In Vitro activity of Melaleuca alternifolia (tea tree) oil on filamentous fungi and toxicity to human cells

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Abstract

Invasive fungal wound infections (IFIs) are increasingly reported in trauma patients and cause considerable morbidity and mortality despite standard of care treatment in trauma centers by experienced medical personnel. Topical agents such as oil of melaleuca, also known as tea tree oil (TTO), have been proposed for adjunctive treatment of IFIs. We evaluated the activity of TTO against filamentous fungi associated with IFIs by testing 13 clinical isolates representing nine species via time-kill assay with seven concentrations of TTO (100%, 75%, 50%, 25%, 10%, 5%, and 1%). To ascertain the safety of topical application to wounds, cell viability assays were performed in vitro using human fibroblasts, keratinocytes, osteoblasts, and umbilical vein endothelial cells with 10 concentrations of TTO (75%, 50%, 25%, 10%, 5%, and 10-fold serial dilutions from 1 to 0.0001%) at five time points (5, 15, 30, 60, and 180 min). Compatibility of TTO with explanted porcine tissues was also assessed with eight concentrations of TTO (100%, 75%, 50%, 25%, 10%, 5%, 1%, and 0.1%) at the time points used for cellular assays and at 24 h. The time-kill studies showed that fungicidal activity was variable between isolates. The effect of TTO on cell viability was primarily concentration dependent with significant cytotoxicity at concentrations of ≥10% and ≥50% for cells lines and whole tissue, respectively. Our findings demonstrate that TTO possesses antifungal activity against filamentous fungi associated with IFIs; furthermore that negligible effects on whole tissues, in contrast to individual cells, were observed following exposure to TTO. Collectively, these findings indicate a potential use of TTO as topical treatment of IFIs.

Key words: cell viability, cytotoxicity, invasive fungal infection, Melaleuca alternifolia, tea tree oil.
Introduction

Invasive fungal wound infections (IFIs) continue to be a source of high morbidity and mortality [1,2]. Populations at increased risk for IFIs include victims of natural disasters and combat-related trauma with rates of up to 3.5% of all trauma admissions in military hospitals [1]. Recent experience with the Joplin, Missouri tornado resulting in 13 proven cases of *Apophysomyces trapeziformis* wound infection and 38% mortality emphasizes the need for awareness of these infections, as well as the initiation of prompt, effective diagnosis and treatment [2,3]. Therapy for these patients relies upon repeated surgical debridement and systemic antifungal therapy. Topical treatment of fungal wound infections has been implemented in some centers using sodium hypochlorite solution (Dakin’s solution) and/or amphotericin B [1, 3–4].

Oil of melaleuca, commonly referred to as tea tree oil (TTO), is a complex mixture of approximately 100 components steam distilled from the native Australian tree *Melaleuca alternifolia*, a member of the Myrtaceae family [5]. The characteristics of commercially available oil of melaleuca, terpinen-4-ol type, are stipulated by the International Organization for Standardization (ISO). While no individual molecule in TTO is responsible for all of its antimicrobial properties, terpinen-4-ol is an active agent and is the main constituent, comprising approximately 40% of the mixture [5,6].

Therapeutic antifungal use of TTO for onychomycosis, tinea pedis, dandruff, and oral candidiasis has been clinically evaluated with mixed results [7–10]. Recent interest in TTO has led to *in vivo* human studies for treatment and prevention of methicillin-resistant *Staphylococcus aureus* colonization [11,12]. Several studies have tested the activity of TTO against various yeasts and dermatophytes, but to our knowledge, there are limited studies evaluating the antifungal activity of TTO against human pathogenic filamentous fungi, other than in *Aspergillus flavus* and *A. fumigatus* [13–15].

TTO can cause allergic and irritant contact dermatitis. It was added to the North American Contact Dermatitis screening panel in 1999, with a prevalence rate of 1.4% in patients referred for patch testing [16–19]. In an Australian population patch tested with a 10% TTO dilution, prevalence of allergic contact dermatitis reaction was 2.9%–4.8% and up to 7.6% in subjects with prior use of TTO [20]. Irritant reactions were noted in 7.2%–10.1% of subjects tested with 100% TTO, but none of the same subjects reacted to 10% TTO [20]. Epidermal effects and skin penetration have been previously determined [21], but toxicity of TTO to human cell lines clinically relevant in IFIs has not been well studied, nor has its safety been rigorously assessed in non-intact skin [22].

There is potential for improvement in topical therapy for IFIs. An ideal agent would be both broadly active against the most commonly reported agents of IFIs, including *Mucorales* spp., *Aspergillus* spp., and *Fusarium* spp., and have less adverse effects on the exposed skin, connective tissue, bone, and blood vessels required for appropriate wound healing [23]. To assess if TTO met these specifications, we designed time-kill studies to assess the activity of various concentrations of TTO against 13 clinical filamentous fungal isolates comprising nine species. We also evaluated the effect of TTO on cellular viability both *in vitro*, using relevant cell lines for wound healing, as well as on explanted porcine tissue, following exposure to multiple different concentrations of TTO.

Materials and Methods

Fungal Isolates

Thirteen clinical mould isolates representing nine species in seven genera [one *Lichtheimia* sp. (previously *Absidia* spp.), three *A. flavus*, one *A. fumigatus*, one *A. terreus*, two *Mucor circinelloides* group, one *Exophiala* spp., one *Apophysomyces* spp., two *Actinomucor elegans*, and one *Fusarium* spp.] were selected from the clinical strain repository at the San Antonio Military Medical Center (Table 1). Fungal isolates in the repository were collected from patients as part of a pathogen surveillance program. All mould isolates were subcultured onto potato flake agar slants for 7–10 days (Remel, Lenexa, KS, USA) prior to testing.

Fungal Time-Kill Study

Time-kill studies were carried out against individual isolates listed above as previously described [4]. Fungal inocula were prepared from subcultures following the Clinical and Laboratory Standards Institute (CLSI) guidelines [24]. Briefly, fungi were grown on potato flake agar slants at 35°C for 7 to 10 d, depending upon sporulation. Standard conidia/hyphae suspensions were prepared in 2 ml sterile water by adjusting the optical density of the suspensions at 530 nm to obtain the proscribed concentrations for each species as indicated [24]. Fungal inocula (1 x 10⁶ cfu/ml) were exposed to various concentrations of TTO (Sigma-Aldrich, St. Louis, MO, USA, product W390215, lot MKBB4099V) (1%, 5%, 10%, 25%, 50%, 75%, 100%) diluted in water containing 0.1% of dimethyl sulfoxide (DMSO; Fisher, Pittsburgh, PA, USA) to determine the in vitro antifungal activity. As TTO includes components immiscible in aqueous solutions, DMSO was used as a solubilizing agent. At specified time points (0, 5, 15, 30, 60, 90 min, and 3, 6, 12, 24 h) aliquots of a 100-fold
Table 1. Fungal Strain Characteristics.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Organism</th>
<th>Specimen type(^a)</th>
<th>Site of Isolation</th>
<th>Antifungal Susceptibility (MIC)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMMC-1</td>
<td>Exophiala sp.</td>
<td>Clinical</td>
<td>Unknown</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-2</td>
<td>Apophysomyces sp.</td>
<td>Clinical</td>
<td>Unknown</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-3</td>
<td>Aspergillus flavus</td>
<td>Clinical</td>
<td>Bronchial wash</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-4</td>
<td>Aspergillus fumigatus</td>
<td>Clinical</td>
<td>Bronchial wash</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-5</td>
<td>Aspergillus flavus</td>
<td>Clinical</td>
<td>Tissue (R. Leg)</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-6</td>
<td>Aspergillus flavus</td>
<td>Clinical</td>
<td>Tissue (Autopsy)</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-7</td>
<td>Aspergillus terreus</td>
<td>Clinical</td>
<td>Bronchial wash</td>
<td>AMB (2ug/mL); VRC (0.25 ug/mL)</td>
</tr>
<tr>
<td>SAMMC-8</td>
<td>Actinomucor elegans</td>
<td>Clinical</td>
<td>Tissue</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-9</td>
<td>Actinomucor elegans</td>
<td>Clinical</td>
<td>Tissue</td>
<td>AMB (0.5 ug/mL); PSC (0.5 ug/mL); VRC (8 ug/mL); Caspofungin (&gt;8ug/mL)</td>
</tr>
<tr>
<td>SAMMC-10</td>
<td>Mucor circinelloides group</td>
<td>Clinical</td>
<td>Tissue</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-11</td>
<td>Lichtheimia sp.</td>
<td>Clinical</td>
<td>Sputum</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-12</td>
<td>Fusarium oxysporum</td>
<td>Clinical</td>
<td>Tissue</td>
<td>-</td>
</tr>
<tr>
<td>SAMMC-13</td>
<td>Mucor circinelloides group</td>
<td>Clinical</td>
<td>Tissue (Ankle)</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Specimen type denotes whether the isolate was collected from the environment or from patients (clinical).

\(^b\) Amphotericin B (AMB), Posaconazole (PSC), Voriconazole (VRC); Antifungal susceptibility testing was ordered at the discretion of the medical provider and performed at the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio following the Clinical and Laboratory Standards Institute’s M38-A2 reference method for broth dilution testing of filamentous fungi.

dilution were plated onto potato flake agar plates to determine antifungal activity. The plates were incubated at 35°C and colony counts determined after incubation for 24 h or as appropriate for slow growing moulds. Assays of each isolate were repeated on two separate occasions.

Cell Lines and Media

Human epidermal keratinocytes (HEK001; ATCC CRL-2404; ATCC, Manassas, VA, USA) were grown in keratinocyte serum-free medium (GIBCO, Grand Island, NY) supplemented with 5 ng/ml of human recombinant epidermal growth factor (EGF) and 2 mM of L-glutamine. Human dermal fibroblasts and osteoblasts (PromoCell, Heidelberg, Germany) were grown in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, 10 U/ml of penicillin, and 10 µg/ml of streptomycin. Human umbilical vein endothelial cells (HUVECs; Invitrogen, Carlsbad, CA, USA) were grown in medium 200 supplemented with low growth serum supplement (Invitrogen), containing a final concentration of 2% v/v bovine serum, 1 µg/ml of hydrocortisone, 10 ng/ml of human EGF, 3 ng/ml of basic fibroblast growth factor, and 10 µg/ml of heparin (Invitrogen). All cell lines were grown and maintained at 37°C in 5% carbon dioxide.

Cellular Viability Assays

Cellular viability assays were performed as previously described [4], which in brief consisted of exposing confluent monolayers of cells to TTO diluted at 10 concentrations (75%, 50%, 25%, 10%, 5%, and 10-fold serial dilutions of 1–0.0001%) in cell media supplemented with 0.1% DMSO for 5, 15, 30, 60, and 180 min. Undiluted TTO was not included due to its corrosive activity on polystyrene as has been described previously [25]. Following exposure, cells were washed, resuspended in phosphate buffered saline (pH 7.4), and cell viability measured using Cell Titer-Fluor assay (Promega, Madison, WI, USA) as recommended by the manufacturer. As a negative control, cells were exposed to media containing 0.1% DMSO and viability reported as a percentage of the nontreated control group. Assays were performed at least twice with a minimum of three technical replicates per test condition.

Tissue Viability Evaluation

Post-mortem skin grafts were harvested from the back of Sinclair Miniature pigs (Sus scrofa) as part of a tissue sharing agreement and in compliance with an approved animal study protocol through the Animal Welfare Act and the implementing Animal Welfare Regulations. Briefly, porcine skin explants were extracted from excised tissue using a 5-mm biopsy punch, tissues were washed with phosphate-buffered saline (PBS), and then sterilized by 15-min exposure to 70% ethanol solution followed by 0.615% sodium hypochlorite solution prepared in PBS [26]. Biopsied tissues (n = 6) were then exposed to eight concentrations of TTO (100%, 75%, 50%, 25%, 10%, 5%, 1%, and 0.1%) at the same time points used for cellular viability assays and an additional time point at 24 h. Following exposure to TTO, tissues were washed twice with PBS, transferred to a
96-well plate, and incubated with MTT cell viability reagent (ATCC, Manassas, VA, USA) for 2 h at 37°C. Following incubation, tissues were then transferred to individual wells of a 96-well plate containing dimethyl sulfoxide (0.07 ml), incubated for 1 h at 37°C, followed by quantification of the solubilized formazan by measuring the absorbance at 540 nm. As a positive control for the MTT assay, a group of tissue samples were heated to 200°C for 5 min. The average of the optical density values of the positive control were then subtracted from the absorbance values obtained for all other samples. Tissue viability was expressed at the ratio between the observed OD \(_{540\text{nm}}\) and the weight of the tissue in grams and reported as a percentage to nontreated control at each time point, as previously described [27,28].

**Statistical calculations**

Multiple linear regression analysis was used to determine whether TTO concentration or exposure time (independent variables) best predicted loss of cell viability in the four human cell lines (the dependent variable), employing the generalized linear model using the reciprocal link function (JMP version 9.0.0). The median lethal doses (LD\(_{50}\)) for all cell lines tested, including keratinocytes, fibroblasts, osteoblasts, and HUVECs, were determined from the resulting statistical model. The overall fungicidal effect of TTO was modeled by regressing the median log-cfu counts from time-kill curves of all fungal isolates tested against time (GraphPad Prism version 5.01). Controls (water and DMSO) were compared at each time point using the Wilcoxon rank-sum test.

**Results**

**Fungal Time-Kill**

Visual inspection of the time-kill curves revealed, both strain and concentration dependent differences in the activity of TTO against the 13 filamentous fungal isolates included in the tests (Fig. 1 and 2, and supplemental information). With the exception of *Exophiala* spp. (SAMMC-1), *Actinomucor* spp. (SAMMC-9), and *Mucor* sp. (SAMMC-10), the log reduction of treatment groups relative to a nontreated control group, water.
Fusarium spp. (SAMMC-11), short exposures to TTO at concentrations up to 100% (v/v) for up to 90 min had minimal to only moderate effect (≤ 2-log reductions) compared to the control groups for the vast majority of fungal strains tested. For Exophiala spp. and Fusarium spp., exposure to 100% TTO resulted in >4-log reduction compared to controls following exposures for as little as 15 min. In contrast, fungicidal activity of TTO at 10% required exposure times of >60 min, whereas only minimal activity up to 90 min was observed following exposure to 1% TTO.

Compared to the short exposure times to TTO, extending the exposure times, from 3 to 24 h demonstrated an enhancement of its fungicidal activity against the majority of strains tested (Fig. 2). For the majority of isolates, fungicidal activity was observed for all of the concentrations tested, 1, 10, and 100% (v/v), following 12–24 h exposures. Of the isolates tested Aspergillus terreus (SAMMC-7) and Absidia spp. (SAMMC-12) seemed to be more tolerant to the activity of TTO requiring higher concentrations for greater log-reductions. Having observed the trend that increasing exposure time of TTO could enhance fungicidal activity at the lower concentrations tested, we performed a linear regression analysis to evaluating the relationship between time and activity of TTO against the clinical isolates tested. As demonstrated in Figure 3, a strong relationship collectively was observed between exposure time and fungicidal activity ($r^2 = 0.99$).

**In Vitro Cell Viability**

In contrast to the antifungal activity of TTO, with all cell lines tested, cell viability decreased with increasing concentrations of TTO starting with the lowest concentration tested (Fig. 4). Fibroblasts remained approximately 90% viable compared with controls at concentrations of 0.1 and 1% v/v TTO. At least 50% of fibroblasts compared with controls remained viable when incubated with 10% v/v TTO, and statistical analysis confirmed LD$_{50}$ to be 12% TTO. No evidence of time-dependent toxicity with fibroblasts was seen at 0.1% v/v TTO. Cell viability was comparable for keratinocytes and osteoblasts.
Figure 3. Relationship between time and fungicidal activity of TTO against filamentous fungi. The median Log CFU of the 13 mould isolates tested herein at each time point was plotted against exposure time and analyzed by linear regression analysis. The high degree of correlation observed ($r^2 = 0.99$) indicates that the fungicidal activity of tea tree oil depends upon exposure time rather than concentration.

Figure 4. Median lethal dose 50 ($LD_{50}$) for human cell lines following exposure to various concentrations of TTO in vitro. Multiple linear regression analysis was used to determine whether TTO concentration or exposure time (independent variables) best predicted loss of cell viability in the four human cell lines. Note that TTO concentration rather than exposure time is a positive predictor for reduction of cell viability.

At least 90% of keratinocytes and osteoblasts remained viable at TTO concentrations of 0.1%, and toxicity increased with concentration, such that approximately 50% of cells remained viable at concentrations of 1%–5% v/v TTO. Approximately 90% of HUVECs remained viable at 0.1% v/v TTO, and greater than 50% of cells remained viable with 1%–10% v/v TTO incubated for up to 30 min. Median lethal dose was 13.4% TTO for HUVECs, which was the highest $LD_{50}$ among the cell lines tested. Combined statistical analysis of all cell lines (200 observations) demonstrated that concentration predominated over exposure time ($P < 0.0001$) as the predictor of toxicity with $LD_{50}$ of 9.9% TTO for all cell lines combined (Fig. 4). Results of individual cell lines are available in the supplementary material.

Figure 5. In vitro evaluation of the effect of TTO, 0.1%–100% (v/v), on the viability of porcine tissue explants following exposure for up to 24 h. Tissue viability is reported as the viability index relative to the control, untreated groups at each time point.

Ex Vivo Tissue Viability

Although TTO was observed to have significant cellular toxicity at concentrations $\geq 10\%$ on the individual cell lines, whole tissues exposed to TTO were capable of tolerating much higher concentrations (Fig. 5). Cell viability was preserved in the ex vivo model compared with the in vitro results, but exposure to concentrations of 50% TTO or higher demonstrated toxicity, with greater cytotoxicity at exposure times $\geq 60$ min. TTO concentrations of $\leq 25\%$ were less toxic, even with prolonged exposure times, with cell viability of 81% relative to control for tissue incubated with 25% TTO for 24 h. Tissue incubated for 24 h with any tested concentration of TTO showed some loss of viability; results ranged from 79% to 84% relative to control for 0.1%–25% TTO, and cell viability decreased to 34% relative to control at 50% TTO.

Discussion

The morbidity and mortality associated with IFIs from combat wounds and other trauma necessitates a continued search for improved therapeutic options. The current recommendation for management of these infections is surgical debridement, but adjunctive systemic and topical antifungal therapy could be especially advantageous for cases involving infections not easily debrided or amputated and for residual infection that is not detected on gross tissue inspection during surgery. The purpose of this study was to explore the antifungal properties and biocompatibility of TTO as a potential topical treatment option for IFIs. To that end, filamentous fungi known to be etiologic agents of IFIs and cell lines relevant to wound healing were tested to determine if TTO showed promise as an effective antifungal agent with a tolerable cytotoxicity profile. Our findings demonstrate that TTO does have antifungal activity against
most of the isolates tested at concentrations that may adversely affect human cells at the individual level, but that are tolerated by whole tissues, extrapolating from \textit{in vitro} and \textit{ex vivo} results. Furthermore, the percent of cell death associated with 1\% TTO \textit{in vitro} is similar to the toxicity seen with 0.00025\% Dakin solution \textit{in vitro}, which is in current clinical use [4]. Cells \textit{in vitro} may be more susceptible to toxicity due to the absence of a basement membrane as supported by our tissue explant results. We noted a contrast in the \textit{ex vivo} and \textit{in vitro} results, with tissue explants showing preserved cell viability compared with cell lines under the same conditions.

The antifungal activity of TTO against a diverse panel of fungal clinical isolates observed in our study, are in contrast to previous studies indicating that TTO is broadly efficacious against fungi although comprehensive studies had not been done with filamentous fungi [17]. Relevant investigations that support our findings have been published as broth microdilution studies of seven different filamentous fungi to determine minimum inhibitory (MIC) and minimum fungicidal concentrations (MFC), as well as time-kill studies with \textit{A. niger} and \textit{A. fumigatus} [14]. The authors found MIC and MFC values were higher with non-dermatophyte filamentous fungi, and \textit{A. niger} had the highest MFCs of all tested isolates. Time-kill studies were done with one concentration, the MFC, for each organism [25] which revealed that \textit{Aspergillus niger} tested with 4\% TTO showed a 2.5 log decline in relative viable count at 8 h [25]. In contrast, \textit{Aspergillus fumigatus} tested with 2\% TTO showed less than a 1.5 log decline in relative viable count in 8 h [25]. Per the authors, no organisms were detected after 24 h of exposure [25], indicating time-dependent fungicidal activity of TTO.

Fibroblast viability after exposure to a range of TTO from 0.125\% to 1\% for 24 h has been examined, and no significant effect was found [29]. This remains consistent with our results as we observed decreased cell viability of 78.5\% with concentrations of 1\% TTO when results from all cells were combined. Furthermore, another study found that TTO was mildly cytotoxic in human epithelial cells and fibroblasts at lower concentrations, but there was marked cytotoxicity at concentrations higher than 100 \(\mu\)g/ml [30]. Interestingly, no time-dependent increase in cytotoxicity was seen with exposure time \(>1\) h [30]; but in our study cell viability continued to decline with longer exposure times, although the time factor was greatly outweighed by the effect of concentration.

Prior research in burns has shown similar cytotoxicity results in human fibroblasts and basal keratinocytes tested with topical agents [31]. Penetration of TTO through intact human epidermal skin under occlusion in an \textit{in vitro} experiment has been shown to be approximately 7\% of the applied concentration of TTO [22]. A randomized, controlled trial tested the use of 10\% TTO cream versus 1\% silver sulfadiazine cream for eradication of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) carriage in wounds and leg ulcers [32]. The authors concluded that TTO was more effective than standard therapy at “superficial body sites including open skin lesions” and no adverse effects were reported [32]. A case series of 10 patients with diabetic foot ulcers treated with debridement followed by a topical, water-based melaleuca oil preparation (Megabac, Nicrosol Laboratories, Brisbane, Australia) and skin grafting has resulted in healing in all cases [33]. A more recent study of 10 patients with skin abscesses/cellulitis due to \textit{Staphylococcus aureus} found improved wound healing when dressings were treated with drops of pure TTO as compared to standard therapy [34]. TTO impregnated dressing (Burnaid, Rye Pharmaceuticals, Roseville, NSW, Australia) has been studied in partial thickness burns in a porcine model [35]. Burns treated with TTO impregnated dressing had no decrease in time to reepithelialization and had scars that were significantly larger than controls \((P = 0.02) [35]. There is no consensus in the literature on the effect of TTO in open wounds, and there are few \textit{in vivo} controlled studies on this topic [23]. Based on our findings in this study, cytotoxicity is still a possible limiting factor for clinical use of TTO in nonintact skin, especially when the viability of cell lines is important for wound healing such as in IFI cases in which wide debridement with large skin and tissue defects are expected. However, the results from our \textit{ex vivo} analysis utilizing whole tissues and the clinical experience published in the literature suggest that \textit{in vitro} experiments may overestimate \textit{in vivo} cytotoxicity. Future studies, employing the use of \textit{in vivo} models may be required to fully evaluate the safety of topical TTO for use on nonintact skin.

Our study was limited by the known variation in the oil composition of TTO. Only 15 of approximately 100 components in TTO are regulated by the ISO [6]. Our product was purchased from a reputable chemical reagent manufacturer (Sigma-Aldrich), sourced from Australia, and met manufacturer’s standards according to the certificate of analysis. Many authors have described the incomplete solubilization of TTO in water [7,25] causing the use of the solvent DMSO to enhance the miscibility of TTO and mitigate the turbidity of the TTO suspension in water. However, this could have changed the properties of the TTO or have had an effect on the cells or fungi. The surfactant Tween was the most common agent used for this purpose in previous studies [7,25]. However, our fungicidal experiments included controls exposed to DMSO which demonstrated statistical equivalence to water at each time point \((P = 0.25—0.95\) by Wilcoxon rank-sum test). Additionally, the most active antimicrobial and largest constituent of TTO,
terpinen-4-ol, is sparingly water soluble [36], so the solubility of pure TTO in water may have less relevance than previously thought.

TTO isolation and storage conditions can affect its components as oxidation of oil is accelerated by exposure to ambient air and sunlight, resulting in decreased terpinene concentrations and increasing concentrations of p-cymene, a degradation product [5]. However, TTO properly stored in cool, dark, dry conditions can be expected to retain its original composition for 10 years or more [5]. The oil procured for this study specified an expiration date period of 5 years beyond the time of our experiments. Cell viability studies were limited by in vitro testing. Though in vitro results provide important insights, it is impossible to extrapolate the clinical relevance of these results to a living organism with certainty.

The potential benefits of topical antimicrobial therapy include direct delivery to the site of infection, bedside application without the need for an operating room, general anesthesia, or surgeon, and avoidance of systemic toxicities [37]. Much remains unproven regarding the efficacy and safety of these agents including those that have been in use for decades. It was recently suggested that lower concentrations of Dakin’s solution than typically used in clinical practice may optimize the balance of effectiveness and toxicity of this agent [5]. Gentian violet and ciclopirox have been used topically for adjunctive treatment in primary cutaneous aspergillosis in infants [38]. Medical grade honey has been reported to have antimicrobial effects, and has been applied topically in burns, but its activity against fungi is unknown [37]. Perhaps most intriguing is the use of topical voriconazole, which has been widely reported in the treatment of ocular fungal infections [39–41] as has the use of topical caspofungin [42]. Furthermore, a 1% topical voriconazole solution prepared by diluting the intravenous formulation and applied twice daily with wet to dry dressing changes has been described in the successful treatment of cutaneous aspergillosis in a bone marrow transplant patient [43]. This patient had an A. flavus infection of the lower extremity thought to represent primary invasive disease, and had failed standard surgical and systemic treatment with amphotericin B and caspofungin [43]. Other case reports describe successful therapy of cutaneous Scedosporium apiospermum infection: one with intravenous voriconazole and surgical debridement, and the other with topical voriconazole monotherapy [44,45].

In this study we evaluated the activity of TTO against a set of filamentous fungal agents associated with IFIs and observed variable fungicidal activity. We also evaluated the viability of cell lines important for wound healing with various concentrations of TTO over time and found that decreased cell viability occurred with all concentrations of TTO, but that cytotoxicity was less pronounced in tissue explants versus cultured cells. Notably, as Dakin’s solution at 0.00025% has also been shown to have comparable in vitro cytotoxicity similar to that observed for TTO, use as a topical agent for IFI is conceivable, given the current usage of Dakin’s solution. However, based on these studies, it is not clear what additional benefit would be derived from TTO over currently employed topical therapy [4]. Notable exceptions may be Fusarium sp. and Exophiala sp., which were exquisitely susceptible. Further studies are needed to define an optimal topical agent active against a broad range of the most common etiologic agents of IFI for use in empiric therapy as culture and species identification are often delayed and these infections have poor prognoses.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary material is available at Medical Mycology online (http://www.mmy.oxfordjournals.org/).

References


