Terpinen-4-ol is the Most Active Ingredient of Tea Tree Oil to Kill Demodex Mites

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Purpose: To determine the active ingredient in tea tree oil (TTO) responsible for its reported killing effect on Demodex mites, the most common ectoparasite found in the human skin extending to the eye.

Methods: Using a reported in vitro killing assay to measure the survival time of adult Demodex folliculorum up to 150 minutes, we have screened serial concentrations of 13 of the 15 known ingredients of TTO (ISO4730:2004) that were soluble in mineral oil and examined their synergistic relationships in killing mites. The most potent ingredient was then tested for its efficacy in killing Demodex in vivo.

Results: All ingredients exhibited a dose-dependent killing effect. Besides Terpinen-4-ol, the order of relative potency did not correlate with the order of relative abundance in TTO for the remaining 12 ingredients. Terpinen-4-ol was the most potent ingredient followed by α-Terpineol, 1,8-Cineole and Sabinene. Terpinen-4-ol, the most abundant ingredient in TTO, was more potent than TTO at equivalent concentrations and its killing effect was even observable at a mere concentration of 1%. Terpinen-4-ol exhibited a significant synergistic effect with Terpinolene, but an antagonistic effect with α-Terpineol in killing mites (both P < 0.05). In vivo, Terpinen-4-ol was shown to eradicate mites.

Conclusions: The above finding suggests that deployment of Terpinen-4-ol alone should enhance its potency in killing Demodex mites by reducing the adverse and antagonistic effects from other ingredients in TTO.

Translational Relevance: Terpinen-4-ol can be adopted in future formulations of acaricides to treat a number of ocular and cutaneous diseases caused by demodicosis.
for wounds and cutaneous infections. Its natural composition is well-characterized by approximately 100 constituents,\textsuperscript{27} but the commercial TTO is regulated by the International Organization of Standardization (ISO4730:2004)\textsuperscript{28} and must contain a specific range of 15 major ingredients as shown in Table 1. Previously, it has been reported that lid scrub with different concentrations of TTO is effective in reducing Demodex mite counts and ocular surface inflammation associated with blepharitis, conjunctivitis, and keratitis.\textsuperscript{20–25,29–33} Although lid scrub with 50\% TTO is effective in killing mites and improving patients’ signs and symptoms, it can cause ocular irritation.\textsuperscript{22,29,34} Thus, it is important to eliminate unwanted chemical ingredients in TTO, and/or reduce the effective concentration of TTO in order to reduce ocular irritation and promote overall safety. As a first step toward this objective, we sought to identify the most active pharmaceutical ingredient in TTO using an in vitro killing assay.

### Materials and Methods

#### Materials

TTO was obtained from the Essential Oil Company (Portland, OR). All TTO ingredients listed from Table 1 were purchased from Sigma-Aldrich (St. Louis, MO) except for $\delta$-Cadinene and Viridiflorol, which were purchased from ISCA Technologies (Riverside, CA). Aseptically, each ingredient was diluted with mineral oil (Sigma-Aldrich) in 7.4-mL amber vials (Fisher-Scientific, Pittsburgh, PA) to the required concentrations of 100\%, 50\%, 25\%, 10\%, 5\%, 2.5\%, and 1\%. The vials were then sealed after being vortexed for 1 minute. In order to study the potential synergistic effect, 50/50 mixtures of Terpinen-4-ol, $\alpha$-Terpineol, Terpinolene, and/or $\gamma$-Terpinene were prepared in 50-mL conical tubes (BD Biosystems, Franklin Lakes, NJ), vortexed for 1 minute each to ensure adequate mixing, and then stored in 7.4-mL amber vials.

#### In Vitro Killing Assay

We employed the same in vitro killing assay to evaluate the above solutions as previously reported,\textsuperscript{34} following the tenets of the Declaration of Helsinki. After the patient had consented with the microscopic diagnosis, epilated eyelashes containing cylindrical dandruff were immediately evaluated under a light microscope using 40$\times$ and 100$\times$ magnifications to detect and count Demodex adult mites, larvae, and eggs. Movements of the legs were used to judge whether mites were alive or dead. Following addition of various solutions, each live adult mite (excluding eggs and larvae, as they are more vulnerable to killing) was monitored for up to 150 minutes. The survival time when the mite ceased any movement was recorded for comparison.

#### In Vivo Killing Assay

A patient with ocular demodicosis had consented and was instructed to use Cliradex (Bio-Tissue, Doral, FL), a lid cleanser containing simply water and Terpinen-4-ol (T40) with a few minor ingredients to help solubilize the T4O. To apply the lid cleanser, the patient would close their eyes and cleanse the eyelid with lateral side to side motions. The skin around the eye was then allowed to air dry for 1 minute before the opening of the eyes. This procedure was practiced twice a day as recommended. Demodex counts from eight epilated eyelashes per visit were recorded to monitor the lid cleanser’s killing effect.

#### Statistical Analysis

All experiments were performed with a sample size of six unless otherwise specified. Data were reported...

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**Table 1. Chromatographic Profile of Tea Tree Oil (From ISO 4730:2004)**

<table>
<thead>
<tr>
<th>Order of Abundance</th>
<th>Components</th>
<th>Maximum %</th>
<th>Minimum %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Terpinen-4-ol</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>$\gamma$-Terpinene</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>1,8-Cineole</td>
<td>15</td>
<td>Trace</td>
</tr>
<tr>
<td>4</td>
<td>$\alpha$-Terpinene</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>$\alpha$-Terpineol</td>
<td>8</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>$\rho$-Cymene</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>$\alpha$-Pinene</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>Terpinolene</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>9</td>
<td>Sabinene</td>
<td>3.5</td>
<td>Trace</td>
</tr>
<tr>
<td>10</td>
<td>$\delta$-Cadinene</td>
<td>3</td>
<td>Trace</td>
</tr>
<tr>
<td>11</td>
<td>Ledene (syn. viridiflorene)</td>
<td>3</td>
<td>Trace</td>
</tr>
<tr>
<td>12</td>
<td>Aromadendrene</td>
<td>3</td>
<td>Trace</td>
</tr>
<tr>
<td>13</td>
<td>Limonene</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>Globulol</td>
<td>1</td>
<td>Trace</td>
</tr>
<tr>
<td>15</td>
<td>Viridiflorol</td>
<td>1</td>
<td>Trace</td>
</tr>
</tbody>
</table>

The ISO 4730:2004 describes the concentration ranges of all 15 major ingredients, which are listed in the order of their relative abundance.
as means ± SD and analyzed with Microsoft Excel (Microsoft, Redmont, WA). The data between groups were evaluated for statistical significance using Student’s t-test and results were reported as P values, where P less than 0.05 were considered statistically significant.

Results

In Vitro Killing of Adult Mites by Individual TTO Ingredients

Previously, we have reported that TTO exhibits a dose-dependent killing effect on adult Demodex mites. To identify the active ingredients in TTO that are responsible for the above killing effect, we obtained all 15 major ingredients in TTO from their commercial sources. During the preparation of test solutions in serial concentrations, we noted that δ-Cadinene and Globulol were not able to be dissolved in mineral oil at any concentration. Because of their relative low abundance in TTO (i.e., ranked as #10 and #14 [Table 1]), we excluded them from the study. Out of the remaining 13 ingredients, we noted that Sabinene and Viridiflorol, which are ranked as #9 and #15 according to their relative abundance in TTO (Table 1), only dissolved at concentrations of 75% or less in mineral oil (Table 2).

Consistent with our previous report, the control (i.e., 100% mineral oil) did not have any killing effect when observed for 150 minutes. Compared with mineral oil, the remaining 13 ingredients exhibited a dose-dependent killing effect (Table 2). We subdivided them into “effective” (<150 minutes) and “ineffective” (>150 minutes) based on the average survival time achieved by the solution with concentrations of 25%. Terpinen-4-ol, which is ranked as #1 based on relative abundance, was found to be the most effective and was the only ingredient in TTO that remained effective at a concentration of 1% (Table 2). Following Terpinen-4-ol, those exhibiting effective killing were ranked as follows: α-Terpineol, 1,8-Cineole, Sabinene, Limonene, Terpinolene, and α-Terpineol, which were ranked as #5, #3, #9, #13, #8, and #14 [Table 1]).

Table 2. Survival Time of Adult Demodex Foliculorum When Exposed to TTO or Its Main Ingredients

<table>
<thead>
<tr>
<th>Order of Potency</th>
<th>Component Name</th>
<th>Survival Time (mean ± SD, min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td>1</td>
<td>Terpinen-4-ol</td>
<td>3.7  ± 0.8</td>
</tr>
<tr>
<td>2</td>
<td>α-Terpineol</td>
<td>3.6  ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>1,8-Cineole</td>
<td>3.8  ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>Sabinene</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>Limonene</td>
<td>13.2 ± 2.3</td>
</tr>
<tr>
<td>6</td>
<td>Terpinolene</td>
<td>4.8  ± 1.7</td>
</tr>
<tr>
<td>7</td>
<td>α-Terpinene</td>
<td>13.6 ± 4.4</td>
</tr>
<tr>
<td>8</td>
<td>Viridiflorol</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>γ-Terpinene</td>
<td>8.3  ± 6.2</td>
</tr>
<tr>
<td>10</td>
<td>β-Cymene</td>
<td>43.9 ± 25.2</td>
</tr>
<tr>
<td>11</td>
<td>Ledene</td>
<td>38.7 ± 12.8</td>
</tr>
<tr>
<td>12</td>
<td>Aromadendrene</td>
<td>60.3 ± 28.5</td>
</tr>
<tr>
<td>13</td>
<td>α-Pinene</td>
<td>78.1 ± 23.9</td>
</tr>
<tr>
<td></td>
<td>δ-Cadinene</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Globulol</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined because the ingredient had a survival time greater than 150 minutes, was not commercially available (δ-Cadinene and Globulol), or could not dissolve in mineral oil at that concentration (Sabinene and Viridiflorol). The order of 13 ingredients tested above is based on their relative effectiveness.

N = 6 unless otherwise stated.

* Values for TTO are taken from Gao et al. 2005.34
and #4, respectively, according to relative abundance in TTO. For the remaining six ingredients that were determined to be “ineffective” because they exhibited no killing effect at the concentration of 25%, their order of potency was determined by the average survival time at the concentrations of 50% and 75%, respectively. Their potency order was Viridiflorol, γ-Terpinene, ρ-Cymene, Ledene, Aromadendrene, and α-Pinene, which were ranked as #15, #2, #6, #11, #12, and #7, respectively, according to relative abundance in TTO (Table 2). These results concluded that Terpinen-4-ol was consistently ranked as #1 according to relative abundance in TTO and relative potency at the concentration of 25%. Except for Terpinen-4-ol, the order of relative potency of the remaining 12 ingredients did not match with that of relative abundance.

Interaction Between Ingredients

We then investigated whether there was any interaction between Terpinen-4-ol and other ingredients in TTO in the killing effect. This was achieved by comparing the average survival time achieved by Terpinen-4-ol alone at 100% compared with that achieved by an equal mixture of Terpinen-4-ol and the other ingredient. The survival times for T4O/Terpinolene and T4O/α-Terpineol were 2.2 ± 0.41 and 7.2 ± 0.79 minutes, respectively. Compared with the survival time shown in Table 2, Terpinen-4-ol exerted a positive synergistic effect with Terpinolene and an antagonistic effect with α-Terpineol in killing mites (both P < 0.05). Terpinen-4-ol did not exhibit any positive or negative effect with γ-Terpinene (4.3 ± 1.7 minutes, P > 0.05), which was determined to be ineffective.

In Vivo Killing Effect of T4O

To establish the clinical and therapeutic value of our findings, we examined whether T4O could eradicate mites in a 61-year-old female presenting with bilateral persistent ocular burning pain and scratchy eyelids for 1 year. She was previously diagnosed with chronic blepharitis and dry eye syndrome. Conventional treatment including topical artificial tears and serum eye drops, hot compresses, insertion of punctual plugs, and oral doxycycline had not shown any significant improvement. Clinical examination revealed the presence of cylindrical dandruff in many lash roots (Fig. 1A, arrow) suggestive of Demodex blepharitis. Microscopic
examination of a total of eight lashes, two epilated randomly from each eyelid, confirmed the presence of 
mites (Fig. 1B) and disclosed the total Demodex mite count of 22. After continuous use of Cliradex lid
leanser for 8 weeks, the patient noted marked resolution of symptoms. Repeated examination 
showed clearer lashes (Fig. 1C) and no mites (Fig. 1D).

Discussion

Among a total of 15 major ingredients in TTO, we have identified Terpinen-4-ol as the most potent (i.e.,
most active) ingredient based on the dose-dependent survival time measured by an in vitro killing assay
(Table 2). Terpinen-4-ol was the only ingredient that is ranked as #1 according to both relative abundance
and relative potency, suggesting that it is primarily responsible for TTO’s Demodex killing effect. Not
surprisingly, Terpinen-4-ol was the only ingredient that exhibited a killing effect at a concentration as low
as 1% in mineral oil. Furthermore, the potency of Terpinen-4-ol at a given concentration was greater
than TTO at an equivalent concentration. For example, 5% Terpinen-4-ol exerted an average survival
time of 32.1 ± 6.6 minutes similar to 34.7 ± 4.3 minutes of 25% TTO (P > 0.05, Table 2), which
should contain 7.5% to 12% Terpinen-4-ol based on the calculated range of abundance in TTO (Table 1).
Such an increasing potency by Terpinen-4-ol alone might be contributed by the exclusion of ingredients
that do not exert comparable potency. Indeed, the order of relative potency did not match with that of
relative abundance for all remaining 12 ingredients that could be dissolved in mineral oil. For example,
γ-Terpinene, which is ranked as #2 in relative abundance, was found to be “ineffective.” Moreover, the
potency of Terpinen-4-ol could be enhanced by a positive synergistic relationship with Terpinolene, but
could also be mitigated by an antagonistic relationship with α-Terpinene (both P < 0.05). One potential
mechanism to explain such an antagonistic relationship is a decreased solubility of T4O when mixed with
other ingredients.35 This finding is important for future selection of excipients to formulate T4O-
containing therapeutics.

Thus, it is highly plausible to promote the potency of killing Demodex mites by using Terpinen-4-ol alone
without other ingredients in TTO. When TTO was used for treatments as topical formulations to the
skin, allergic reactions were observed.36-44 These reactions have been linked to prolonged storage of
TTO and the oxidation products formed within which generate p-cymene (an ineffective ingredient) and
other compounds like peroxides, epoxides, and endoperoxides.45,46 These studies also suggested that
the most important allergens could be terpinolene, α-
terpinene, ascaridol and 1,2,4-trihydroxymethane, of
which the first two were “effective” in killing mites as
determined by our study. We, thus, strongly suspect
that elimination of other active and ineffective ingredients could reduce irritation and allergic reactions
that have been reported for TTO in previous studies.

In conclusion, our results showed that Terpinen-4-
ol is the most active ingredient in TTO in exerting Demodex mite-killing effects. This finding is parti-
cularly exciting, as other studies have shown that Terpinen-4-ol is also the most active ingredient in
TTO to exert antibacterial47–49 and antifungal50,51 effects. Such a wide range of antimicrobial effects of
Terpinen-4-ol have been shown against several microbes responsible for hospital-acquired infections
and ocular surface infections including Methicillin-
resistant Staphylococcus aureus (MRSA), Methicillin-
sensitive Staphylococcus aureus (MSSA), Coagulase-
negative Staphylococi (CoNS), and Pseudomonas
aeruginosa.47,52–56 Although the antimicrobial effect
of Terpinen-4-ol has been attributed to the compro-
mised cytoplasmic membrane,57 it remains unclear
how it exerts its miticidal effect. Additionally,
Terpinen-4-ol also possesses anti-inflammatory prop-
erties by suppressing superoxide production and pro-
inflammatory cytokines,58,59 evidenced by a notable
resolution of ocular irritation and inflammatory signs
and vision improvement in some patients presumably
due to reduction of corneal inflammation and neovascularization.20,29 Furthermore, the improve-
ment was coupled with a stable lipid tear film (verified
by tear interference images) presumably due to
improvement of meibum lipids in 6 of 22 eyes.29
Collectively, these data support the inclusion of
Terpinen-4-ol as the most active pharmaceutical
ingredient in future formulations to treat a number
of ocular and cutaneous diseases caused by demodi-
cosis that may be associated with or without
concomitant bacterial or fungal infections.

Acknowledgments

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