The Essential Oil of *Melaleuca alternifolia* (Tea Tree Oil) and Its Main Component, Terpinen-4-ol Protect Mice from Experimental Oral Candidiasis

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The therapeutic efficacy of tea tree oil (TTO), *Melaleuca alternifolia*, and its main component, terpinen-4-ol, were evaluated in a murine oral candidiasis model. Prednisolone-pretreated mice were orally infected with a fluconazole-susceptible (TIMM 2640) or a resistant (TIMM 3163) strain of *Candida albicans* to induce oral candidiasis. TTO or terpinen-4-ol was administered with a cotton swab 3 h and 24 h after *candida* infection. These treatments clearly showed a decrease in the symptom score of tongues and in the viable *candida* cell number in the oral cavity at 2 d after azole-susceptible *C. albicans* infection, although the degree of the efficacy was less than that of fluconazole. Even against oral candidiasis caused by azole-resistant *C. albicans*, TTO and terpinen-4-ol were similarly effective, while fluconazole appeared ineffective. These results suggest that TTO and terpinen-4-ol may have the potential of therapeutic ability for mucosal candidiasis which may also be applicable to *C. albicans* oral candidiasis induced by the azole-resistant strain.

**Key words** *Candida albicans*; essential oil; *Melaleuca alternifolia*

*Candida albicans* is a major cause of oral and esophageal infections in immunocompromised patients with hyposalivation, diabetes mellitus and prolonged use of antibiotics or immunosuppressive drugs.1,2) It is also now known that up to 90% of patients with human immunodeficiency virus infection or AIDS suffer from oropharyngeal candidiasis.3) Oral candidiasis can usually be managed by topical delivery of azole-type antifungal agents, but long-term use of antifungal azoles may result in azole-resistant strains.4–8) Therefore, the development of new types of antifungal agents other than azoles is needed.

One of the essential oils, tea tree oil (TTO), *Melaleuca alternifolia*, has long been a folk medicine among Australian aborigines, and use of this remedy has recently expanded worldwide as an aroma therapy against superficial infections with inflammation and for oral hygiene. Several reports have described that this oil has not only antibacterial but also antifungal activities,4–8) suggesting that it could be applicable to oral candidiasis.

In a previous paper,9) we reported a murine experimental oral candidiasis model which made it possible to estimate therapeutic efficacy of a natural product by two parameters: reduction of colony forming units (CFU) of *C. albicans* in the mouth and scores of the clinical manifestation of candida-infected tongues. Here, we show that TTO and its major component, terpinen-4-ol, display significant therapeutic efficacy in this model and that oral treatment with TTO has therapeutic activity even against oral candidiasis caused by an azole-resistant strain of *C. albicans*.

**MATERIALS AND METHODS**

**Organisms** *C. albicans* strains TIMM 2640, a clinically isolated strain,9) and TIMM 3163, an azole-resistant strain10) clinically isolated from patients with AIDS, were maintained at the Teikyo University Institute of Medical Mycology. These strains were stored at −80°C in Sabouraud dextrose broth (Becton Dickinson, MD, U.S.A.) containing 0.5% yeast extract (Becton Dickinson) and 10% glycerol in our laboratory until the experiment was performed. *C. albicans* was grown on candida GS agar plate (Eiken Chemical Co., Ltd., Tokyo, Japan) at 37°C for 24 h and the cells were harvested, suspended to at 2.5×107 cells/mL in RPMI-1640 medium containing 2.5% fetal calf serum (FCS) for oral inoculation.

**Agents** TTO was purchased from Hyperplants, Ltd. (Tokyo) and terpinen-4-ol was purchased from Sigma Chemical Co. (MO, U.S.A.). The major constituents of TTO are as follows: terpinen-4-ol 37.7%, γ-terpinene 21.25%, α-terpinene 10.5%, terpinolene 3.65%, 1–8 cineole 3.65%, α-terpinenol 2.75%, α-pinene 2.65%, para-cymene 2.3% and other minor (<2%) components. Fluconazole was purchased from Pfizer Japan Inc. (Tokyo).

**Animals** All animal experiments were performed according to the guidelines for the care and use of animals approved by Teikyo University and also guidelines for animal experiments conducted at research institutions by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Six week-old female ICR mice (Charles River Japan, Inc., Kanagawa, Japan) were used for all animal experiments. The photoperiods were adjusted to 12h of light and 12h darkness daily, and the environmental temperature was constantly maintained at 21°C. The mice were kept in cages housing 5–6 animals and were given access to food and water ad libitum.

**Minimal Inhibitory Concentration (MIC) Determination**
The MICs of TTO and terpinen-4-ol and fluconazole against \textit{C. albicans} TIMM 2640 and TIMM 3163 were determined by the broth microdilution method (M27-P protocol) according to the National Committee for Clinical Laboratory Standards (NCCLS).^{11}

**Oral Candidiasis in Mice** The experimental procedure of the oral candidiasis model was described previously.\(^9\) Briefly, immuno-suppressed mice were induced by subcutaneous treatment with a 100 mg/kg dose of prednisolone (Mitaka Pharmaceutical Co., Tokyo) 1 d prior to oral infection. Tetra-cycline hydrochloride (Takeda Shering Purau Animal Health Co., Osaka, Japan) in drinking water at a dose of 0.08% was given to the animals 1 d before infection. Mice were then anesthetized by intramuscular injection with 50 \(\mu\)L of 0.2% chlorpromazine chloride (Wako Pure Chemical Industries, Ltd., Osaka) in each femur, and were orally infected with about 2.5\(\times\)10\(^2\) cells/mL viable cells of \textit{C. albicans} TIMM 2640 or TIMM 3163, respectively, in RPMI-1640 containing 2.5% FCS. Oral infection was performed by means of a cotton swab (baby cotton buds; Johnson and Johnson, Co., Tokyo) rolled over all parts of the mouth. The cell number of \textit{Candida} inoculated in oral cavity was calculated to be approximately 1\(\times\)10\(^6\) cells/mouse by the difference in viable cell number associated to cotton swabs before and after oral inoculation, as described previously.\(^9\)

**Antifungal Treatment** TTO or terpinen-4-ol was suspended in 1% Tween 80 and applied with a cotton swab 3 h and 24 h after \textit{C. albicans} inoculation. This oral application schedule was suggested by previous therapeutic studies of farnesol, a quorum sensing molecule.\(^12\) One percent Tween 80 was applied to the control group using the same method. Fluconazole was administered in drinking water at 50 \(\mu\)g/mL from 3 h after inoculation throughout the experiments.

**Scoring of the Tongue's Fur and Squamous Layer and Quantitation of Oral Infection** Groups of mice were sacrificed, and the lesions of each tongue were observed macroscopically and scored from 0 to 4 based on the severity and extent of whitish, card-like patches on the surface as follows: 0, normal; 1, white patches less than 20%; 2, white patches less than 90% but more than 21%; 3, white patches more than 91% and the squamous layer; 4, thick fur on more than 91% and the squamous layer.

In order to measure the number of viable cells, the oral cavity (i.e. cheek, tongue, and soft palate) was swabbed using a cotton swab. After swabbing, the end of the swab was cut off and then placed in a tube containing 5 mL sterile saline. The \textit{candida} cells were resuspended by mixing on a vortex mixer before culture in 100-fold dilution on candida GS plate for 20 h at 37°C, after which the CFU were counted.

**Histological Finding** For histological study, the tongues were fixed in 20% formalin solution and embedded in paraffin. Five-micron sections were obtained from the paraffin block and stained with hematoxylin and eosin stain and periodic acid-Schiff (PAS) stain.

**Statistical Analysis** The lesion scores were compared using the non-parametric Mann–Whitney \(U\) test. The data of the log\(_{10}\)CFU of \textit{C. albicans} isolated from the mouths of the experimental groups were compared using Student’s \(t\)-test. \(p\) values of <0.05 were considered significant

**RESULTS**

**Inhibitory Effect of TTO, Terpinen-4-ol and Fluconazole in Vitro** The effects of TTO, terpinen-4-ol and fluconazole on the growth in \textit{vivo} of fluconazole-susceptible and -resistant \textit{C. albicans} strains were first examined following the NCCLS (Table 1). The MICs of TTO, terpinen-4-ol and fluconazole against TIMM 2640 were 20 mg/mL, 5 mg/mL and less than 1 \(\mu\)g/mL, respectively. The MICs of these substances against the azole-resistant strain TIMM 3163 were 5 mg/mL, 1.25 mg/mL and more than 64 \(\mu\)g/mL, respectively. This clearly shows that these two strains of \textit{C. albicans} were similarly susceptible to 1.25–20 mg/mL of TTO or terpinen-4-ol, although TIMM 3163 was resistant to a pharmacological concentration of fluconazole.

**Protective Activity of TTO and Terpinen-4-ol against Experimental Oral \textit{C. albicans} (TIMM 2640) Infection** We examined the protective effects of these agents in the murine oral candidiasis model. Figure 1 indicates the typical tongue lesions in macro- and microscopically. Control mice had clinically manifested lesions on the lingual mucosa, consisting of a white patchy area of smooth mucosa and well-delineated atrophic areas on the dorsal portions of tongues 2 d after infection as depicted in Fig. 1(a) (Score 3). Histological

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**Table 1.** The Effects of TTO, Terpinen-4-ol and Fluconazole on the Growth in Vitro of Fluconazole-Susceptible (TIMM2640) and -Resistant (TIMM3163) \textit{C. albicans} Strains

<table>
<thead>
<tr>
<th>Concentration of TTO</th>
<th>0.08 mg/mL</th>
<th>0.16 mg/mL</th>
<th>0.31 mg/mL</th>
<th>0.63 mg/mL</th>
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<td>TIMM2640</td>
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<th>2 (\mu)g/mL</th>
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Anti-\textit{Candida} efficacy of TTO and terpinen-4-ol to azole-susceptible (TIMM2640) and -resistant (TIMM3163) strain was evaluated following the broth microdilution method (M27-P protocol) according to the NCCLS. Each of MIC was as follows: TTO (TIMM2640: 20 mg/mL, TIMM3163: 5 mg/mL), terpinen-4-ol (TIMM2640: 5 mg/mL, TIMM3163: 1.25 mg/mL), fluconazole (TIMM2640: 1 \(\mu\)g/mL, TIMM3163: >64 \(\mu\)g/mL).
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Study of dorsal tongues of control mice showed thick *candida* mycelia in the lesions near oral epithelium and an accumulation of inflammatory cells was observed. (Fig. 1(b)).

In contrast, tongues of 4% TTO-treated mice macroscopically appeared to be healthy and without lesions (Score 0) as shown in Fig. 1(c). There were also no PAS-positive fungal-like hyphae and no accumulation of inflammatory cells in the tongues of 4% TTO-treated mice (Fig. 1(d)).

Figure 2 shows that oral treatment with more than 1% of TTO reduced the symptomatic score of tongues and significantly lowered the number of CFUs recovered from the oral cavity. On the other hand, 0.5% of TTO showed no significant effect (data not shown). These findings indicated that TTO protected the mice from severe oral infection of *C. albicans*.

In the case of fluconazole treatment, no Candida organism was detected in the oral cavities of any tested animals and their normal glossy dorsal tongue surfaces were observed on day 2.

Therapeutic activity of the major component of TTO, terpinen-4-ol is shown in Fig. 3. This component also demonstrated that it reduced the symptom score and log$_{10}$ CFU significantly at the concentration of 1 and 4% to the same as TTO.

The effective timing of oral administration of TTO was examined (Fig. 4). Compared to the case of two applications (3, 24 h) following *candida* inoculation, a single application at 3 h similarly decreased the tongue’s score, but that at 24 h did not. This indicated that early application of TTO was necessary for sufficient efficacy.

**Protective Activity of TTO against Oral Candidiasis with Azole-Resistant *C. albicans* (TIMM 3163)** Finally, the therapeutic efficacy of TTO and terpinen-4-ol against oral candidiasis infected by azole-resistant *C. albicans* TIMM 3163 was examined with this model. As shown in Fig. 5, administration of 4% of TTO significantly lowered the lesion score in the oral cavity 2 d after oral *Candida* inoculation. Also, it decreased more of the CFU than telpinen-4-ol although there...
was no significance. Meanwhile, terpinen-4-ol also showed therapeutic activity, although the CFU was not decreased significantly. Fluconazole that was applied at 10 or 20 mg/kg showed no therapeutic activity against infection with the azole-resistant strain TIMM 3163.

DISCUSSION

TTO has been traditionally used in the aboriginal society in Australia and more recently has become a popular essential oil used as a non-ethnic remedy for world wide. We demonstrated that oral doses of TTO or terpinen-4-ol had a protective effect against oral *C. albicans* infection in mice. From the viewpoint of research for effective chemotherapeutics against oral candidiasis, the most important finding presented here is that even oral candidiasis caused by an azole-resistant strain of *C. albicans* also can be inhibited by this treatment with TTO.

We think that one of the active components in TTO must be terpinen-4-ol since this terpenoid alcohol makes up 37.7% of this oil and its therapeutic activity appeared to be the same as that of TTO, while no increase in any specific activity could be observed. It is therefore possible that TTO may contain other active components than terpinen-4-ol.

TTO administration 3 h after infection protected the mice from severe symptoms of oral candidiasis, but did not do so at 24 h (Fig. 4a). This result suggests that early administration of TTO is highly effective. It does not mean that the therapeutic activity of TTO is limited to the marginal regions of the tongue surface, because we reported that *C. albicans* invaded the surface epithelium of tongues 3 h after inoculation in this experimental model. Terpinen-4-ol is very easily infused into skin or mucosal tissues because it is hydrophobic with a low molecular weight of 154.24 daltons. We therefore speculate that this terpenoid for oral use can be effective at the point where *candida* hyphae invade the surface of the oral mucosa as shown in Fig. 1.

The mechanisms underlying the protective action of TTO against oral candidiasis remain to be clarified. Several reports have shown that TTO has anti-*Candida* activity *in vitro*. Our results indicated that MIC of TTO for *Candida albicans* was 5–20 mg/mL which was close to the data previously described. The effective concentration 1–4% (10–40 mg/mL) of TTO against for oral candidiasis was also nearly the same as its MIC. So we can presume that the direct anti-fungal action of this oil may account for its therapeutic activity. The direct antifungal activity of TTO may be explained by disturbance of plasma membrane as reported in antibacterial activity of terpinen-4-ol or by change in gene-expression as reported in the case of anti-*Candida* activity of farnesol, a terpene-alcohol like terpinen-4-ol. We also think that some possible contribution of components other than terpinen-4-ol in protective function of TTO should be checked.

The correlation between therapeutic efficacy and *in vitro* anti-*Candida* activities of TTO and fluconazole was also confirmed by the experimental results with azole-resistant *C. albicans* TIMM 3163. Figure 5 indicates that TTO was similarly effective against oral candidiasis with *C. albicans* (TIMM 3163), but fluconazole was not.

We can also note that TTO displayed remarkable therapeutic efficacy in the macroscopic lesional pathogenesis of tongues as estimated by the score. This protective activity may be explainable by the oil’s anti-inflammatory activities, since few inflammatory cells were observed on the surface tissues of tongues of TTO-treated mice as shown in Fig. 1. Anti-inflammatory actions of TTO, including inhibition of the production of inflammatory cytokines and inhibition of...
histamine induced inflammation has been reported in various reports.\textsuperscript{17,18}

TTO has been mainly used topically for human patients with microbial infectious diseases and inflammatory lesions, for at least 80 years. Anecdotal evidence obtained over this period suggests that topical use is safe and adverse events have been minor and infrequent. On the other hand, TTO can be toxic if ingested: the 50\% lethal dose in a rat model was reported to be 1.9 g/kg.\textsuperscript{19} Thus, its oral use by patients must be done only under the instruction of a practitioner. Under these conditions, TTO has to date been tested clinically against vulvovaginal candidiasis or to improve the oral health status of hospice patients, and has demonstrated positive efficacy.\textsuperscript{20,21} We hope that it will contribute to clinical therapy as a new regimen in the near future, especially in cases of azole-resistant \textit{C. albicans}.

REFERENCES


3) Lopez-Ribot JL, McAtee RK, Pe


