# The Antibacterial Activity of Fragrance Ingredients against *Legionella pneumophila*

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**In the current study we investigated the antibacterial activity of fragrance ingredients against *Legionella pneumophila*, a causative agent of severe pneumonia. Among the 41 different fragrance ingredients tested, we found that the natural fragrance ingredients oakmoss (OM) and birch tar oil (BT), which contain many components, exhibit potent antibacterial activity. The minimum inhibitory concentration (MIC, % (v/v)) of OM and BT were 0.0020 and 0.0024, respectively and were lower than that of cinnamic aldehyde (0.0078), which has been previously shown to possess high antimicrobial activity. In a time–kill assay of OM and BT at MIC and two times MIC, the colony forming units (CFU) of the microbe were reduced to between $10^{-3}$ to $10^{-4}$ of the original CFU after 1 h co-incubation. After this time, the CFU gradually decreased in number, but remained above detection levels even after a 48-h co-incubation, except for BT at two times MIC. In contrast, at a concentration of 0.1% OM and BT (approximately 50 times MIC), CFU were not detected after co-incubation for 1 h. Another 18 fragrance ingredients including ketone, aldehyde, lactone, acid, phenol derivative, aliphatic alcohol and quinoline also exhibited a lesser degree of antibacterial activity against *L. pneumophila* at a MIC of less than 0.10.**

**Key words** antibacterial activity; fragrance ingredient; *Legionella pneumophila*; oakmoss; birch tar oil

Due to their antimicrobial activity, fragrance ingredients have been used from ancient times as antiseptics and preservatives, as well as aromatic agents. Antimicrobial activity against bacteria and fungi has been demonstrated for many kinds of fragrance ingredients.[1—5] Several Gram-negative and -positive bacteria including *Escherichia coli*, *Staphylococcus aureus* and the genus *Salmonella* have been shown to be susceptible to antibacterial activity from fragrance ingredients. However, antibacterial activity against *Legionella pneumophila*, a causative agent of water-born, severe pneumonia has not been extensively investigated.[6—8]

In our ongoing research into the antimicrobial activity of fragrance ingredients, we have established an improved method for the estimation of antimicrobial activity of volatile and hydrophobic fragrance ingredients.[9] Through this method, we have identified 101 of the 999 kinds of fragrance ingredients tested that demonstrate potent antimicrobial activity against representative strains of Gram-negative and Gram-positive bacteria and fungi.[10] The 101 fragrance ingredients exhibit antimicrobial activity at a minimum inhibitory concentration (MIC, % (v/v)) of less than 0.10 against at least one of the following six microbes: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspegillus niger* and *Candida albicans*. In the present study, 41 of these 101 fragrance ingredients were investigated for their antibacterial activity against *L. pneumophila*, in the aim of developing a novel disinfectant that would be appropriate for sterilizing water systems used in the community. The 41 fragrance ingredients were selected largely based on their reported antimicrobial activity,[10] specifically against Gram-negative bacteria.

## MATERIALS AND METHODS

**Fragrance Ingredients** All fragrance ingredients used in this study were provided by Ogawa & Co., Ltd., Chiba, Japan. These fragrance ingredients do not contain any additive preservative or antimicrobial agent.

**Bacterial Strain and Cultivation** *Legionella pneumophila* strain JCM7571 (Philadelphia 1) was used for the estimation of antibacterial activity of fragrance ingredients. The bacterial strain was cultured on BCYE-$\alpha$ (Becton Dickinson, MD, U.S.A.) plates at 37 °C for 72 h. The bacteria samples were then suspended in saline (for MIC determination) or PBS (for time–kill assay) and the optical density at 600 nm was adjusted to 0.1, a value that is known to contain $1.7\times10^8$ colony forming unit (CFU)/ml of bacteria.

**MIC Estimation** MIC was estimated in accordance with the modified broth micro-dilution method as previously described[9] based on the standard method described by the Clinical and Laboratory Standards Institute (CLSI).[11] Given the susceptibility of *L. pneumophila* to detergents, Tween 80 was not used in this experiment. The fragrance ingredients were diluted 1 : 4 with dimethylsulfoxide (DMSO) and then further diluted with BYE-$\alpha$ broth (Becton Dickinson) to achieve a final concentration of 1.0% (v/v). Serial dilution of the fragrance ingredients with BYE-$\alpha$ was carried out using a deep-well plate (BM6022, BM Equipment Co., Tokyo, Japan). Then, 50 $\mu$l of each diluted fragrance sample was transferred to the well of a micro-plate and 50 $\mu$l of the bacterial suspension that had been adjusted to $6.0\times10^5$ CFU/ml with BYE-$\alpha$ was also added to each well. The MIC was estimated following incubation at 37 °C for 48 h using a previously described method based on the standard CLSI procedure.[11]

**Time–Kill Assay** Time–kill assays were performed using previously described standard CLSI methods.[12] Bacterial suspensions diluted with BYE-$\alpha$ to $3.0\times10^5$ CFU/ml were pre-incubated in a rotary shaker (NR-3, TITEC, Saitama, Japan) with shaking (150 rpm) at 37 °C for 12 h.

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These samples were then co-incubated with fragrance ingredient solutions adjusted to 1.0% in BYE-$\alpha$ to give the final concentration of MIC and two times MIC, or by adding 25% solutions in DMSO to give a final concentration of 0.1%. Aliquots of 100 $\mu$l of the culture before (0 h, positive control) and after (1, 3, 6, 12, 24, 48 h) the addition of fragrance ingredient were used to estimate CFU on BYE-$\alpha$ plates with adequate dilution using buffered saline supplemented with 0.01% gelatin. Three plates were used for one sample and the estimation of CFU was repeated separately. The CFU values are expressed as mean$\pm$standard deviations based on CFU values obtained from six plates.

## RESULTS

The antibacterial activity (MIC, % (v/v)) of the 41 selected fragrance ingredients against *L. pneumophila* is outlined in Table 1. Among the 6 kinds of natural fragrance ingredients tested, oakmoss (OM) and birch tar oil (BT) exhibited markedly potent antibacterial activity against *L. pneumophila*. The MIC values for OM and BT were 0.0020 and 0.0024 respectively, less than one-third of the 0.0078 MIC value recorded for cinnamic aldehyde, a well known naturally occurring fragrance ingredient that also exhibits potent antimicrobial activity. In contrast, the antibacterial activity of the 4 other natural fragrance ingredients, hiba oil (MIC, 0.13), palmarosa cymbopogon oil (MIC, 0.50), palmarosa oil (MIC, 0.50) and allspice oil (MIC, 0.19) was considerably less than that of OM and BT. Compared to OM and BT, the MIC values of these natural fragrance ingredients were approximately 50 to 200 times higher. Additional fragrance ingredients investigated in this study were classified into groups based on their chemical characteristics and were found to possess antibacterial activity against *L. pneumophila* at MIC values ranging between 0.01 and 0.1. These groups (number of active/tested fragrance ingredients contained) included ketones (1/3), aldehydes and acetals (7/8), lactones (4/6), acids (2/2), phenol derivatives (2/2), aliphatic alcohols (3/8) and quinolines (1/2). Antibacterial activity was not recorded in the terpenic alcohol group.

The antibacterial effects of OM and BT were also analyzed in time–kill assays and the results were compared to time–kill assays for cinnamic aldehyde and chlorhexidine gluconate (MIC, 0.00013), a common disinfectant used for standard hygiene purposes. Figure 1A shows the bactericidal effect associated with cinnamic aldehyde on *L. pneumophila*. The CFU value was reduced to below detection levels after co-incubation for 3 h at two times MIC and after 12 h at MIC. A similar bactericidal effect was also observed for chlorhexidine gluconate (Fig. 1B). Similarly, the CFU value was reduced to below detection limits after co-incubation for 3 h at a concentration of 0.1% (approximately 800 times MIC), which is the usual concentration of chlorhexidine gluconate, and after 24 h at MIC. The antibacterial effects of OM (Fig. 1C) and BT (Fig. 1D) showed a different pattern from those of cinnamic aldehyde and chlorhexidine gluconate. For OM and BT, CFU was rapidly reduced to $10^{-3}$ to $10^{-4}$ of the original CFU value after 1 h co-incubation. Although the CFU value continued to decrease gradually thereafter, with the exception of the case of BT at two times MIC, the CFU did not decrease to below detection limits, even after 48 h of co-incubation. On the other hand, when the concentrations of OM and BT were increased to 0.1%, which corresponds to approximately 50 times the MIC of these fragrance ingredients, the CFU value was reduced to below detection levels after 1 h co-incubation, and was similar to the result observed for cinnamic aldehyde at two times MIC and 0.1% chlorhexidine gluconate. Thus, the current study demonstrates that OM and BT exhibit both bactericidal and bacteriostatic effects towards *L. pneumophila* depending on the concentration.

Table 1. Minimum Inhibitory Concentration (MIC) of Fragrance Ingredients against *Legionella pneumophila*

| Fragrance ingredient | MIC[a] | |
|---|---|---|
| | % (v/v) | $\mu$g/ml[b] |
| Natural fragrance ingredients | | |
| Oakmoss | 0.0020 | 22.3 |
| Birch tar oil | 0.0024 | 27.2 |
| Hiba oil | 0.13 | 1240 |
| Palmarosa cymbopogon oil | 0.50 | 4470 |
| Palmarosa oil | 0.50 | 4420 |
| Allspice oil | 0.19 | 1980 |
| Ketones | | |
| Anisyl acetone | 0.13 | 1362 |
| Ethyl maltol | 0.11 | 1100 |
| Methyl Lavender Ketone® | 0.047 | 423 |
| Aldehydes and acetals | | |
| Anis aldehyde | 0.039 | 438 |
| Canthoxal | 0.035 | 365 |
| Cinnamic aldehyde | 0.0078 | 82.0 |
| Cortex Aldehyde® | 0.039 | 440 |
| Helional® | 0.016 | 186 |
| Melozone® | 0.16 | 1650 |
| Floralozon® | 0.055 | 525 |
| Vanillin | 0.039 | 390 |
| Terpenic alcohols | | |
| Apo Petchone® | 0.28 | 2800 |
| Citronellol | 0.50 | 4280 |
| Geraniol | 0.22 | 1940 |
| Terpineol | 0.13 | 1220 |
| Lactones | | |
| $\gamma$-Decalactone | 0.13 | 1240 |
| $\delta$-Decalactone R-80 | 0.063 | 612 |
| $\delta$-Decalactone | 0.063 | 612 |
| Jasmolactone | 0.063 | 630 |
| $\gamma$-Nonalactone | 0.13 | 1260 |
| $\delta$-Undecalactone | 0.016 | 151 |
| Acids | | |
| Oenanthic acid | 0.016 | 147 |
| Tiglic acid | 0.063 | 630 |
| Phenol derivatives | | |
| Carvacrol | 0.055 | 539 |
| Eugenol | 0.063 | 673 |
| Aliphatic alcohols | | |
| Undecyl alcohol | 0.050 | 417 |
| Cinnamyl alcohol | 0.098 | 1020 |
| Floralol® | 0.19 | 1792 |
| 3,6-Nonadienol | 0.28 | 2438 |
| *cis*-6-Nonenol | 0.50 | 4250 |
| Peomosa® | 0.094 | 951 |
| Phenyl hexanol | 0.13 | 1251 |
| 9-Decen-1-ol | 0.50 | 4230 |
| Quinolines | | |
| *iso*-Butyl quinoline | 0.50 | 4230 |
| *p*-Methyl quinoline | 0.063 | 674 |

*a*) Values are expressed as mean values obtained across three independent experiments. *b*) Concentrations in $\mu$g/ml were calculated based on the specific gravities of individual fragrance ingredients.

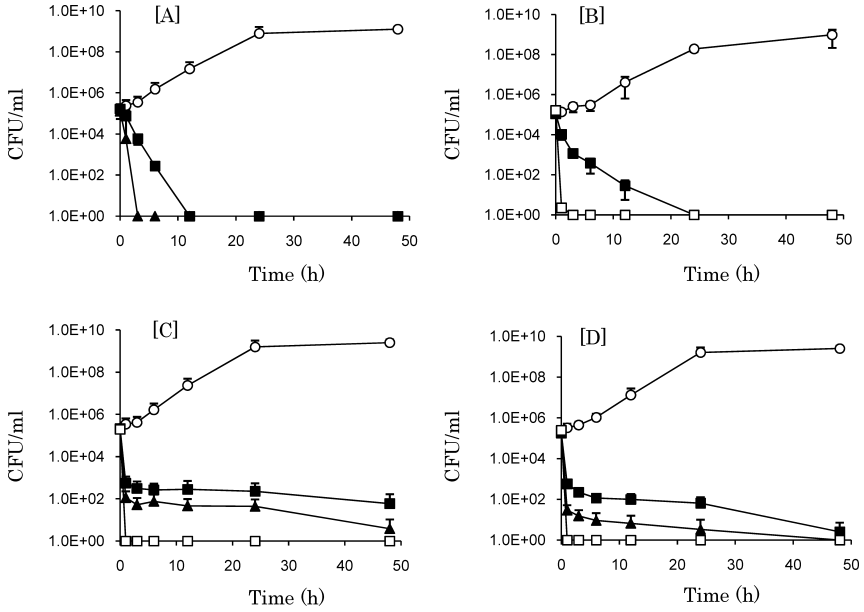


Fig. 1. Time–Kill Curves of *L. pneumophila* in the Presence of MIC (■) and Two Times MIC (▲) of Cinnamic Aldehyde [A] , MIC (■) and 0.1% (□) of Chlorhexidine Gluconate [B], MIC (■), Two Times MIC (▲) and 0.1% (□) of Oakmoss [C] and Birch Tar Oil [D]

The open circle (○) indicates the positive control.

## DISCUSSION

In the present study, we demonstrated that the natural fragrance ingredients OM and BT exhibit potent antibacterial activity against *L. pneumophila*. This activity appears to be higher than that of cinnamic aldehyde, and is also considerably higher than activity levels reported against other species of Gram-negative bacteria.[10] Morita *et al.* reported that hinokitiol and its related compounds exhibit high antibacterial activities against two strains of *L. pneumophila*.[6,7] The MIC for these compounds against the *L. pneumophila* strain SG 3 was assayed by the agar dilution method and was demonstrated to be in the range of 12.5 to 25.0 $\mu$g/ml. These values are similar to those for OM and BT (22.3 and 27.2 $\mu$g/ml, respectively) obtained in this study even though the actual method of MIC estimation differed. In addition, several fragrance ingredients other than OM and BT also exhibited antibacterial activities in a range of MIC values between 0.01 and 0.1.

The antibacterial effects of OM and BT on *L. pneumophila* were found to be both bactericidal and bacteriostatic. When low concentrations (MIC and two times MIC) of OM and BT were administered in time–kill assays, the CFU were rapidly reduced to $10^{-3}$ to $10^{-4}$ of the original CFU value, and were then maintained over time. On the other hand, when high concentrations (0.1%) of OM and BT were administered, the CFU was rapidly reduced to below detection levels. These results lead to the hypothesis that as OM and BT are natural fragrance ingredients comprising a number of components, with a specific component or components exhibiting bactericidal effects and another component exhibiting bacteriostatic effects that may be acting simultaneously. A variety of carbonyl, phenol, acid and depside compounds are reported components of OM,[13—15] while various phenol derivatives form the major component of BT.[16,17] Therefore, a serial investigation involving the separation and characterization of these components, and the analysis of antibacterial activity of individual components may more precisely define the mode of antibacterial activity for OM and BT. In addition, such experiments may lead to the future usage of OM, BT, and other candidates presented in this study as disinfectants for water systems in the community.

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