Lemongrass and Perilla Essential Oils Synergistically Increased Antimicrobial Activity

Sanae A. Ishijima, Kunio Ezawa, and Shigeru Abe

Teikyo University Institute of Medical Mycology

Abstract

We postulated that disinfection of viable *Trichophyton* species in shoes would help reduce the number of patients with tinea pedis in Japan and that this might be accomplished safely using volatile components of essential oils. As vapor of lemongrass (*Cymbopogon citratus*) oil and citral have strong antimicrobial activities against *Trichophyton*, we examined the conditions under which lemongrass oil or citral show optimal antimicrobial activity in shoes. First, we investigated whether or not a strong antimicrobial effect could be obtained by combining with terpene aldehydes or aromatic aldehydes. When combined with citral, perillaldehyde showed superior antimicrobial activity to citronellal, cinnamaldehyde, cuminaldehyde, hydroxycitronellal, and vanillin. The combined effects of citral and perillaldehyde against *Trichophyton mentagrophytes*, *Bacillus subtilis*, and *Candida albicans* as volatile components dotted on filter paper placed away from the petri dish inoculated with fungi or bacteria were examined. Citral (2.5 mg/mL) and perillaldehyde (2.5 mg/mL) showed a greater inhibitory effect on growth of *C. albicans* than either solution alone in the aromatogram (disc diffusion) descent method (fractional inhibitory concentration [FIC] index of 0.58). Citral (2.5 mg/mL) and perillaldehyde (1.25 mg/mL) vapors in a closed box synergistically inhibited growth of *B. subtilis* and *T. mentagrophytes* (FIC indexes of 0.5 and 0.38, respectively). These results suggested that this combination would be safe and useful for disinfection of shoes.

**Key words**: citral, perillaldehyde, terpene aldehyde, tinea pedis, *Trichophyton*

Introduction

Despite the development of novel antifungal agents, there has been no reduction in the incidence of tinea pedis in Japan\(^1\,\,^2\). This is probably because many infections occur from the external environment. To prevent infection from the environment, it is necessary to identify areas where the responsible *Trichophyton* species are abundant and to eradicate the fungus.

Fungi that cause tinea pedis have been reported in bath mats\(^3\), room dust\(^4\), socks\(^5\), and shoes\(^6\,\,^8\). We postulated that reinfection from within the shoes is a major cause of tinea pedis\(^6\) as *Trichophyton* spp. have been cultured from factory workers’ shoes worn for long periods during the day\(^7\). The effects of disinfection of the inside of shoes on the onset of tinea pedis should be investigated to verify our hypothesis. We postulated that it would be desirable to disinfect the inside of work-specific shoes that cannot be washed. To test this, we previously developed an in-shoe sterilization method using volatile essential oils and their components and reported the sterilization of shoes with lemongrass oil and its volatile component, citral\(^9\,\,^10\). More than 30 essential oils have been shown to have strong antimicrobial activity\(^11\,\,^{15}\), and their antimicrobial active ingredients often contain terpenes or other aromatic constituents with functional groups, such as aldehyde or ketone\(^6\), which have a high affinity for the cell membranes of bacteria and fungi and affect membrane fluidity and integrity\(^17\,\,^{20}\). The major advantage of the use of volatile components as disinfectant is their penetrative ability to kill not only microbes attached to the surface but also those hidden in deep layers of the shoe leather.

For sterilization, it is necessary to use essential oils or volatile components at low concentrations, as high concentrations are known to cause skin irritation. The International Fragrance Association (IFRA) standards for the safety of ingredients contained in cosmetics\(^21\) are accepted worldwide. The specific conditions under which such volatile components...
can achieve antimicrobial activity at low concentrations must be determined to meet the IFRA standards. To reduce their concentrations, we examined mixtures of essential oil components that could be used to obtain higher antimicrobial effects than when used alone.

In the present study, we examined the antimicrobial activities of plant-derived volatile components and tested their utility for the disinfection of shoes. Lemongrass oil and citral have high antimicrobial activity against Trichophyton spp. in a closed box. Perilla essential oil and perillaldehyde also have anti-Trichophyton activity in the vapor phase. We therefore assessed the antimicrobial activities of the combination of citral and perillaldehyde, the major components of these two essential oils.

Materials and methods

Reagents

Citral (CAS: 5392-40-5), cuminaldehyde (CAS: 122-03-2), and perillaldehyde (CAS: 6611-91-2) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) at purities of at least 98.0%, 97.0%, and 87.0%, respectively, as determined by gas chromatography (GC). Citronellal (CAS: 106-23-0), cinnamaldehyde (CAS: 101-39-3), hydroxycitronellal (CAS: 107-75-5), and vanillin (CAS: 121-33-5) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) at purities of at least 85%, 98%, and 98%, respectively, as determined by GC, and vanillin was obtained at a purity of at least 98% as determined by thin layer chromatography (TLC) titration.

Detection of the combined effects of perillaldehyde and citral on growth of Candida albicans TIMM 1768 or Bacillus subtilis JCM2499 by the conventional aromatogram (disc diffusion) method

The C. albicans strain TIMM1768, recovered from a patient with candidiasis, was stored at −80°C in Sabouraud dextrose broth (SDB; Becton Dickinson, Sparks, MD, USA) containing 10% glycerol. For aromatogram preparation, Candida cells were grown on Sabouraud dextrose agar (SDA; Becton Dickinson) at 37°C for 20 hours, harvested with a microspatula, and resuspended in inoculum medium consisting of SDB. C. albicans TIMM 1768 cells (2 × 10^6) or 100 µL of a tenfold diluted solutions of McFarland 0.5 (1 × 10^8/mL) B. subtilis JCM2499 cell suspension were inoculated onto the surface of SDA plates 90 mm in diameter. Aliquots of 50 µL of perillaldehyde serially diluted in 40% isopropanol and 40% isopropanol control were dropped onto filter paper discs 8 mm in diameter attached with double-sided tape to the center of the petri dish lid. All plates were sealed and cultured at 37°C for 20 hours. Digital images of the Candida growth inhibition circles on the agar plates were taken using a digital camera positioned parallel to the agar surface. An analysis of the area of growth inhibition was performed automatically using the Lenaraf 220b software program (Vector Japan, Tokyo, Japan). We determined the fractional inhibitory concentration (FIC) for each volatile component and the FIC Index (Σ FIC) for the combination using the following formula: Σ FIC = FIC A + FIC B, where FIC A is the minimum inhibitory concentration (MIC) of volatile component A in the combination (A1) /MIC of volatile component A alone (A0), and FIC B is the MIC of volatile component B in the combination (B1)/MIC of volatile component B alone (B0). The effects of antimicrobial combinations were graded using the Σ FIC as follows: synergistic, ≤0.5; additive, 0.5–1; indifferent, 1–4; and antagonistic, >4.

Detection of the combined antimicrobial effects of perillaldehyde and citral on growth of Trichophyton mentagrophytes TIMM 2789 or B. subtilis JCM2499 by the closed box method

T. mentagrophytes TIMM2789 was inoculated onto SDA plates 35 mm in diameter at 1 × 10^7 conidia/plate. McFarland 0.5 (1 × 10^8/mL) B. subtilis JCM2499 cell suspension was diluted to 30 CFU/100 µL on SDA plates. Two plates were set on the bottom of a closed box (145 × 100 × 90 mm; approximately 1.3 L). Citral, perillaldehyde, or a mixture of the two solutions (200 µL) diluted with 40% isopropanol was dropped at the center of a filter paper disc 90 mm in diameter attached to the lid of the closed box, and the lid was immediately closed. The closed box was allowed to stand at 23°C–26°C for 16 hours. The petri dishes were taken out from the closed box, covered with a lid, sealed with film (Parafilm; Bemis Company, Inc., Neenah, WI, USA), and cultured at 30°C for 144 hours. The degree of growth of Trichophyton mycelia that appeared on the medium in the petri dish was scored on a 4-point scale from 0 to 3 as follows: 0, no growth; 1, recognizable Trichophyton mycelia; 2, Trichophyton mycelia covering >50% of the surface of the medium; 3,
Trichophyton mycelia covering 100% of the surface of the medium.

For B. subtilis, the petri dishes were removed from the closed box, covered with a lid, sealed with film, and cultured at 37°C for 24 hours. The number of colonies that appeared in the medium on the petri dish was counted and scored on a 4-point scale as follows: 0, no growth; 1, 1–5 colonies; 2, 6–10 colonies; 3, >11 colonies.

Results

Combined effects of citral and terpene aldehydes or aromatic aldehydes from essential oils on growth of B. subtilis

The combined antibacterial effects of citral and cinnamaldehyde, cuminaldehyde, citronellal, hydroxycitronellal, perillaldehyde, or vanillin were determined by the aromatogram method on the same agar medium. Typical images of the effects of citral and perillaldehyde or citral and cuminaldehyde are shown in Figs. 1A and 1B, respectively. Various concentrations (0%, 2.5%, 5%, and 10%) of perillaldehyde or cuminaldehyde were added to four of five small filter paper discs around the center of two plates, and a different concentration of citral (0% and 5%) was added to the center filter paper disc in each plate. Bacillus cells grew as a white film on the agar surface after 20-hour incubation at 37°C. A circle equivalent in size to the area of growth inhibition was superimposed on the photographs shown in Figs. 1A1, 1A2, 1B1, 1B2, 1C1, 1C2, 1C3, 1C4, 1D1, 1D2, 1D3, 1D4.
1B1, and 1B2, and its diameter was measured as the parameter of antimicrobial activity (Figs. 1A4 and 1B4). The results indicated that the area of growth inhibition seen with 5% citral in 40% isopropanol added to the center of the agar medium was increased in the presence of perillaldehyde or cuminaldehyde. Similarly, citral and cinnamaldehyde, citronellal, hydroxycitronellal, or vanillin showed combined anti-
Bacillus
effects. Measurements of the areas of inhibition and ratios of enhancement of growth inhibition are summarized in Table 1. Perillaldehyde showed the highest ratio of increase in area of growth inhibition compared with citral alone (2.0), followed by cuminaldehyde (1.6), and vanillin (1.5). These observations indicated that the combination of citral and perillaldehyde had the strongest synergistic antimicrobial activity among the combinations tested.

**Combined effects of citral and terpene aldehydes or aromatic aldehydes from essential oils on growth inhibition of C. albicans**

Next, we investigated whether or not the combined use of citral and other aldehydes would show effects on C. albicans similar to those seen with B. subtilis as the target organism. Fig. 1C shows the area of growth inhibition of C. albicans TIMM1768 by the combination of citral and perillaldehyde. We examined the antifungal effects of combinations of different components, i.e., citral and perillaldehyde, vanillin, or hydroxycitronellal. In addition, we also examined the effects of Perilla essential oil and lemongrass oils by the conventional aromatogram method on similar agar plates. Typical images of Candida growth inhibition are shown in Fig. 1C. Various concentrations (0%, 2.5%, 5%, and 10%) of perillaldehyde were added to four of the five small filter paper discs around the center of three plates as shown Fig. 1C4. Various concentrations of citral (0%, 2.5%, and 10%) were added to the center filter paper disc on each plate. The Candida cells grew as a white film on the agar surface after 20-hour incubation at 37°C. The area of inhibition in the presence of perillaldehyde increased, and the number of Candida cells in the entire agar plate decreased when 10% citral was dropped onto the central filter paper disc (Fig. 1C3). Slight growth of the white yeast film was observed on the left, and Candida growth inhibition could not be measured on the 10% citral plate (Fig. 1C3).

Table 2 summarizes the measurements of the areas of Candida growth inhibition by citral and perillaldehyde,

| Table 1. Combined effect of citral and perillaldehyde on growth of B. subtilis determined by the aromatogram method |
|--------------------------------------------------|
| terpene aldehyde | conc. (%) | 40% isopropanol | 5% citral in 40% isopropanol | Expansion ratio of area of growth inhibition |
|------------------|-----------|-----------------|-----------------------------|---------------------------------------------|
| perillaldehyde   |           |                 |                             |                                             |
| 2.5              | 9         | 13              | 1.3                         |                                             |
| 5                | 10        | 16              | 1.6                         |                                             |
| 10               | 10        | 20              | 2.0                         |                                             |
| cinnamaldehyde   |           |                 |                             |                                             |
| 2.5              | 21        | 25              | 1.2                         |                                             |
| 5                | 28        | 30              | 1.1                         |                                             |
| 10               | 29        | 34              | 1.2                         |                                             |
| cuminaldehyde    |           |                 |                             |                                             |
| 2.5              | 12        | 15              | 1.3                         |                                             |
| 5                | 17        | 27              | 1.6                         |                                             |
| 10               | 20        | 30              | 1.5                         |                                             |
| vanillin         |           |                 |                             |                                             |
| 2.5              | 9         | 12              | 1.3                         |                                             |
| 5                | 10        | 15              | 1.5                         |                                             |
| 10               | 12        | 18              | 1.5                         |                                             |
| citronellal      |           |                 |                             |                                             |
| 0.63             | 9         | 10              | 1.1                         |                                             |
| 1.3              | 11        | 17              | 1.5                         |                                             |
| 2.5              | 18        | 21              | 1.2                         |                                             |
| hydroxycitronellal |         |                 |                             |                                             |
| 2.5              | 11        | 8               | 0.7                         |                                             |
| 5                | 14        | 9               | 0.6                         |                                             |
| 10               | 18        | 14              | 0.8                         |                                             |

*Comment: First letter of first word should be capitalized: e.g. Terpene aldehyde, Perillaldehyde, Conc., etc. (same for Table 2).*
vanillin, and hydroxycitronellal. As shown in Table 2, dropping 2.5% citral on the central filter paper disc increased by 1.3 times the area of growth inhibition around the peripheral filter paper discs onto which perillaldehyde had been dropped (2.5%, 5%, and 10%). Experiments with lemongrass oil and Perilla oil showed similar results, i.e., the area of growth inhibition was increased by 1.3 times with addition of 5% Perilla oil (Table 2). Similarly, combination effects were also detected by this method for citral and vanillin or citral and cinnamaldehyde using Candida cells (data not shown).

The aromatogram descent method was then performed to quantify the volatile effect of citral dropped on the central disc. Subsequent experiments were performed using only citral and perillaldehyde as they showed the greatest combined effect.

**Determination of the combined effect of citral and perillaldehyde on growth inhibition of C. albicans**

An area of growth inhibition was formed under the filter paper disc onto which citral, perillaldehyde, or a mixture of both had been added. The mixture of perillaldehyde and citral resulted in a larger area of inhibition than either solution alone (Fig. 2A). The concentration of citral and perillaldehyde showing 3-cm² and 5-cm² growth inhibition areas were plotted on Fig. 2C, and the FIC index was determined as an index of the combined effect. As a result, FIC indexes of 0.58, 0.65, and 0.7 were obtained, which made it clear that an effect equal to or greater than the additive effect but less than the synergistic effect was obtained. A similar weak effect of the combination of cuminaldehyde and citral was also detected (data not shown).

**Determination of the combined effect of citral and perillaldehyde on quantitative growth inhibition of B. subtilis**

We used the closed box method for the quantitative evaluation of the volatile antibacterial effects of citral and perillaldehyde on B. subtilis. The antibacterial effects of the solution at various mixing ratios of citral and perillaldehyde were scored on a 4-point scale from 0 to 3 in accordance with the number of B. subtilis colonies formed on the agar plates. Plots for each concentration giving a growth score of 1 are shown in Fig. 3. The combination of 1.25 mg/mL perillal-
Aldehyde and 2.5 mg/mL citral showed an FIC index of 0.5, indicating a synergistic antibacterial effect; and the combinations of 2.5 mg/mL perillaldehyde and 1.25 mg/mL citral or 0.63 mg/mL perillaldehyde and 5 mg/mL citral both showed an FIC index of 0.63, indicating an additive antibacterial effect.

### Determination of the combined effect of citral and perillaldehyde on quantitative growth inhibition of *T. mentagrophytes*

The closed box method was also used for the quantitative evaluation of the volatile antifungal effects of citral and perillaldehyde on *T. mentagrophytes* TIMM2789. The antifungal effects of the solutions at various mixing ratios of citral and perillaldehyde were scored on a 4-point scale from 0 to 3 in accordance with the area of *Trichophyton* growth on the agar plates. Plots for each concentration giving a growth score of 0 are indicated in Fig. 4. The combination of 1.25 mg/mL perillaldehyde and 0.63 mg/mL citral showed an FIC index of 0.38, indicating a synergistic antifungal effect; and the combination of 0.63 mg/mL perillaldehyde and 2.5 mg/mL citral showed an FIC index of 0.63, indicating an additive antifungal effect.

### Discussion

The combination of citral and perillaldehyde was shown to have synergistic antibacterial effects on *B. subtilis* and *T. mentagrophytes* and additive antifungal effects on *C. albicans*. The antimicrobial activity of the combination of citral and perillaldehyde for *C. albicans* was demonstrated not only by the standard aromatogram method, but also by the aromatogram descent method, which can directly detect antifungal activity of volatile agents. Furthermore, the vapors of this combination in the closed box method also showed strong antimicrobial activity against *B. subtilis* and *T. mentagrophytes*, which are the causative microorganisms in shoe contamination. The combined use of citral and perillaldehyde showed greater antimicrobial activity than combinations of citral with other aromatic compounds. The combination of citral and perillaldehyde in the vapor phase had antifungal effects against the filamentous fungus *Trichophyton*. These observations raise questions regarding why the combination of citral and perillaldehyde showed stronger antimicrobial activity than other combinations. There have been a number of studies regarding the mechanisms underlying the antimicrobial activities of essential oils and their components, but the detailed antimicrobial mechanisms of action of each component have not yet been elucidated. Terpenes are components of essential oils that have been shown to have strong antimicrobial activity and have high affinity for cell membranes, considered to be similar to effects of hopane in bacteria or ergosterol in fungi based on their structural properties. The functional groups of

| Table 2. Combined effect of citral and perillaldehyde on growth of *C. albicans* determined by the aromatogram method |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| A                               | Diameter of area of growth inhibition (mm) | Expansion ratio of area of growth inhibition |
| terpene aldehyde conc. (%)      | 40% isopropanol | 2.5% citral in 40% isopropanol | 5% citral in 40% isopropanol |
| perillaldehyde                  | 2.5 13 17 NT 1.3 | | |
| 5                               | 17 21 NT 1.3 | | |
| 10                              | 22 29 NT 1.3 | | |
| vanillin                        | 2.5 18 NT 23 1.3 | | |
| 5                               | 20 NT 26 1.3 | | |
| 10                              | 22 NT 29 1.3 | | |
| hydroxycitronellal              | 2.5 14 NT 12 0.9 | | |
| 5                               | 15 NT 15 1.0 | | |
| 10                              | 18 NT 18 1.0 | | |
| B                               | Diameter of area of growth inhibition (mm) | Expansion ratio of area of growth inhibition |
| 2nd essential oil conc. (%)     | 40% isopropanol | 2.5% lemongrass oil in 40% isopropanol | 5% lemongrass oil in 40% isopropanol |
| Perilla oil                     | 2.5 9.0 9.5 NT 1.1 | | |
| 5                               | 12.0 15.7 NT 1.3 | | |
| 10                              | 15.1 21.3 NT 1.4 | | |
terpenes affect their antimicrobial activity. The order of antimicrobial activity of terpenes in accordance with functional group is aldehyde > alcohol > ketone. Terpene aldehyde seems to penetrate across and disrupt the integrity of the cell membrane and the mitochondrial membrane where ATP synthesis occurs, resulting in disruption of ATP synthesis.

On the basis of these reports, we believe that even small volatile molecules with the same aldehyde group may have different antimicrobial activities due to differences in the structures to which the substituents connect; therefore their combinations would result in increased antimicrobial activity. We screened combinations of citral with six terpene or aromatic aldehydes that have been reported to have strong antimicrobial activity, i.e., citronellal, cinnamaldehyde, cuminaldehyde, hydroxycitronellal, perillaldehyde, and vanillin; and our results indicated that the strongest effect was obtained with the combination of citral with perillaldehyde. The combined antimicrobial effect of the combination of citral and terpene aldehyde increased in order of molecular weight (MW); i.e., perillaldehyde (MW 150.22) < citronellal (MW 154.25) < hydroxycitronellal (MW 172.27). Perillaldehyde, which has the smallest molecular weight and is therefore the most volatile of the terpene aldehydes tested, showed highest antimicrobial activity. The antimicrobial effect of the combinations of citral and aromatic aldehydes did not appear to differ in accordance with MW as with terpene aldehydes; i.e., cinnamaldehyde (MW 132.13), cuminaldehyde (MW 148.21), and vanillin (MW 152.15) had comparable effects. We speculated that the position of the aldehyde group close to the isoprene skeleton or benzene ring may be important for the action of the aldehyde group, but further studies are required to verify this suggestion.

Here, we discuss whether the combination of citral with perillaldehyde can be used in the eradication of microbial contaminants in shoes. A size-26-cm man’s shoe has a volume of about 0.6 L, which is about half the volume of the closed box used in our experiments (1.3 L).

Under our experimental conditions, the inner wall of the
closed box was covered with aluminum foil to minimize adsorption of components into the plastic box, and the tight seal increased the antimicrobial efficiency. In shoes, however, a larger volume of fluid would be needed because of the amount of adsorption to the inner wall of the shoe and the loss of vapor from the open end of the shoe. Thus, it is necessary to take the problems of absorption and diffusion of active components into consideration.

Previously, we examined a method of sterilizing the inside of shoes using lemongrass oil or citral, and established a method for eradication of Trichophyton by the combined application of lemongrass and Perilla oils to shoes. These experiments showed that the antimicrobial activity in the shoe was reduced to 25% compared with the results in the closed box test due to adsorption into the shoe material and loss of vapor from the open end of the shoe.

Although higher concentrations of antimicrobial essential oil components may show stronger activities against bacteria or fungi, they may also result in adverse effects on humans, such as inflammatory irritation of the skin. The IFRA standard (Amendment 49) places limits on these components in accordance with usage for parts of the body or degree of contact. Foot care products, which are the most relevant classification for this shoe sterilization method, are included in category 4 as “fine fragrance”. We considered it appropriate for the disinfectant method in shoes described in this paper to be based on this category 4 standard of 0.6% for citral and 0.3% for perillaldehyde. Based on the results shown in Fig. 3, the combined usage of 0.0625% – 0.5% citral and 0.0625% – 0.25% perillaldehyde has effective antibacterial activity while remaining within the IFRA safe limit. Similarly, these concentration ranges were suggested to have antifungal activity against Trichophyton (Fig. 4). These results suggest that the combined usage of citral and perillaldehyde can sufficiently and safely eradicate the bacteria responsible for odor in shoes and the fungi responsible for tinea pedis.

Tinea pedis is known to spread through contact with infected human skin scales. Thus, the eradication of these sources of Trichophyton in the environment will reduce the incidence of tinea pedis. The combination of citral and perillaldehyde can be used in small amounts to resolve the problem of reinfection with Trichophyton from the environ-
Antimicrobial effects of volatile essential oils can be facilitated by creating a closed environment for a short period of time by putting caps on the shoes, which can then be opened to enable elimination of the residual volatile components by diffusion into the air. Citral and perillaldehyde may also have further applications in management of hygiene in everyday life.

Acknowledgments

We would like to express our gratitude to Dr. M Hiruma (Hiruma Clinic), Dr. M Yamazaki (TIMM), Dr. T Takizawa (TIMM), and Dr. K Sano (Soda Aromatic Co. Ltd.) for making meaningful suggestions in compiling this paper.

Conflicts of interest

None.

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