

## Article

# 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  $\alpha$ -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  $\alpha$ -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.

## Introduction

*Demodex* mites (*Demodex folliculorum* and *Demodex brevis*) are the most common ectoparasites infesting the pilosebaceous unit of the skin. Unbeknownst to many, *Demodex* prevalence increases with age and is observed in 84% of the population at age 60 and 100% of the population over the age of 70.<sup>1</sup> Uncontrolled skin *Demodex* infestation (demodicosis) has been implicated in several diseases in the skin, including rosacea, with papulopustular skin lesions and perifollicular inflammatory infiltrate.<sup>2–9</sup> Because the eye is surrounded by protruding body parts such as the nose, the brow, and the cheek, it is not as accessible to daily hygiene like the rest of the body. Therefore, once demodicosis occurs in the face, it will spread and flourish in the eyelids. It has been

estimated that demodicosis is the most common, but often overlooked, cause of 29% to 74% of eyes with chronic blepharitis,<sup>10–17</sup> which constitutes 37% and 47% of the patients seen in clinical practices of ophthalmologists and optometrists, respectively,<sup>18</sup> and has a high incidence in elderly populations.<sup>12,19</sup> We, and others, have also shown that ocular demodicosis, verified by microscopic examination and the counting of mites on epilated eye lashes, is strongly correlated with ocular surface inflammation associated with blepharitis, conjunctivitis, meibomian gland dysfunction, and even sight-threatening keratitis,<sup>20–25</sup> not to mention an overlooked risk factor of pterygium recurrence.<sup>26</sup>

Tea tree oil (TTO) is a natural essential oil steam-distilled from the Australian native plant *Melaleuca alternifolia* and has long been used by the Aborigines

for wounds and cutaneous infections. Its natural composition is well-characterized by approximately 100 constituents,<sup>27</sup> but the commercial TTO is regulated by the International Organization of Standardization (ISO4730:2004)<sup>28</sup> 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.<sup>20–25,29–33</sup> Although lid scrub with 50% TTO is effective in killing mites and improving patients' signs and symptoms, it can cause ocular irritation.<sup>22,29,34</sup> 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,<sup>34</sup> 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

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

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

The ISO 4730:2004 describes the concentration ranges of all 15 major ingredients, which are listed in the order of their relative abundance.

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 T40. 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

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

Order of Potency	Component Name	Survival Time (mean $\pm$ SD, min)						
		Concentration of Component						
		100%	50%	25%	10%	5%	2.5%	1%
1	Tea tree oil*	3.7 $\pm$ 0.8	14.8 $\pm$ 9.5	34.7 $\pm$ 4.3	>150		ND	
2	Terpinen-4-ol	3.6 $\pm$ 1.1	4.5 $\pm$ 1.0	8.3 $\pm$ 3.1	12.3 $\pm$ 8.8	32.1 $\pm$ 6.6	55.5 $\pm$ 10.2	87.6 $\pm$ 13.5
3	$\alpha$ -Terpineol	3.8 $\pm$ 0.8	12.5 $\pm$ 2.9	22.8 $\pm$ 3.9	43.4 $\pm$ 4.3	105.8 $\pm$ 9.7		ND
	1,8-Cineole	13.5 $\pm$ 2.0	18.8 $\pm$ 4.1	23.5 $\pm$ 3.9	44.4 $\pm$ 7.2	>150		ND
				(N = 5)				
4	Sabinene	ND	18.8 $\pm$ 1.3	38 $\pm$ 5.3	47.3 $\pm$ 10.5	52.5 $\pm$ 10.4	81 $\pm$ 19	>150
5	Limonene	13.2 $\pm$ 2.3	22.8 $\pm$ 1.3	55.2 $\pm$ 9.9	>150		ND	
			(N = 5)	(N = 5)				
6	Terpinolene	4.8 $\pm$ 1.7	31.0 $\pm$ 8.9	63.2 $\pm$ 16.3	>150		ND	
7	$\alpha$ -Terpinene	13.6 $\pm$ 4.4	21.0 $\pm$ 2.2	61.6 $\pm$ 11.6	>150		ND	
				(N = 5)				
8	Viridiflorol	ND	35.3 $\pm$ 15.9	>150			ND	
9	$\gamma$ -Terpinene	8.3 $\pm$ 6.2	75.9 $\pm$ 29.8	>150			ND	
10	$\rho$ -Cymene	43.9 $\pm$ 25.2	94 $\pm$ 7.4	>150			ND	
11	Ledene	38.7 $\pm$ 12.8	>150			ND		
12	Aromadendrene	60.3 $\pm$ 28.5	>150			ND		
13	$\alpha$ -Pinene	78.1 $\pm$ 23.9	>150			ND		
	$\delta$ -Cadinene				ND			
	Globulol				ND			

ND, not determined because the ingredient had a survival time greater than 150 minutes, was not commercially available ( $\delta$ -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.<sup>34</sup>

as means  $\pm$  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.

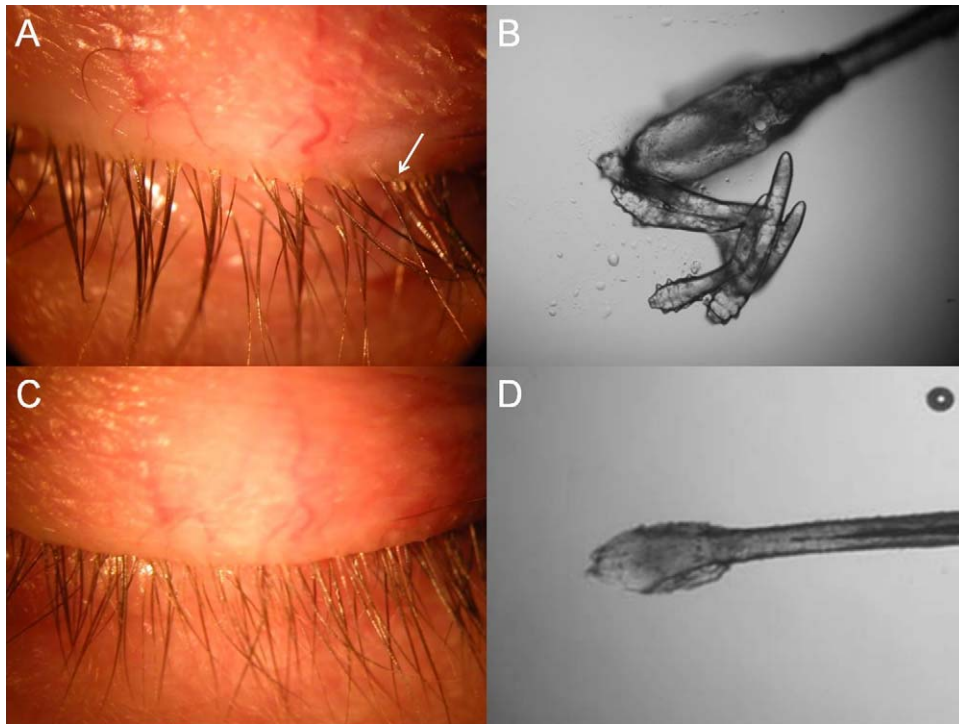
## Results

### 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.<sup>34</sup> 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  $\delta$ -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,<sup>34</sup> 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:  $\alpha$ -Terpineol, 1,8-Cineole, Sabinene, Limonene, Terpinolene, and  $\alpha$ -Terpinene, which were ranked as #5, #3, #9, #13, #8,



**Figure 1.** In vivo effect of Terpinen-4-ol on eradication of *Demodex* mites. Before treatment, cylindrical dandruff was found in many lashes (A, arrow) and mites were detected under microscopic examination of the epilated lash (B). After treatment with the T4O lid cleanser, the lashes were clean (C) and no mite was detected in the epilated lash (D).

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,  $\gamma$ -Terpinene,  $\rho$ -Cymene, Ledene, Aromadendrene, and  $\alpha$ -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/ $\alpha$ -Terpineol were  $2.2 \pm 0.41$  and  $7.2 \pm 0.79$  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  $\alpha$ -Terpineol in killing mites (both  $P < 0.05$ ). Terpinen-4-ol did not exhibit any positive or negative effect with  $\gamma$ -Terpinene ( $4.3 \pm 1.7$  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.<sup>20</sup> 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 cleanser 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  $32.1 \pm 6.6$  minutes similar to  $34.7 \pm 4.3$  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,  $\gamma$ -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  $\alpha$ -Terpineol (both  $P < 0.05$ ). One potential mechanism to explain such an antagonistic relationship is a decreased solubility of T4O when mixed with other ingredients.<sup>35</sup> 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.<sup>36-44</sup> 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.<sup>45,46</sup> These studies also suggested that the most important allergens could be terpinolene,  $\alpha$ -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 particularly exciting, as other studies have shown that Terpinen-4-ol is also the most active ingredient in TTO to exert antibacterial<sup>47-49</sup> and antifungal<sup>50,51</sup> 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 Staphylococci (CoNS), and *Pseudomonas aeruginosa*.<sup>47,52-56</sup> Although the antimicrobial effect of Terpinen-4-ol has been attributed to the compromised cytoplasmic membrane,<sup>57</sup> it remains unclear how it exerts its mitocidal effect. Additionally, Terpinen-4-ol also possesses anti-inflammatory properties by suppressing superoxide production and pro-inflammatory cytokines,<sup>58,59</sup> 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.<sup>20,29</sup> Furthermore, the improvement 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.<sup>29</sup> 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 demodicosis that may be associated with or without concomitant bacterial or fungal infections.

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