Safety and efficacy of 4-terpineol against microorganisms associated with blepharitis and common ocular diseases

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ABSTRACT
Objective Microbial infection has been reported to cause blepharitis, conjunctivitis and keratitis. We evaluated the safety and efficacy of a foam formulation of 2% 4-terpineol (T4O) against common ocular microorganisms.
Material and methods The antimicrobial effect of a 2% T4O formulation was evaluated by the United States Pharmacopeia 51 (USP <51>) antimicrobial effectiveness test for 14 and 28 days, as well as by a Time Kill Study (ASTM E2315) with a 60 s exposure time. Its potential of causing skin and ocular irritation was evaluated by the Repeated Insult Patch Test and the Hen’s Egg Chorioallantoic Membrane Test, respectively.
Results and discussion It was seen that 2% T4O formulation did not cause ocular irritation, skin irritation, sensitisation or allergic contact dermatitis in human subjects. Most importantly, it killed microorganisms listed in USP <51> at both 14 and 28 days and exerted a rapid killing effect within 60 s against 13 bacteria, 1 fungus and Acanthamoeba castellanii.
Conclusion The above finding suggests that 2% T4O formulation is safe and effective in killing microorganisms related to common ocular and skin infective diseases.
Translational relevance Although the clinical efficacy in treating ocular disease was not directly studied, this foam formulation containing 2% T4O, based on the in vitro results of this work, demonstrated that it can potentially be used as a preservative-free cleansing agent for ocular hygiene maintenance due to its ability to exert a broad-spectrum antimicrobial effect without causing ocular or skin irritation.

INTRODUCTION
The eye is continuously exposed to the external environment and is therefore highly susceptible to a multitude of pathogens. The eyelid margin is a particularly favourable environment for the colonisation of pathogens due to the protection of eyelashes and associated adnexal glands. Overproliferation of pathogens in this locale can cause two of the most common eye diseases, that is, blepharitis and meibomitis of the eyelids, which are prevalent eye diseases that constitute at least 37% and 47% of patients seen in clinical practices of ophthalmologists and optometrists, respectively,1 and are commonly associated with Staphylococcus aureus, Propionibacterium acnes, Candida spp, Penicillium spp and Moraxella catarrhalis.6,7 In fact, these microorganisms are isolated in approximately 50% of the swabs taken from the conjunctiva and tears, and >50% from the eyelids.5 In addition, fungi and Demodex mites are also found respectively in 79% and 42% of patients with blepharitis.4 Due to their prominence, these microorganisms and pathogens may also invade the ocular surface to cause conjunctivitis, keratitis, and even sight-threatening endophthalmitis.6,7 Topical antibiotics or steroids may be used to address the aforementioned diseases; however, these methods may facilitate biofilm formation as well as lead to the emergence of resistant bacterial strains. In addition, the long-term use of topical steroids has been associated with the risk of elevated intraocular pressures and cataract formation.5 Due to these short comings, ocular hygiene of

Key messages
► Many studies have previously suggested a possible role for the topical application of tea tree oil (TTO) as an antiseptic; however, despite years of use, irritation remains as an issue when TTO is applied to human skin. As we recently demonstrated that 4-terpineol (T4O), the major component of TTO is effective in killing Demodex Mite, we expanded the study and demonstrated that:
  – The formulation of 2% T4O is safe. It does not cause ocular irritation, skin irritation, sensitisation, or allergic contact dermatitis
  – The formulation of 2% T4O is effective in rapidly killing microorganisms associated with ocular diseases. Particularly, it can also kill Acanthamoeba castellanii which is responsible for difficult to treat cases of infectious keratitis.
► Based on the results of this work, we demonstrated that T4O at low concentration can potentially be used in a formulation for ocular hygiene maintenance due to its ability to exert a broad-spectrum antimicrobial effect without causing ocular or skin irritation.
the eyelids is another preferred measure for reducing microbial colonisation. In this regard, an ocular hygiene agent containing 4-terpineol (T4O), a major component purified from a naturally occurring essential oil of tea tree oil, can be an attractive option since it has been shown to be effective at concentrations between 0.125% and 8% against various microorganisms responsible for infections, such as *S. aureus*, *Pseudomonas aeruginosa* and coagulase-negative staphylococci (CoNS). Moreover, T4O has also been shown to exert an anti-fungal effect against fungi such as *Candida spp*, *Saccharomyces cerevisiae*, *Trichophyton rubrum* and *Penicillium spp* at concentrations of 0.125% to 0.5%. Recently, we have also reported that T4O at a concentration as low as 1% is effective in exerting a miticidal effect against *Demodex* mites, which play a role in blepharitis, unexplained keratitis, superficial corneal vascularity, marginal infiltration, phlyctenule-like lesions, nodular scarring and rosacea. In addition to its antimicrobial properties, T4O also possesses anti-inflammatory properties by suppressing superoxide production and proinflammatory cytokines. This overwhelming therapeutic potential prompted us to develop a formulation of 2% T4O for ocular hygiene and evaluate its safety and efficacy.

**MATERIAL AND METHODS**

**Preparation of 2% T4O formulation**

T4O was obtained from Takasago (CAS: 562-74-3) and contract-prepared by Formulated Solutions (Largo, Florida, USA) to a final concentration of 2% as a foam formulation and stored in a 35×95 mm closed aerosol canister (CCL Container, Hermitage, Pennsylvania, USA) by adding the following excipients: 90.8% water, 2% cocamidopropyl betaine (Glenn Corporation, Lake Elmo, Minnesota, USA), 2% glycercin, 1% caprylic/capric triglyceride, 1% butylene glycol, 0.2% allantoin, 0.75% polysorbate 20% and 0.25% sorbitan oleate (all from Univar USA, Redmond, Washington, USA).

**USP <51> antimicrobial effectiveness test**

The antimicrobial efficacy of 2% T4O and a control with no T4O (blank) were evaluated against microorganisms listed in Chapter 51 of the United States Pharmacopeia (USP <51>) by Alcamo Coporation (Wilmington, North Carolina, USA). The control and 2% T4O formulation were separately evaluated following the same procedures/techniques. Briefly, five standard microorganisms, that is, *Escherichia coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027), *S. aureus* (ATCC 6538), *Candida albicans* (ATCC 10231) and *Aspergillus brasiliensis* (ATCC 16404), were inoculated separately and grown at 30°C–35°C on Soybean-Casein Digest Agar, while yeast and mould were grown at 20°C–25°C on Sabouraud Dextrose Agar. On culturing completion, the above-mentioned microorganisms were then added to 0.5%–1% of the volume of 2% T4O at respective concentrations of 3×10^3, 2×10^3, 1.4×10^3, 4.1×10^2 and 3.9×10^1 CFU/mL (colony-forming unit/microlitre). Subsequently, microorganism reduction was evaluated at days 14 and 28 by calculating the log 2.0 reduction rate. Acceptance for bacterial species is based on a not <2.0 log reduction at 14 days compared with baseline, and no increase observed from 14 to 28 days. For yeast and moulds, the acceptance criteria are based on no increase at 14 and 28 days compared with baseline.

**Rapid time kill study**

The time kill study was conducted via ASTM E2315 standards by Accugen Laboratories (Willowbrook, Illinois, USA) against various microorganisms. Beforehand, purity of all microorganisms was assured by confirming microorganisms’ characteristics by Gram stain and colony morphology. Microorganisms were then incubated at 35°C–37°C aerobically with 5% CO2 for aerobic bacteria, at 35°C–37°C under anaerobic conditions for anaerobic bacteria, and at 25°C–28°C for yeast and mould. Appropriate agar, sterile deionised water and phosphate buffer were used to support the growth of each microorganism. Once the microbial population reached at least 10^7 CFU/mL, 0.5 mL of the inoculum suspension was added to 10 mL of 2% T4O or 10 mL of sterile phosphate buffer as the control. Tubes were then vortexed thoroughly to mix the organisms and placed at ambient temperature (21°C) for 60s. The inoculum suspensions were enumerated by using pour plate method and colonies were counted to calculate the concentration of viable cells. We then transformed the measured initial and final populations, inoculum suspension and test recoveries to log10 reduction scale as such: Log10 reduction (LR)=mean log10 (microbial population) – mean log10 (surviving test population). Percent reduction (%)=100× (1 – LR). Surviving organisms were identified by gram stain.

**Repeated Insult Patch Test (RIPT) for skin irritation**

RIPT was conducted in accordance with the intent and purpose of Good Clinical Practice described in Title 212 of the US Code of Federal Regulations by Essex Testing Clinic (Verona, New Jersey). Following obtaining the informed consent from 58 subjects, the procedure was carried out in two stages. The first stage was the induction phase, in which a 4cm² square of cotton fabric patch moistened by 0.2 mL of 2% T4O was applied to the back of each subject between the scapulae and the waist for 24 hours. This application was repeated every Monday, Wednesday and Friday until a total of 9 applications was completed. The site was scored prior to the next patch application. On completion of the induction phase, with a rest period of 2 weeks without any applications, the second stage, that is, the challenge phase was conducted by applying the same patch to a previously unpatched test site for 24 and 72 hours. All subjects were instructed to report any delayed skin reactivity that occurred after the final challenge patch reading. Dermal responses for both the induction and challenge phases of the study were scored according to the following 6-point scale: 0=no evidence of any
effect, + = barely perceptible (minimal, faint, uniform or spotty erythema), 1 = mild (pink, uniform erythema covering most of the contact site), 2 = moderate (pink-red erythema uniform in the entire contact site), 3 = marked (bright red erythema with/without petechiae or papules) and 4 = severe (deep red erythema with/without vesiculation or weeping).

**Hen’s Egg Chorioallantoic Membrane test for acute ocular irritation**

The ocular irritation evaluation was performed by MB Research Laboratories (Spinnerstown, Pennsylvania, USA) per Protocol No. 47: Hen’s Egg Test Chorioallantoic Membrane (HET-CAM) test (Invitotox 1992) recommended by ICCVAM as an alternative to the Draize eye irritation evaluation in rabbits. In this test, 0.9% saline (Hospira, Lake Forest, Illinois, USA) was used as vehicle control and two positive controls were selected per the aforementioned HET-CAM protocol: 0.1 N NaOH and 1% (W/V) sodium dodecyl sulfate (SDS, Fisher, Waltham, Maryland, USA) in distilled water. The test substance was prepared by adding 1 mL 2% T4O with 9 mL of 0.9% saline. Hen eggs were incubated at 32–37°C for 10 days. After this period, they were each inspected to determine the viability of the embryo and were examined for any abnormalities prior to the test. Subsequently, 300 µL of the test solutions and the vehicle control, that is, 0.9% saline, was pipetted onto the chorioallantoic membrane of the hen’s egg (n = 6 for each group). Irritation potential was classified by a scheme which depended on two components. The first was the calculated irritation score (IS). The IS was based on the time until adverse reactions (haemorrhage, vessel lysis and coagulation) were first observed. In this experiment, the eggs were observed continuously for 5 min for the appearance of lysis (L), haemorrhage (H) and/or coagulation (C) to determine the IS using the formula given below. The time for each reaction to occur was recorded in seconds (sec) and the degree of severity of each reaction (L, H, C) was graded as 0 = no reaction, 1 = slight reaction, 2 = moderate reaction and 3 = severe reaction.

\[
IS = \left(\frac{300 - secL}{300}\right) \times 5 + \left(\frac{300 - secH}{300}\right) \times 7 + \left(\frac{300 - secC}{300}\right) \times 9
\]

The second component of irritation potential was a determination of the severity (slight, moderate or severe) of adverse reactions after 1 and 5 min. The irritation threshold (TH) was defined as the lowest concentration at which slight reactions occur.

**Statistical analysis**

Data were reported as means ± SD and analysed with using SPSS software, V.24.0. The data between groups were evaluated for statistical significance using Student’s t-test and results were reported as p values, where p < 0.05 were considered statistically significant.

**RESULTS**

2% T4O formulation exerts a broad-spectrum antimicrobial effects on USP <51> microorganisms

Although the control and the 2% T4O formulation were evaluated separately by 12 months, the same procedures were followed without deviation; therefore, the two results are comparable. The control with no T4O did not exert any inhibitory effects on these microorganisms, while the CFU/mL was <100 for *E. coli*, *C. albicans*, *P. aeruginosa*, *S. aureus* and 1.0 × 10³ CFU/mL for *A. brasiliensis* after exposure to 2% T4O for 14 days (table 1). Their corresponding log reduction at day 14 were >3.6, >3.6, >3.4, >3.1 and 2.6, respectively, indicating that 2% T4O formulation was effective in killing these microorganisms. At day 28, the CFU/mL of all species including *A. brasiliensis* was <100, confirming that there was no growth increase from day 14 to 28. Collectively, these data indicated that 2% T4O formulation was effective in killing followed by inhibiting the microbial growth. Consequently, these results also support the notion that 2% T4O alone could establish and maintain sterility of this foam formulation so that no additional preservatives were required to be used. This finding has direct clinical importance, for example, to avoid preservative-induced ocular toxicity.

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>ATCC#</th>
<th>Initial concentration (CFU/mL)</th>
<th>Time point</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>14 day</td>
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<tr>
<td></td>
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<td><em>Escherichia coli</em></td>
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<td><em>Pseudomonas aeruginosa</em></td>
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<td>2.3 × 10⁵</td>
<td>&lt;100</td>
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<td><em>Staphylococcus aureus</em></td>
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<td>1.4 × 10⁶</td>
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<td><em>Candida albicans</em></td>
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<td>4.1 × 10⁵</td>
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<tr>
<td><em>Aspergillus brasiliensis</em></td>
<td>16404</td>
<td>3.9 × 10⁵</td>
<td>1.0 × 10³</td>
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</table>

2% T4O formulation exerts a broad-spectrum antimicrobial effect on microorganisms related to common eye diseases within 60 s. ATCC, American Type Culture Collection; CFU, colony-forming unit; USP, United States Pharmacopeia.
an antimicrobial effect against other microorganisms related to ocular/skin infections (as listed in Table 2) including 15 bacterial species (8 Gram-negative, 7 Gram-positive), 2 fungi (Trichophyton interdigitale and A. brasiliensis) and Acanthamoeba castellani. Within 60 s of exposure to 2% T4O formulation, Acinetobacter baumanii, Clostridium prefringens, Haemophilus influ-


<table>
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<tr>
<th>Microorganism</th>
<th>ATCC#</th>
<th>Avg. CFU/mL Control</th>
<th>Avg. CFU/mL Post 60 s exposure to 2% T4O</th>
<th>Log10 reduction</th>
<th>Reduction (%)</th>
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<tr>
<td>Acinetobacter baumanii</td>
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<td>7.8×10^4</td>
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<td>Serratia marcescens</td>
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<td>Aspergillus brasiliensis</td>
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<td>1.8×10^5</td>
<td>2.0×10^4</td>
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</table>

2% T4O is safe and does not cause skin and eye irritation.
ATCC, American Type Culture Collection; CFU, colony-forming unit.

The IS was 0.0 for all 55 subjects, indicating that 2% T4O did not cause any skin irritation in human volunteers.

The ocular irritation was evaluated by the HET-CAM test. The results showed the appearance of lysis and haemorrhage in the positive controls, that is, 1% (w/v) SDS and 0.1N NaOH, and the appearance of coagulation in the 0.1N NaOH positive control. The mean calculated IS was 10.4±0.17 for 1% SDS and 16.8±0.18 for 0.1N NaOH. In contrast, the score for 2% T4O was 0.0 and threshold concentration was found to be >10%, which was the same as the vehicle control, that is, 0.9% saline. These data show that the irritation potential of 2% T4O is comparable to 0.9% saline and significantly <1% SDS (p<0.001) and 0.1N NaOH (p<0.001).
Effective ocular hygiene will not only reduce microbial colonisation in the skin and lid margin but also prevent spreading microbes to the ocular surface causing conjunctivitis, keratitis and even sight-threatening endophthalmitis. In this regard, the foam formulation of 2% T4O can be an effective and viable option to achieve this objective as it demonstrated cidal activity against all microorganisms listed in USP <51> at both 14 and 28 days and exerted a rapid killing effect within 60 s against a number of Gram-positive and Gram-negative ocular isolates, such as *A. baumannii* that causes endophthalmitis infection, *Propionibacterium acnes* that causes chronic blepharitis, endophthalmitis, Meibomian gland dysfunction (MGD) and dry eye.

We thus expanded our analysis to test the effectiveness of 2% T4O against additional microorganisms including *S. hominis* and *S. haemolyticus* where their CFU/mL were reduced by 58% and 29%, respectively, within 60 s. Lastly and most importantly, our data demonstrated that *A. castellanii* which is one of the most common causes of contact lens-related infectious keratitis has been previously shown to be killed by both 5% TTO and T4O.

We therefore expanded our study to test the effectiveness of 2% T4O against additional microorganisms including *S. hominis* and *S. haemolyticus* where their CFU/mL were reduced by 58% and 29%, respectively, within 60 s. Lastly and most importantly, our data demonstrated that *A. castellanii* which is one of the most common causes of contact lens-related infectious keratitis has been previously shown to be killed by both 5% TTO and T4O. These results, together with our recent report that T4O is also effective in killing *Demodex* mites and suggesting 2% T4O has a broad antimicrobial spectrum against eyelid-associated bacteria, fungi, amoeba and parasites. In addition, while we understand future studies will be needed to further determine antimicrobial resistance for those surviving microbes, 2% T4O does also achieved killing >99% of all microorganisms listed in USP <51> indicating that such a foam formulation does not require the addition of other preservatives such as benzalkonium chloride which has been shown to potentially cause ocular surface toxicity. On the contrary, although clinical efficacy of 2% T4O in treating ocular disease was not directly studied and the formulation was not directly tested on eyelid skin and the eyelid margin, 2% T4O formulation does exert antimicrobial activity against various microbes and it is unlikely to cause skin or ocular irritation as demonstrated by RRIPT and Het-Cam test. These benefits are similar to the other researchers’ finding that T4O also exerts anti-inflammatory actions by selectively regulating cell function, in particular monocyte activity, and downregulating immune responses to foreign antigens in the skin. Collectively, we believe a foam formulation with 2% T4O can be used as an effective measure for ocular hygiene against potential microbial colonisation.

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