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**Original**

# Effects of Oakmoss Components on Extra- and Intracellular *Legionella pneumophila* and Its Host *Acanthamoeba castellanii*

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***Acanthamoeba castellanii*** **is a ubiquitous organism found in environmental water. The amoeba is pathogenic to toward humans and is also a reservoir of bacteria of the genus *Legionella*, a causative agent of legionellosis. Oakmoss, a source of natural fragrance ingredients, and its components are antibacterial agents that are specifically active against the genus *Legionella*. In the present study, oakmoss and its components were investigated for their inhibitory effects on total (extra- and intracellular) *Legionella pneumophila* within *A. castellanii* and on *L. pneumophila* within *A. castellanii*. Among the oakmoss components, 3-hydroxy-5-methylphenyl 2,4-dihydroxy-6-methylbenzoate (1), 3-methoxy-5-methylphenyl 2,4-dihydroxy-6-methylbenzoate (2), and 8-(2,4-dihydroxy-6-(2-oxoheptyl)phenoxy)-6-hydroxy-3-pentyl-1H-isochromen-1-one (8) reduced the number of total bacteria (extra- and intracellular) in a test culture and also exhibited high amoebicidal activity against *L. pneumophila* within *A. castellanii* at concentrations lower than their $IC_{50}$ values for *A. castellanii*. In contrast, 6,8-dihydroxy-3-pentyl-1*H*-isochromen-1-one (5) reduced the total number of *L. pneumophila* and, also that of total bacteria after 24 h of treatment ($P < 0.05$), whereas the compound did not exhibit amoebicidal activity against *L. pneumophila* within *A. castellanii* at concentrations lower than its $IC_{50}$ value against *A. castellanii*. Thus, it is suggested that these oakmoss components could be good candidates for disinfectants to protect from *Legionella* infection.**

*Key words* : *Legionella pneumophila* / *Acanthamoeba castellanii* / Oakmoss / Antibacterial activity / Amoebicidal activity.

## INTRODUCTION

Legionellosis (Legionnaires' disease) is a severe form of pneumonia or non-pneumonic Pontiac fever caused by gram-negative bacteria belonging to the genus *Legionella*, which are widely distributed in natural water environments and artificial water supply systems. *Legionella pneumophila* is the type species of this genus and the main causative agent of legionellosis caused by the inhalation of an aerosol generated from *L. pneumophila*-contaminated water (Greub and Raoult, 2004; Molmeret et al., 2005; Rowbotham, 1980; Winiecka-Krusnell and Linder, 2001). In the natural environment, *L. pneumophila* exists as free-living plankton and/or as an intracellular parasite of protozoans, such as *Acanthamoeba* spp. (Rowbotham, 1980). *Acanthamoeba* spp. are free-living amoebae, which are commonly found in various environmental water sources throughout the world. They are known to be causative agents of *Acanthamoeba* keratitis, amoebic pneumonitis, and skin inflammation in humans (Khan, 2006; Szénási et al., 1998) and also host *L. pneumophila*. Therefore, *L. pneumophila* is often isolated from the same location from which the amoebae are isolated (Rowbotham, 1980; Sasaki et al., 2003;

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Thomas et al., 2006) for instance, from soil, river and tap water samples; hot springs; swimming pools; and in some cases, contact lens care solutions (Edagawa et al., 2009; Ettinger et al., 2003; Gianiazzi et al., 2009; Hsu et al., 2009; Huang and Hsu, 2010; Jeong et al., 2007; Kilvington et al., 2004; Rowbotham, 1980).

Intracellular *L. pneumophila* is protected from adverse conditions (Thomas et al., 2004) and shows decreased sensitivity to disinfectants not only because of its intracellular location but also consequent to phenotypic modifications (Bandyopadhyay et al., 2004; Garduño et al., 2002). In addition, *L. pneumophila* grows in biofilm, which makes the bacterium resistant to a variety of disinfectants (Barker et al., 1992; Cooper and Hanlon, 2010; Wright et al., 1991). It is, therefore, very difficult to control the number of *L. pneumophila* in natural environments and artificial water supply systems using common disinfectants. Chlorine disinfectants have been generally used to control *L. pneumophila* contamination; however, these disinfectants are not sufficiently effective against amoeba- and biofilm-associated *L. pneumophila* (Aragó et al., 2015; Dupuy et al., 2011; Garcia et al., 2007; Kim et al., 2002). In addition, Barker et al. (1992 and 1995) have reported that *L. pneumophila* grown in amoeba was less sensitive than *L. pneumophila* from cultures in broth to disinfectants and antibiotics because these bacteria undergo phenotype modifications upon intracellular growth. *L. pneumophila* replicated from *Hartmannella vermiformis* possess chlorine resistant (Chang et al., 2009).

Oakmoss, a natural fragrance ingredient, and the compounds isolated from oakmoss have been found to show specific antibacterial activity against bacteria of the genus *Legionella* (Nomura et al., 2012). These compounds also exhibit antibiofilm-forming activity against *L. pneumophila* (Nomura et al., 2013), amoebicidal activity against *A. castellanii*, and inhibitory effects on the uptake of *L. pneumophila* into *A. castellanii* cells (Nomura et al., 2015). In the present study, we focused on didepside and isochromen derivatives, which showed particularly strong antibacterial activity against planktonic *L. pneumophila*. Therefore, we examined the antibacterial activity of oakmoss and its components against total (extra- and intracellular) *L. pneumophila* within *A. castellanii* with the aim of developing a novel disinfectant that could prevent legionellosis by controlling the numbers of *L. pneumophila* and the host for its multiplication, *A. castellanii*.

## MATERIALS AND METHODS

### Fragrance ingredients and antibacterial agents

Absolute Mousse De Chene Selecta (OM; Charabot, Grasse, France) and Oakmoss Absolute AT 086 (OMAT;

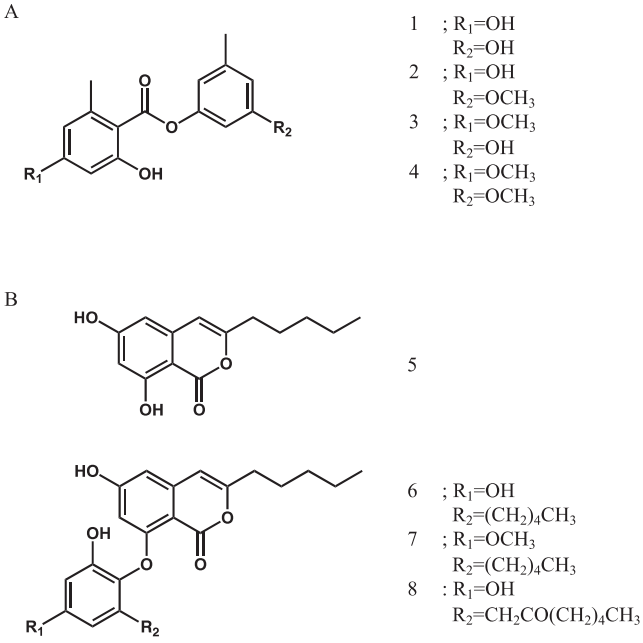


**FIG. 1**. The structure of didepside (A) and isochromen derivatives (B).

H. Reynaud & Fils, Monterun Les Bains, France) were provided by Ogawa & Co., Ltd. (Chiba, Japan) and stored at 4℃ until use. The following compounds were isolated from OM and OMAT as previously reported (Nomura et al., 2012) and used in this study (Fig. 1): 3-hydroxy-5-methylphenyl 2,4-dihydroxy-6-methylbenzoate (1), 3-methoxy-5-methylphenyl 2,4-dihydroxy-6-methylbenzoate (2), 3-hydroxy-5-methylphenyl 2-hydroxy-4-methoxy-6-methylbenzoate (3), 3-methoxy-5-methylphenyl 2-hydroxy-4-methoxy-6-methylbenzoate (4), 6,8-dihydroxy-3-pentyl-1*H*-isochromen-1-one (5), 8-(2,4-dihydroxy-6-pentylphenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one (6), 8-(2-hydroxy-4-methoxy-6-pentylphenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one (7), and 8-(2,4-dihydroxy-6-(2-oxoheptyl)phenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one (8). Sodium hypochlorite (Wako Pure Chemical Co., Ltd., Osaka, Japan) was used as a common disinfectant against *Legionella* spp. The chlorine concentration was measured by the diethyl-p-phenylenediamine method (The Pharmaceutical Society of Japan, 2005).

### Bacterial and amoeba strains

The bacterial strain, *L. pneumophila* Brenner et al. ATCC BAA-74, used in this study was provided by American Type Culture Collection (ATCC; Manassas, VA, USA). *L. pneumophila* was grown on buffered charcoal yeast extract agar supplemented with α-ketoglutarate containing L-cysteine (BCYE-α) or in BYE-α broth

(BCYE-α without charcoal and agar).

The pathogenic strain of *A. castellanii* ATCC 30234 was obtained from ATCC. The amoeba was cultured as adherent cells in peptone-yeast extract-glucose (PYG) medium in a cell culture flask at 25℃. Trophozoites in the exponential growth stage were collected by centrifugation at 250×*g* for 5 min and washed three times with Acanthamoeba (Ac) buffer (PYG medium without peptone and yeast extract). The amoebae were suspended in Ac buffer, and their concentration was determined by counting cells in a Neubauer counting chamber under an inverted microscope (DM2500, Leica Microsystems, Wetzlar, Germany). Then, the amoeba suspension was diluted with Ac buffer to give a final concentration of $1\times10^5$ cells/mL and used immediately.

**Broth dilution susceptibility testing**

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined using a modification of the broth microdilution method (Nomura et al., 2015), which is based on the standard method employed by the Clinical and Laboratory Standards Institute (CLSI, 2000).

**Cytotoxicity assay**

The cytotoxicity was carried out by MTT assay. THP-1 cells TIB-202 was provided by ATCC. The cell was cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Corp., CA, USA), 50 μM 2-mercaptoethanol, 0.1 mg/mL streptomycin (Wako) and 100 U/mL benzylpenicillin (Wako) at 37℃ with 5% $CO_2$. Cells were plated at $1\times10^6$ cells/mL and each sample at various concentrations (0.01, 0.1, 1. 10, 100 and 1000 μg/mL) was then added. After incubation for 24 h, MTT solution was added to each well (final concentration of MTT; 300 μg/mL) and incubated for an additional 3 h. The supernatant was removed, and water insoluble dark blue formazan crystals formed in viable cells were solubilized in DMSO, and the absorbance was measured at 570 nm using microplate reader (SpectraMax M5, Molecular Devices Japan, Tokyo, Japan). Cell survival was determined by comparing the absorbance values obtained for treated and untreated cells. The cytotoxicity was expressed as the concentration of sample that inhibited 50% of cell growth ($IC_{50}$).

**The skin sensitization testing**

The skin sensitization was determined using a modification of the h-CLAT protocol (Sakaguchi et al., 2006). THP-1 cells was plated at $1\times10^6$ cells/mL and treated using a concentration of each samples at $0.5\times IC_{50}$ for 24 h and 48 h. After incubation, Fc receptor blocking procedure was conducted: 0.01% of Globlins Chon fraction II, III (Shigma-Aldrich, Germany) were added for 10 min on ice. The cells were stained using a FITC-conjugated monoclonal anti-human CD54 antibody (clone: 6.5B5) for DAKO (Glostrup, Denmark) and FITC-conjugated monoclonal anti-human CD86 antibody (clone; Fun-1) from BD Pharmingen (San Diego, CA, USA). A FITC labeled-mouse IgG1 (clone; DAK-G01) form DAKO used as an isotype control. After staining, the cells analyzed using flow cytometry (Epics XL, Beckman Coulter, USA). For dead cells was gated out, propidium iodide solution was used and a total of 10,000 living cells were analyzed. Relative fluorescence intensity (RFI) was used as an indicator of CD54 and CD86 expression and was calculated by the following formula: RFI (%)=(MFI of sample-treated cells - MFI of sample-treated isotype control cells) / (MFI of vehicle control cells - MFI of vehicle isotype control cells)×100. The RFI values of ≥200% (CD54) and/or ≥150% (CD86) were judged to be positive for skin sensitization. (MFI = mean fluorescence intensity)

**Preparation of *L. pneumophila* within *A. castellanii***

A cell suspension of *A. castellanii* ($1\times10^5$ cells/mL, 100 μL) was placed in each well of a 96-well of microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and the amoebae cells were allowed to adhere to the wells and equilibrated at 37℃ for 1 h before adding the bacterium. *L. pneumophila* was incubated in BYE-α broth at 37℃ for 24 h, and the bacterial cells, harvested by centrifugation at 3,220×*g* for 5 min, were resuspended in sterile saline at a final concentration of $2\times10^7$ CFU/mL. After addition of 10 μL of the bacterial suspension to each well containing the amoeba cells (multiplicity of infection of 20), the plates were spun at 250×*g* for 20 min for the bacteria to come in contact with the amoeba cells and then incubated at 37℃ for 1 h. The amoeba cells were then washed three times with Ac buffer, and incubated at 37℃ for 1 h in Ac buffer containing 100 μg/mL gentamicin to kill extracellular bacteria, followed by additional three washes with Ac buffer.

**Evaluation of antibacterial effect**

Antibacterial effects of the oakmoss and their components against total (extra- and intracellular) *L. pneumophila* were estimated as follows. Diluted sample solutions (100 μL) of the oakmoss and their components in Ac buffer (2×, 1× or 0.5×MIC) were added to microtiter wells containing the *L. pneumophila* within *A. castellanii* cells prepared as described above. As a reference disinfectant, chlorine was used at a concentration of 0.4 μg/mL which is the recommended concentration used for disinfection of public bath or hot spring facilities (Ministry of Health, Labour and Welfare, 2001)

to avoid an outbreak of legionellosis. The plates were incubated at 37℃ for 24 h. After the incubation, culture supernatants were transferred to new plates, and the remaining amoeba cells were lysed by adding 100 µL of Ac buffer containing 0.04% Triton X-100. The reference chlorine was neutralized with 0.3% sodium thiosulfate before the culture supernatants were transferred to new plates. Aliquots of the amoeba cell lysate and culture supernatants were mixed and immediately diluted with buffered saline supplemented with 0.01% gelatin (BSG) to stabilize the bacterial cells (Guling and Doyle, 1993). Then, the mixtures were plated on BCYE-α plated and incubated at 37℃ for 72 h for colony enumeration. The data are reported as the mean±standard deviation of three separate experiments.

For evaluation of antibacterial effects against intracellular *L. pneumophila*, mixed cultures of amoeba cells and solutions of the oakmoss components were incubated as described above. After the incubation, the culture supernatants were removed, and the remaining amoeba cells were washed three times with Ac buffer and incubated at 37℃ for 1 h in 100 µL of Ac buffer containing 100 µg/mL gentamicin. The amoeba cells were then washed three times with Ac buffer and lysed as described above. Aliquots of the amoeba cell lysate diluted with BSG to stabilize the bacterial cells were plated on BCYE-α and incubated at 37℃ for 72 h for colony enumeration. The data are reported as the mean ±standard deviation of three separate experiments.

### Evaluation of effects on the survival rate of *L. pneumophila* within *A. castellanii*

*L. pneumophila* within *A. castellanii* cells in 100 µL of Ac buffer were treated with 100 µL of a test sample solution at a concentration of 0.5–2×MIC at 37℃ for 24 h. After the incubation, the culture supernatants were removed and the plates were washed three times with Ac buffer. The amoeba cells were stained with 0.2 % trypan blue (Wako Pure Chemical Ind., Ltd.), and the number of living amoeba cells was counted under a light microscope (IX50, Olympus Co., Tokyo, Japan). The survival rate of amoeba cells was calculated using the following equation: Survival rate (%) = (number of living cells in sample-treated wells) / (number of living cells in sample untreated wells)×100. For each assay, the viability of amoeba cells was evaluated using two replicates containing samples at the same concentrations. The viability of amoeba cells was analyzed using the Student's t-test, and the data are presented as the mean ±standard deviation of three separate experiments.

## RESULTS

### Antibacterial activity against *L. pneumophila*

The antibacterial activities (MIC and MBC) of the oakmoss and their components are presented in Table 1. The four didepside derivatives and four isochromen derivatives isolated from oakmoss exhibited antibacterial activities against *L. pneumophila* ATCC BAA-74, with the MICs ranging from 2.0 to 32.0 µg/mL and the MBCs ranging from 4.0 to greater than 256.0 µg/mL. Among these compounds, 5 (isochromen derivative) showed markedly high antibacterial activity; its MIC and MBC values were 2.0 µg/mL and 4.0 µg/mL, respectively. The two types of oakmoss (OM and OMAT) also showed antibacterial activities, but the MIC and MBC values of OMAT were lower than those of OM.

### Cytotoxicity and skin sensitization

The cytotoxicity and skin sensitization of the oakmoss and their components are presented in Table 2. The didepside derivatives and isochromen derivatives exhibited cytotoxicity against THP-1 cell, with the $IC_{50}$ values ranging from 30.0 to 191.9 µg/mL, and the $IC_{50}$ values of oakmoss (OM and OMAT) were 157.4 and 42.7 µg/mL. Furthermore, the skin sensitization of oakmoss and their components was evaluated by an alternative method, the h-CLAT protocol, using a human cell line. In which expression of CD54 observed in addition of compound 1 and 4 (didepside derivatives), pronounced on a skin sensitization affect of these compounds, but not CD86. Neither CD54 nor CD86 was expressed with the addition of the oakmoss, didepside derivatives (compound 2 and 3) and all of isochromen derivatives. Thus, these components did not show skin sensitization.

### Antibacterial effects on total (extra- and intracellular) *L. pneumophila*

The antibacterial effects on total (extra- and intracellular) *L. pneumophila* are shown in Fig. 2. Two of each didepside (compounds 1 and 2) and isochromen (compounds 5 and 8) derivatives reduced the number of total *L. pneumophila* after 24 h of incubation with *L. pneumophila* within *A. castellanii*. When the *L. pneumophila* within *A. castellanii* cells were incubated with compounds 1 or 2 at a concentration of 2×MIC, the mean numbers of *L. pneumophila* were reduced to 2.5 $\pm 4.0\times10^4$ and $1.0\pm1.2\times10^5$ CFU/mL, respectively, compared with the control ($2.0\pm1.5\times10^6$ CFU/mL). In the case of the isochromen derivatives (compounds 5 and 8), the mean numbers of *L. pneumophila* were reduced to $1.0\pm0.9\times10^2$ and $1.3\pm1.1\times10^3$ CFU/mL, respectively, after 24 h of compound incubation with *L. pneumophila* within *A. castellanii*. The antibacterial effects of these compounds on total *L. pneumophila* were stronger than those of the didepside derivatives (compounds 1 and 2). In contrast, the other didepside (compounds 3 and 4) and isochromen (compounds 6

**TABLE 1**. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) values of components isolated from oakmoss and showing antibacterial activities against *L. pneumophila* and amoebicidal activities against *A. castellanii*.

| | *L. pneumophila* Brenner et al. ATCC BAA-74 | | *A. castellanii* ATCC 30234 |
|---|---|---|---|
| | MIC (μg/mL)[a)] | MBC (μg/mL)[a)] | $IC_{50}$ (μg/mL)[b)] |
| Natural fragrance ingredients | | | |
| OM | 32.0 | 212.7 | 81.3±5.8 |
| OMAT | 16.0 | 128.0 | 69.3±16.9 |
| Didepside derivatives | | | |
| 1 | 8.0 | 32.0 | 29.6±3.0 |
| 2 | 16.0 | 85.3 | >100.0 |
| 3 | 8.0 | 53.3 | >100.0 |
| 4 | 8.0 | >256.0 | >100.0 |
| Isochromen derivatives | | | |
| 5 | 8.0 | 26.7 | 22.8±4.6 |
| 6 | 2.0 | 4.0 | >100.0 |
| 7 | 32.0 | >256.0 | >100.0 |
| 8 | 16.0 | 32.0 | 82.9±6.9 |

[a)] MIC and MBC values are the means obtained from three independent experiments, each performed in triplicate.
[b)] $IC_{50}$ values are referenced from Nomura et al. (2015).

**TABLE 2**. The cytotoxicity against THP-1 cell and skin sensitization.

| | $IC_{50}$ (μg/mL) | CD54 | | CD86 | | Skin sensitization |
|---|---|---|---|---|---|---|
| | | 24 h | 48 h | 24 h | 48 h | |
| Natural fragrance ingredients | | | | | | |
| OM | 157.4 | − | − | − | − | |
| OMAT | 42.7 | − | − | − | − | |
| Didepside derivatives | | | | | | |
| 1 | 35.2 | + | + | − | − | Positive |
| 2 | 34.7 | − | − | − | − | |
| 3 | 30.0 | − | − | − | − | |
| 4 | 191.9 | + | + | − | − | Positive |
| Isochromen derivatives | | | | | | |
| 5 | 34.5 | − | − | − | − | |
| 6 | 45.6 | − | − | − | − | |
| 7 | 33.6 | − | − | − | − | |
| 8 | 33.2 | − | − | − | − | |

+ : Positive, − : Negative

and 7) derivatives did not show significant antibacterial effects on either extra- or intracellular *L. pneumophila*. Sodium hypochlorite, a common disinfectant, did not show antibacterial effects on extra- or intracellular *L. pneumophila* even at the commended concentration (0.4 μg/mL) recommended for disinfection to avoid an outbreak of legionellosis.

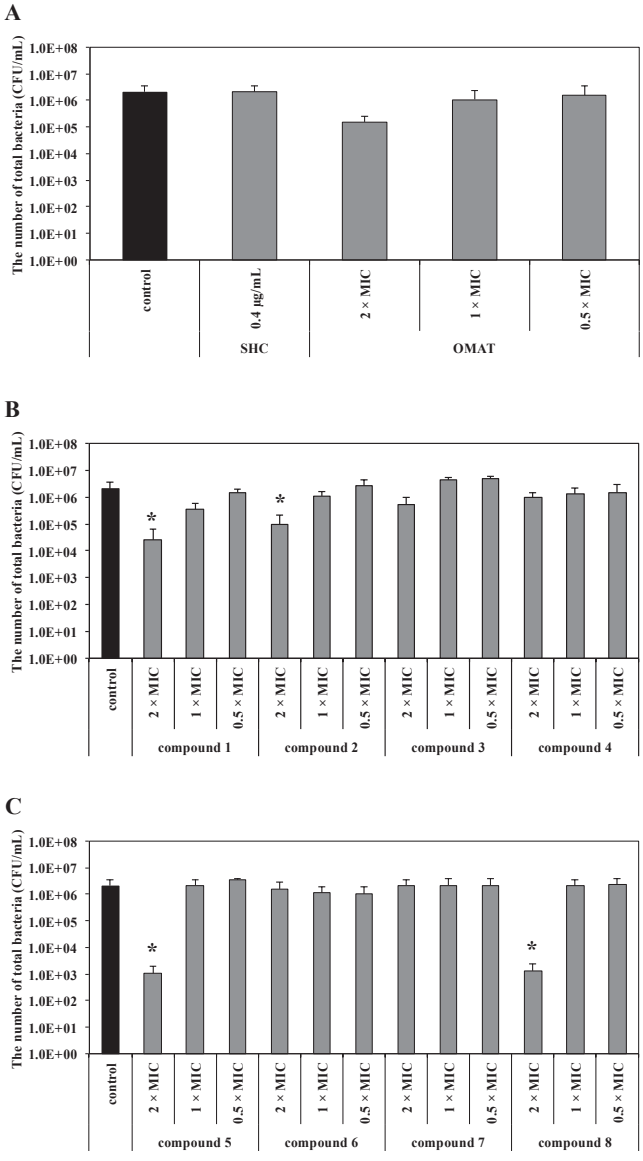


**FIG. 2**. Antibacterial effects on total (extra- and intracellular) *Legionella pneumophila*. Amoebae infected with bacterial cells were incubated in Ac buffer containing (A) sodium hypochlorite (SHC) and OMAT, (B) didepside derivatives (compounds 1, 2, 3, and 4), and (C) isochromen derivatives (compounds 5, 6, 7, and 8). The MIC values were OMAT, 16.0 μg/mL; compound 1, 8.0 μg/mL; compound 2, 16.0 μg/mL; compound 3, 8.0 μg/mL; compound 4, 8.0 μg/mL; compound 5, 8.0 μg/mL; compound 6, 2.0 μg/mL; compound 7, 32.0 μg/mL; and compound 8, 16.0 μg/mL. SHC was used at a concentration of 0.4 μg/mL. ($^*P < 0.05$)

### Antibacterial effects on *L. pneumophila* within *A. castellanii*

The antibacterial effects of compounds 1, 2, 5, and 8 on intracellular *L. pneumophila* are shown in Fig. 3. Similar to the results obtained for total *L. pneumophila*, two of each didepside (compounds 1 and 2) and isochromen (compounds 5 and 8) derivatives reduced the numbers of intracellular *L. pneumophila*. Compounds 1 and 2 reduced the mean numbers of intracellular *L. pneumophila* to $4.0 \pm 3.5 \times 10^1$ and $1.4 \pm 2.1 \times 10^2$ CFU/mL, respectively, at a concentration of 2×MIC, and $4.4 \pm 2.3 \times 10^2$ and $2.7 \pm 0.1 \times 10^3$ CFU/mL, respectively, at a concentration of 1×MIC. Similarly, compounds 5 and 8 reduced the mean numbers of intracellular *L. pneumophila* to $2.1 \pm 2.7 \times 10^2$ and $7.3 \pm 6.5 \times 10^2$ CFU/mL, respectively. Sodium hypochlorite was ineffective against intracellular *L. pneumophila*.

### Effects on the survival rate of *L. pneumophila* within *A. castellanii*

Amoebicidal effects of compounds 1, 2, 5, and 8 were examined against *L. pneumophila* within *A. castellanii*. Fig. 4 shows the survival rates of *L. pneumophila* within *A. castellanii* after 24 h of incubation with these compounds. The two didepside derivatives (compounds 1 and 2) and an isochromen derivative (compound 8) exhibited high amoebicidal effects against *L. pneumophila* within *A. castellanii* at a concentration of 2×MIC, even though their 2×MIC values are lower than the respective $IC_{50}$ values of against *A. castellanii* (see Table 1). The amoeba survival rates in the presence of these compounds ranged from 24.4±5.2% to 44.4±14.3% at a concentration of 2×MIC. Compound 5 (isochromen derivative) did not exhibit any amoebicidal effect on *L. pneumophila* within *A. castellanii*; on the contrary, the survival rate (ranging from 101.3±13.5% to 121.9±13.3%) was almost equivalent to or slightly higher than that in the control. Sodium hypochlorite showed no effect on the survival rate of *L. pneumophila* within *A. castellanii*.

## DISCUSSION

We have previously reported that natural fragrance ingredient oakmoss and several of their components exhibit specific antibacterial activities against *L. pneumophila*, and some of them are antiamoebic agents (Nomura et al., 2012; Nomura et al., 2013; Nomura et al., 2015). In the present study, we examined the antibacterial effects of oakmoss and their components on *L. pneumophila* within *A. castellanii*.

The oakmoss (OM and OMAT), four didepside derivatives, and four isochromen derivatives exhibited antibacterial activities against planktonic *L. pneumophila*; in particular, the MIC and MBC values of compound 6 were the lowest among the compounds (see Table 1). Compound 4 and 7 only showed bacteriostatic activity. Furthermore, the oakmoss and their components were evaluated cytotoxicity (Table 2). The three didepside derivatives (compound 1, 2 and 3) and all of isochromen

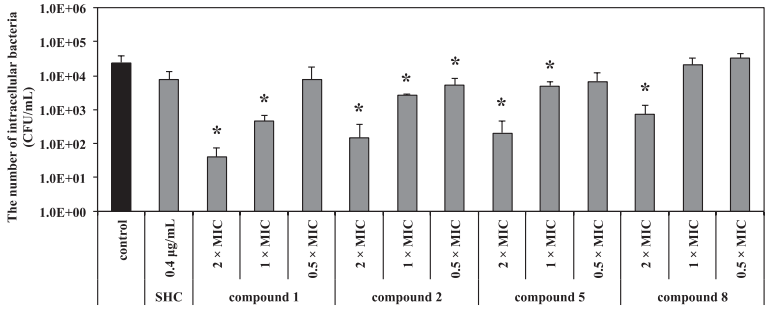


**FIG. 3**. Antibacterial effects on *L. pneumophila* within *A. castellanii*. Amoebae infected with bacterial cells were incubated in Ac buffer containing compounds 1, 2, 5, 8, and sodium hypochlorite (SHC). The MIC values were as follows: compound 1, 8.0 μg/mL; compound 2, 16.0 μg/mL; compound 5, 8.0 μg/mL; and compound 8, 16.0 μg/mL. SHC was used at a concentration of 0.4 μg/mL. ($^{*}P < 0.05$)

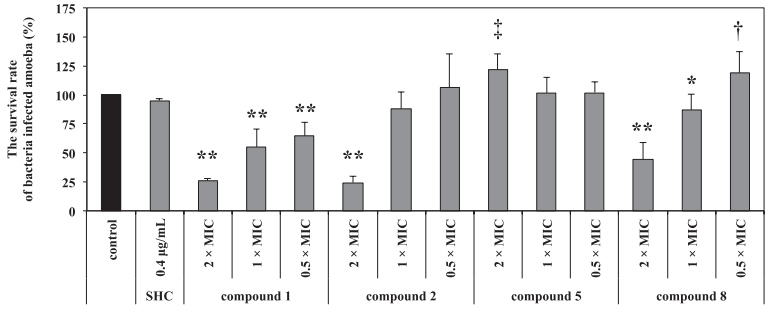


**FIG. 4**. Amoebicidal effects on *L. pneumophila* within *A. castellanii*. *L. pneumophila* within *A. castellanii* was incubated in Ac buffer containing compounds 1, 2, 5, 8, and sodium hypochlorite (SHC). The MIC values were as follows: compound 1, 8.0 μg/mL; compound 2, 16.0 μg/mL; compound 5, 8.0 μg/mL; and compound 8, 16.0 μg/mL. SHC was used at a concentration of 0.4 μg/mL. ($^{**, ‡}P < 0.001$, $^{*, †}P < 0.01$)

derivatives showed cytotoxicity against THP-1 cell, but their $IC_{50}$ values were higher than MICs of their components against planktonic *L. pneumophila*. In addition, oakmoss and their components were evaluated skin sensitization. Compound 1 and 4 of didepside derivative showed skin sensitization at a concentration of 0.5 $\times IC_{50}$, but other didepside derivatives (compound 2 and 3), isochromen derivatives and oakmoss did not show skin sensitization (Table 2). Thus, the results were considerable that the oakmoss and its components exhibit antibacterial activity without human skin affect even when used at the concentration of antiseptic use.

Chlorine disinfectants are commonly used to control *L. pneumophila* and prevent legionellosis. However, García et al. (2007) reported that intracellular *L. pneumophila* within *Acanthamoeba polyphaga* and *L. pneumophila*-infected *A. polyphaga* exhibited high resistance to chlorine disinfectants. In other cases, chlorine disinfectants were only mildly effective against amoeba-associated *L. pneumophila* (Aragó et al., 2015; Dupuy et al., 2011; Kim et al., 2002). Barker et al. (1992 and 1995) and Chang et al. (2009) were reported that *L. pneumophila* grown in amoeba was less sensitive than planktonic *L. pneumophila* to disinfectants, containing chlorine, and antibiotics. In the present study, we also observed that sodium hypochlorite did not show any marked effects on extra- and intracellular *L. pneumophila* and on *L. pneumophila* within *A. castellanii*. Therefore, development of new type of disinfectant(s), which is really effective for not only control of free-living *L. pneumophila* and amoebae but also for growth inhibition of *L. pneumophila* within *Acanthamoeba* cells, is desired, instead of chlorine disinfectants. We evaluated the effects of long-term exposure to oakmoss and its components on total (extra- and intracellular) *L. pneumophila* and *L. pneumophila* within *A. castellanii*. Compound 6 did not reduce the number of total *L. pneumophila* but exhibited the highest bactericidal activity against free-living planktonic *L. pneumophila*,

showing that *L. pneumophila* cells proliferating within *A. castellanii* cells are much more resistant to this compound. Compounds 1, 2, and 8 reduced the numbers of total (extra- and intracellular) and intracellular *L. pneumophila* and, also reduced the numbers of *L. pneumophila* within *A. castellanii*. Since the concentrations of these compounds used were lower than their $IC_{50}$ values, it was suggested that cells of *A. castellanii* infected with *L. pneumophila* were somewhat more susceptible to these compounds than non-infected cells were. The reduction of the numbers of total (extra- and intracellular) and intracellular *L. pneumophila*, caused by these compounds, might be due to the reduction of the number of *L. pneumophila* within *A. castellanii*. Compound 5 exhibited antibacterial activity against total (extra- and intracellular) and intracellular *L. pneumophila* but did not show any amoebicidal activity, although the $IC_{50}$ value of this compound is lower than those of compounds 1, 2, and 8. It was suggested that compound 5 inhibited bacterial growth within *A. castellanii* by passing through the amoeba membrane without causing any damage to the amoeba because this compound reduced the numbers of both total and intracellular *L. pneumophila* to the same CFU levels, and the amoebae survived slightly more in the presence of the compound than control. Although the antiamoebic mechanism of these compounds is not clear at present, the cell membrane of *A. castellanii* might be altered by infection with *L. pneumophila* to become susceptible or resistant to the respective compounds. It was reported that *L. pneumophila* replicated from amoeba possess chlorine resistant (Chang et al., 2009). It is suggested that compound 5 may be a useful disinfectant to replace the chlorine disinfectants, because compound 5 showed strong antibacterial activity against extracellular *L. pneumophila* (Fig. 2 and 3).

As we have previously reported, the oakmoss components show antibacterial activities against *Legionella* spp. (Nomura et al., 2012), antibiofilm forming activities, and bactericidal activities against *L. pneumophila* in biofilm (Nomura et al., 2013), as wells as amoebicidal activities against *A. castellanii* and inhibition of the uptake of *L. pneumophila* into *A. castellanii* (Nomura et al., 2015). In addition, as described above, the oakmoss components exhibit antibacterial activities against total (extra- and intracellular) *L. pneumophila* and antiamoebic activities against *L. pneumophila* within *A. castellanii*. Although compound 1 and 4 induced skin sensitization, a minimal concentration of each compound showing the toxicity was higher than that exhibiting the antibacterial activity against extra- and intracellular *L. pneumophila*. It is therefore suggested that the use of these compounds in combination may lead to the development of a new and safety disinfection procedure for controlling multiple stages of the *L. pneumophila* life cycle as well as *A. castellanii*.

## CONFLICT OF INTEREST

The authors have no conflicts of interest directly relevant to the content of this article.

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