centrifugation, resuspended in PI (20 μM in PBS), and incubated for 15 min at 37°C. These were then transferred to a black 96-well plate for quantification in a fluorescent plate reader (Ex_{485}/Em_{620}). Each assay was performed on at least two independent occasions in triplicate. Error bars represent the ±standard error of the mean.

cells to 35 and 15%, respectively (**Figure 4A**). At $0.5 \times MIC_{50}$ T-4-ol displayed no toxicity (98% viable) against PRF compared with TTO (63% viable). Both 0.5× T-4-ol and 0.5× TTO were non-toxic to OKF6 cells (>100% viable).

Given that the concentration of $1 \times MIC_{50}$ is cytotoxic, all subsequent work was performed with $0.5 \times MIC_{50}$. Transcriptional expression of IL-8 was assessed by qPCR, using zymosan as a potent cell inflammatory agonist. Generally, greater IL-8 mRNA levels were observed at 4 h compared to 24 h. TTO and T-4-ol pre-treatment of the cells had no effect on the induction of the IL-8 gene when compared to the control (**Figure 4B**). In zymosan-stimulated cells pre-treatment with TTO did not reduce IL-8 expression significantly at either 4 or 24 h ($p > 0.05$). Although T-4-ol showed up to a twofold reduction in IL-8 expression in cells at both 4 and 24 h, though this reduction was not statistically significant ($p > 0.05$).

Analysis of IL-8 protein levels after 4 and 24 h stimulation with zymosan showed that neither TTO or T-4-ol were able to significantly affect IL-8 levels in culture fluids ($p > 0.05$) of untreated and zymosan-stimulated oral keratinocytes (data shown for zymosan only). Nevertheless, a significant (50%) reduction in IL-8 expression was observed in culture fluids of TTO treated zymosan-stimulated cells ($p < 0.05$), and a modest reduction of approximately 14% of IL-8 was noted for T-4-ol treated zymosan-stimulated cells (**Figure 4C**). These compounds had no effect on the IL-8 ELISA (data not shown).

DISCUSSION

Increasing use of conventional antifungal agents, such as azoles, in parallel with larger groups of susceptible individuals (aging population and more common immunosuppressive therapies) has resulted in the emergence of multidrug-resistant *Candida* strains (Sanglard and Odds, 2002; Akins, 2005; Cannon et al., 2007; Niimi et al., 2010). Non-compliance due to toxic side-effects and palatability may also exacerbate this escalating trend. Consequently, there is a demand for novel therapies to manage these infections. Our current and previous data investigating the effect of TTO on a wide variety of yeast species clearly demonstrate that it is effective against *C. albicans* planktonic and biofilm cells (Bagg et al., 2006).

Given the complex chemical composition of TTO we aimed to investigate key individual components to assess specific activities.

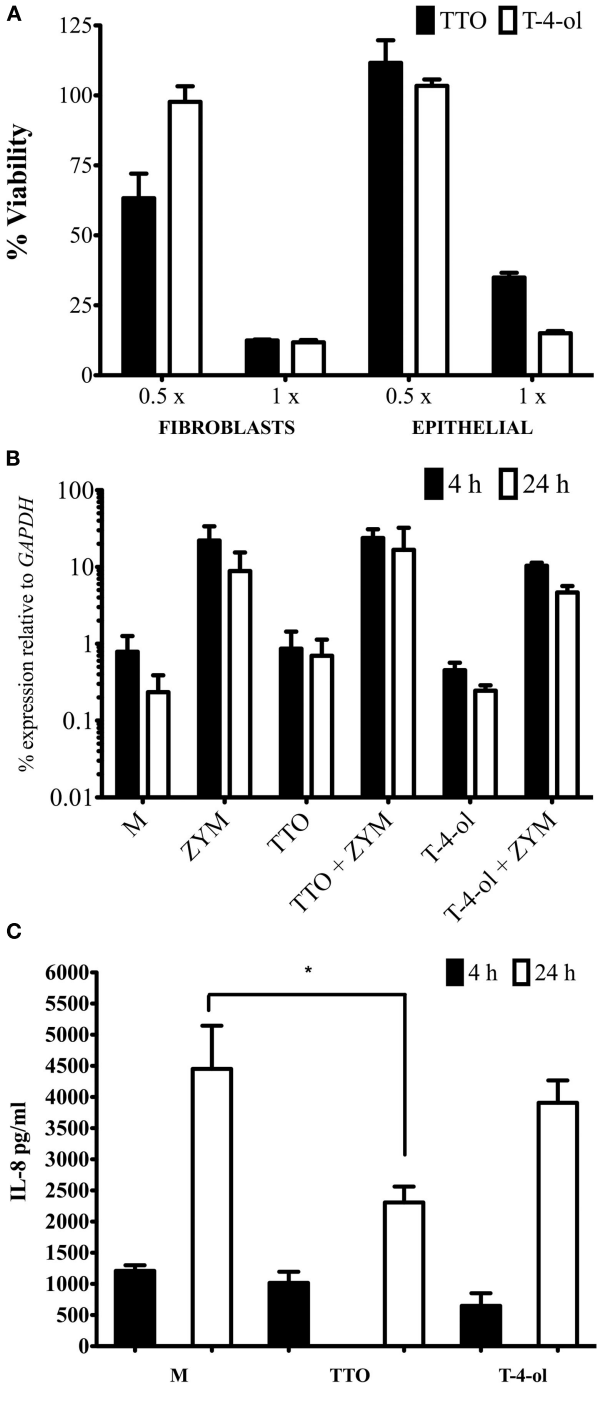

FIGURE 4 | Tea tree oil and T-4-ol are biologically active against mammalian cells. (A) PR fibroblast and OKF6 epithelial cells were grown until 90–100% confluent. These were exposed to 0.5 and 1 × MIC_{50} of each compound for 2 min. Cell viability was assessed using an XTT assay after incubation in 5% CO_2 at 37°C for 2 h. Viability was calculated based on unexposed control cells. For cytokine studies OKF6 cells were grown in 12 well tissue culture trays treated with TTO and T-4-ol at 0.5 × MIC_{50} for 2 min, washed then stimulated with zymosan (50 μg/ml). Cells were processed for **(B)** qPCR analysis and **(C)** supernatant was processed for protein analysis of IL-8. Each assay was performed on three independent occasions in triplicate. *$p < 0.05$. Error bars represent the ±standard error of the mean.

T-4-ol and α-terpineol exhibited the greatest and comparable antifungal activity against both planktonic and sessile cells, which has been confirmed from reports elsewhere (Hammer et al., 2003a; Mondello et al., 2006; Terzi et al., 2007). Moreover, time-kill studies showed a rapid and sustained level of activity of both compounds, significantly superior to TTO at 2 × MIC_{90} after 60 min. This superior activity was less evident during the early biofilm inhibition studies (0–2 h), however, at 4 h TTO showed a significant decline in its ability to inhibit biofilm growth. Both T-4-ol and α-terpineol have hydroxyl groups in their chemical structures, making them moderately water-soluble. This allows them to diffuse through water, enter, and destabilize cell membranes, resulting in osmotic shock (Straede et al., 2007). This was evident from analysis of cell membrane integrity using a PI uptake assay and microscopic examination by SEM.

T-4-ol and α-terpineol both showed excellent activity, but the remainder of the studies compared TTO with T-4-ol, primarily because of the high bioavailability of the latter within TTO and its overall antifungal profile (Carson et al., 2006). Cytotoxicity was observed at MIC_{50} levels for both TTO and T-4-ol, whereas at 0.5 × MIC_{50} (concentrations able to inhibit *C. albicans* growth) OKF6 cells remained viable. Previous reports have demonstrated varying levels of cytotoxicity to primary fibroblasts and primary epithelial cells, with a 1 h exposure to 0.03%TTO shown to be toxic (Soderberg et al., 1996). Further reports indicated that a 4 h exposure to 0.28% TTO was required to inhibit the HeLa epithelial cell line (Hayes et al., 1997). In addition, it was shown previously that TTO was highly toxic to monocytes and neutrophils, but after a prolonged 20 h exposure (Hart et al., 2000). The relevance of this length of exposure is difficult to interpret. Collectively, these studies highlight the importance of the cell line being tested and in what context. Given that our primary interests lay in developing a mouthwash to prevent candidal growth as opposed to treating an active infection, a 2 min exposure of sub-inhibitory concentrations was deemed optimal.

These data were used to assess the impact of TTO-based compounds on inflammation, using IL-8 as a biomarker. Both transcriptional and protein analysis showed that IL-8 was regulated by both compounds, with TTO showing protein reduction of approximately 50% 24 h post treatment, whereas T-4-ol only caused a 14% reduction. However, at 4 h post treatment T-4-ol inhibited IL-8 protein production by 53% opposed to 16% for TTO, although neither was significant. The differences observed may be accounted for by the quantitative composition of TTO, which contains approximately 40% T-4-ol. A previous study of LPS stimulated monocytes demonstrated a significant IL-8 suppression by 0.052% T-4-ol after 40 h (Hart et al., 2000). The discordance with our data can be explained through differences in concentration and exposure time. Irrespective, both studies indicate that these molecules have the potential capacity to suppress inflammatory mediators, which are common within the oral cavity of OPC sufferers. Indeed, there is *in vivo* evidence to support this. Several murine studies have shown inhibitory effects on inflammatory processes, including reduced contact hypersensitivity (Brand et al., 2002a), reduced histamine-induced edema (Brand et al., 2002b), and blocking of zymosan-induced inflammation by inhaled TTO (Golab and Skwarlo-Sonta, 2007). In humans,

nickel-induced contact hypersensitivity has been reduced by the topical application of 100% TTO (Pearce et al., 2005). Both TTO (Koh et al., 2002) and T-4-ol (Khalil et al., 2004) have also been shown to reduce histamine-induced weal and flare reaction in human skin. Conversely, other studies have implicated TTO as being pro-inflammatory (de Groot and Weyland, 1992; Rutherford et al., 2007). Given the apparent contradictory reports in the literature it seems prudent to focus on utilizing the most biologically active and abundant compound from TTO, i.e., T-4-ol. This will enable investigators to determine accurately the medicinal benefits of pure T-4-ol and exclude deleterious effects caused by the other terpenes that comprise TTO.

In summary, these studies have added to the body of *in vitro* evidence indicating that TTO and some of its individual components, specifically T-4-ol, exhibit strong antimicrobial efficacy against fungal biofilms. Furthermore, this has also demonstrated a potential biofilm inhibiting activity, suggesting that this agent may be suitable for use in prophylactic oral hygiene products such as mouth rinses and denture cleansers, as well as treatment for established OPC infections. The use of T-4-ol, a single component from TTO, has advantages over the complete essential oil in terms of product safety and consistency. Oral candidosis is a continuing problem for cancer patients, as well as other groups of immunocompromised hosts. In the face of increasing resistance to azoles and other established antifungal drugs, the need for novel preventive and therapeutic agents has never been greater. The weight of laboratory data that has now accumulated and the anecdotal reports of clinical efficacy suggest that clinical trials of TTO components, particularly T-4-ol, would be merited.

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