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Original

Effects of Oakmoss and its Components on *Acanthamoeba castellanii* ATCC 30234 and the Uptake of *Legionella pneumophila* JCM 7571（ATCC 33152）into *A. castellanii*

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***Acanthamoeba castellanii*, a ubiquitous organism in water environments, is pathogenic toward humans and also is a host for bacteria of the genus *Legionella*, a causative agent of legionellosis. Oakmoss, a natural fragrance ingredient, and its components are antibacterial agents specifically against the genus *Legionella*. In the present study, oakmoss and its components were investigated for their amoebicidal activity against *A. castellanii* ATCC 30234 and the inhibitory effect on the uptake of *L. pneumophila* JCM 7571（ATCC 33152）into *A. castellanii*. The oakmoss and its components 3-hydroxy-5-methylphenyl 2,4-dihydroxy-6-methylbenzoate（5）, and 6,8-dihydroxy-3-pentyl-1*H*-isochromen-1-one（12）exhibited high amoebicidal activity（IC_{50} values；10.5 ± 2.3, 16.3 ± 4.0 and 17.5 ± 2.8 μg/mL, respectively）after 48 h of treatment, which were equivalent to that of the reference compound, chlorhexidine gluconate. Pretreatment of *L. pneumophila* with sub-minimal inhibitory concentration of oakmoss, compound 5, 3-hydroxy-5-methylphenyl 2-hydroxy-4-methoxy-6-methylbenzoate（10）and 8-（2,4-dihydroxy-6-pentylphenoxy）-6-hydroxy-3-pentyl-1*H*-isochromen-1-one（14）obviously reduced the uptake of *L. pneumophila* into *A. castellanii*（$p < 0.05$）. The inhibitory effect of compound 5 on the uptake of *L. pneumophila* was almost equivalent to that of ampicillin used as a reference. Thus, the oakmoss and its components were considered to be good candidates for disinfectants against not only genus *Legionella* but also *A. castellanii*.**

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

INTRODUCTION

Acanthamoeba is a free-living amoeba that is commonly found in various environmental water sources throughout the world. These organisms have been successfully isolated from soil, river and tap water samples, hot springs, swimming pools and 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；Thomas et al., 2006）. *Acanthamoeba* are known to cause *Acanthamoeba* keratitis, amoebic pneumonitis and skin inflammation in humans（Khan, 2006；Szénási et al., 1998）.

Acanthamoeba have at least two developmental stages；the trophozoite, a vegetative feeding form, and the cyst, a resting form. Cysts exhibit resistance to disinfectants（Khunkitti et al., 1998；Turner et al., 2000）. The trophozoite, a metabolically active stage, feeds on bacteria and multiplies by binary fission. In particular, *Acanthamoeba* are hosts of *Legionella pneumophila*, a waterborne pathogenic bacterium responsible for Legionnaires' disease（Greub and Raoult, 2004；Molmeret et al., 2005；Rowbotham, 1980；Winiecka-Krusnell and Linder, 2001）. *L. pneumophila* is

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often isolated from the same location from where the amoebae are isolated (Rowbotham, 1980; Sasaki et al., 2003; Thomas et al., 2006). Intracellular *L. pneumophila* is protected from adverse conditions (Thomas et al., 2004), and shows decreased sensitivity to disinfectants not only because of their intracellular location but also as a result of phenotypic modifications (Bandyopadhyay et al., 2004; Garduño et al., 2002).

It is very important to control the number of *L. pneumophila* in natural and manmade water systems to prevent outbreaks of legionellosis. Various disinfectants have been used to control *L. pneumophila*, but control is very difficult because *L. pneumophila* grows in biofilm or in amoeba cells making it resistant to a variety of disinfectants (Barker et al., 1992; Cooper and Hanlon, 2010; Kim et al., 2002; Wright et al., 1991). Since *L. pneumophila* multiplies in *Acanthamoeba* cells, it is important to control the cell and also to inhibit the uptake of the bacteria into *Acanthamoeba* cells.

Oakmoss, a natural fragrance ingredient, and compounds isolated from oakmoss are known antibacterial agents that are specifically active against bacteria of the genus *Legionella* (Nomura et al., 2012). These compounds also exhibit anti-biofilm forming activity against *L. pneumophila* (Nomura et al., 2013). In the current study, we examined the amoebicidal activity of oakmoss and its components against *A. castellanii* and their inhibitory effects on the uptake of *L. pneumophila* into *A. castellanii* with an aim of developing a novel disinfectant that could prevent legionellosis by controlling the numbers of both *L. pneumophila* and *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, H. Reynaud & Fils, Monterun Les Bains, France) were provided by Ogawa & Co., Ltd. (Chiba, Japan) and stored at 4℃ until use. Chlorhexidine gluconate (CHG) and ampicillin (ABPC) (Wako Pure Chemical Co., Ltd., Osaka, Japan) were used as reference compounds. The following compounds which have been isolated from OM and OMAT as previously reported (Nomura et al., 2012) were used; 5-methylbenzene-1,3-diol (**1**), 3-methoxy-5-methylphenol (**2**), 2,6-dihydroxy-4-methylbenzaldehyde (**3**), methyl 2,4-dihydroxy-6-methylbenzoate (**4**), 3-hydroxy-5-methylphenyl 2,4-dihydroxy-6-methylbenzoate (**5**), ethyl 2,4-dihydroxy-6-methylbenzoate (**6**), methyl 2,4-dihydroxy-3,6-dimethylbenzoate (**7**), isopropyl 2,4-dihydroxy-6-methylbenzoate (**8**), 3-methoxy-5-methylphenyl 2,4-dihydroxy-6-methylbenzoate (**9**), 3-hydroxy-5-methylphenyl 2-hydroxy-4-methoxy-6-methylbenzoate (**10**), ethyl 2-hydroxy-4-methoxy-6-methylbenzoate (**11**), 6,8-dihydroxy-3-pentyl-1*H*-isochromen-1-one (**12**), ethyl 3-formyl-2,4-dihydroxy-6-methylbenzoate (**13**), 8-(2,4-dihydroxy-6-pentylphenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one (**14**), isopropyl 3-formyl-2,4-dihydroxy-6-methylbenzoate (**15**), 3-methoxy-5-methylphenyl 2-hydroxy-4-methoxy-6-methylbenzoate (**16**), 8-(2-hydroxy-4-methoxy-6-pentylphenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one (**17**), 2,5-dimethylbenzene-1,3-diol (**18**), 3-chloro-2,6-dihydroxy-4-methylbenzaldehyde (**19**), 8-(2,4-dihydroxy-6-(2-oxoheptyl)- phenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one (**20**).

Legionella strain, *Acanthamoeba* strain and culture

Legionella pneumophila JCM 7571 (ATCC 33152) used in this study was grown on buffered charcoal yeast extract agar supplemented with α-ketoglutarate and L-cysteine (BCYE-α) or in BYE-α broth (BCYE-α without charcoal and agar).

The pathogenic strain of *Acanthamoeba castellanii* ATCC 30234 was obtained from the American Type Culture Collection. The amoeba was cultured as adherent cells in PYG (Peptone-Yeast extract-Glucose) medium using 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 buffer (Ac buffer; PYG medium without proteose peptone and yeast extract). The amoebae were suspended in the Ac buffer and the concentration was determined by counting cells using the Neubauer counting chamber under an inverted microscope (DM2500, Leica Microsystems, Wetzlar, Germany) and then, the amoeba suspension was diluted with Ac buffer to give a final concentration of 4×10^6 cells/mL, 2×10^5 cells/mL or 1×10^5 cells/mL, and used immediately.

Estimation of amoebicidal activity

Oakmoss and its components were dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mg/mL (CHG and ABPC were dissolved in water). These samples were diluted with Ac buffer to 200, 20 and 2 μg/mL. DMSO showed no effect on the number of the amoebae at the concentration used in this study (data not shown). The amoebicidal activity of samples was determined by the alamarBlue® assay as previously described (McBride et al., 2005; Martín-Navarro et al., 2008). A total of 50 μL of *A. castellanii* suspension (4×10^6 cells/mL) was seeded in duplicate on a 96 well microtiter plate (Greiner Bio-One Co. Ltd., Frickenhausen, Germany) and the amoebae cells were allowed to adhere to the wells at 37℃ for 3 h. Then, 50 μL of the

test sample solutions in Ac buffer were added to each well and incubation was done at 37℃ for 24 or 48 h. At 6 h prior to the end of incubation, 10 μL of alamarBlue® reagent (Invitrogen, Carlsbad, CA, USA) was added to each well and the plates were further incubated at 37℃ for 6 h in the dark. The intensity of the fluorescence of the reduced alamarBlue® dye was measured using a SpectraMax M5 (Molecular Devices Japan, Tokyo, Japan) at an excitation wavelength of 560 nm and emission wavelength of 590 nm. The data are reported as the mean±standard deviation of three separate observations.

Effects of oakmoss and its components on the uptake of *L. pneumophila* into *A. castellanii*

The MIC of ABPC against *L. pneumophila* JCM 7571 (ATCC 33152) was estimated according to the modified broth microdilution method (Takeda et al., 2008) based on the standard method of Clinical and Laboratory Standards Institute (CLSI, 2000). The MICs of OMAT and its components against *L. pneumophila* JCM 7571 (ATCC 33152) were previously reported (Nomura et al., 2012). The experiments were performed by a partially modified method of Lück et al. (1998) and Moffat and Tompkins (1992). For measurement of the inhibitory effect on the uptake of *L. pneumophila* into *A. castellanii*, the amoebae and the bacterial cells were co-incubated by the following methods a) – c).

In method a), *A. castellanii* was suspended in Ac buffer at a concentration of 1×10^5 cells/mL and 100 μL of the suspension was then placed in each well of a 96 well microtiter plate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan). The amoebae were allowed to adhere to the wells and equilibrated at 37℃ for 1 h before the bacteria were added. *L. pneumophila* was incubated in BYE-α broth containing 0.25×MIC of ABPC, OMAT, compound **5**, **10**, **12** or **14** for 24 h, and the bacterial cells harvested by centrifugation at 3220×g for 5 min was suspended in sterile saline at a final concentration of 1×10^7 CFU/mL. Ten μL of the bacterial suspension was added to each well containing *A. castellanii* (a multiplicity of infection (MOI) of 10).

In method b), *A. castellanii* cells were suspended in Ac buffer at a concentration of 2×10^5 cells/mL and 50 μL of the suspension was then placed in each well of a 96 well microtiter plate. The amoebae were allowed to adhere to the wells and equilibrated at 37℃ for 1 h before the bacteria were added. *L. pneumophila* cultivated in BYE-α broth was diluted with sterile saline to give a concentration of 1×10^7 CFU/mL. Into the each well containing amoebae, 10 μL (MOI 10) of the bacterial suspension and 40 μL of a solution of ABPC, OMAT, compound **5**, **10**, **12** or **14** (0.5×MIC, final concentration 0.25×MIC) were added.

In method c), *A. castellanii* was incubated in PYG medium containing 0.1×MIC of ABPC, OMAT, compound **5**, **10**, **12** or **14** for 4 days, and the suspension was centrifuged at 250×g for 5 min. The *A. castellanii* pellet was suspended in Ac buffer at a concentration of 1×10^5 cells/mL and 100 μL of the suspension was then placed in each well of a 96 well microtiter plate. The amoebae were allowed to adhere to the wells and equilibrated at 37℃ for 1 h before the bacteria were added. Then, 10 μL of the bacterial suspension prepared as described in b) was added (MOI 10).

All of the microtiter plates prepared as a) – c) were spun at 250×g for 20 min to make the bacteria contact the amoebae and incubated at 37℃ for 1 h. At the end of the infection period, the amoebae cells were washed three times with Ac buffer, and incubated at 37℃ for 1 h in Ac buffer containing 100 μg/mL gentamicin to kill the extracellular bacteria. The amoebae cells were washed again, and lysed by adding final 0.04% Triton X-100. Aliquots of the amoebae cell lysates were immediately diluted with buffered saline supplemented with 0.01% gelatin to stabilize the bacterial cells (Guling and Doyle, 1993), plated on BCYE-α plates and incubated at 37℃ for 72 h for colony enumeration. All assays were performed in triplicate and the numbers (CFU/mL) of viable intracellular bacteria were analyzed using the Student's *t*-test, and data are reported as the mean ± standard deviation of three separate observations.

RESULTS

Amoebicidal activity of oakmoss and its components against *A. castellanii*

The amoebicidal activity of oakmoss and its components was examined by estimating the IC_{50} value against *A. castellanii* trophozoites. The survival rate was estimated by the alamarBlue® assay. In this experiment, oakmoss and its twenty components were selected: twelve phenol derivatives (compounds **1**, **2**, **3**, **4**, **6**, **7**, **8**, **11**, **13**, **15**, **18** and **19**), four didepside derivatives (compounds **5**, **9**, **10** and **16**) and four isochromen derivatives (compounds **12**, **14**, **17** and **20**). All of them have previously been reported to exhibit antibacterial activity against *L. pneumophila* (Nomura et al., 2012). Amoebicidal activities of oakmoss and its components against *A. castellanii* are shown in Table 1. Six phenol derivatives (compounds **6**, **7**, **8**, **13**, **15** and **19**), one didepside derivative (compound **5**) and two isochromen derivatives (compounds **12** and **20**), had amoebicidal activity after 48 h of treatment. Compounds **5**, **8**, **12** and **19** exhibited the highest amoebicidal activity, with IC_{50} values of 29.6±3.0, 34.4 ±4.1, 22.8±4.6 and 34.9±6.0 μg/mL (for 24 h) and

TABLE 1. Cytotoxicity of oakmoss and its components against *A. castellanii* ATCC 30234.

	IC_{50} (μg/mL)	
	24 h	48 h
Natural fragrance ingredient		
OM	81.3±5.8	24.8±4.9
OMAT	69.3±16.9	10.5±2.3
Phenol derivatives		
1	>100.0	>100.0
2	>100.0	>100.0
3	>100.0	>100.0
4	>100.0	>100.0
6	>100.0	21.1±8.8
7	91.0±0.7	23.9±1.6
8	34.4±4.1	26.7±2.6
11	>100.0	>100.0
13	>100.0	30.5±12.9
15	65.8±7.6	30.5±2.9
18	>100.0	>100.0
19	34.9±6.0	24.7±2.9
Didepside derivatives		
5	29.6±3.0	16.3±4.0
9	>100.0	>100.0
10	>100.0	>100.0
16	>100.0	>100.0
Isochromen derivatives		
12	22.8±4.6	17.5±2.8
14	>100.0	>100.0
17	>100.0	>100.0
20	82.9±6.9	47.8±3.1
Other compounds		
ABPC[a)]	>100.0	>100.0
CHG[b)]	31.4±1.2	13.2±4.8

a) ampicillin. b) chlorhexidine gluconate.

16.3±4.0, 26.7±2.6, 17.5±2.8 and 24.7±2.9 μg/mL (for 48 h), respectively. The amoebicidal activities of **5** and **12** were almost equivalent to those of the reference compound CHG, which is known to exhibit high amoebicidal activity (Borazjani et al., 2000). The two oakmoss samples (OMAT and OM) themselves exhibited amoebicidal activity. The IC_{50} value of OMAT after 48 h of treatment was less than that of OM ($p < 0.01$). The amoebicidal activity of OMAT after 48 h of treatment was equivalent to that of CHG, although the amoebicidal activity of OMAT after 24 h of treatment was less than that of CHG ($p < 0.05$). Other phenol, didepside and isochromen derivatives did not exhibit any amoebicidal activity even at the concentration of 100 μg/mL.

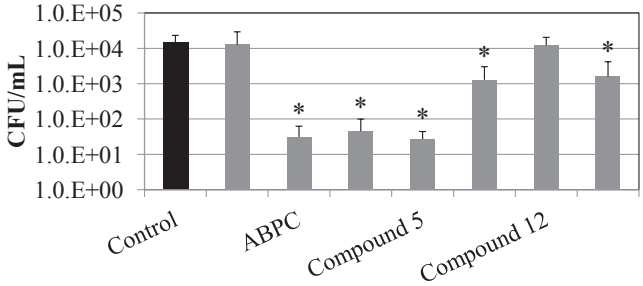

FIG. 1. Effects of oakmoss and its components on the uptake of *L. pneumophila* JCM 7571 (ATCC 33152) into *A. castellanii* ATCC 30234 at subinhibitory concentrations. *A. castellanii* was added to *L. pneumophila*, which was cultured in the presence of each sample. The plate was incubated at 37℃ for 1 h. The MIC values were ABPC; 2.0 μg/mL, OMAT; 10.7 μg/mL, compound **5**; 8.0 μg/mL, compound **10**; 4.0 μg/mL, compound **12**; 1.7 μg/mL and compound **14**; 2.0 μg/mL. Values were analyzed using Student's *t*-test and data were presented as the mean ± standard deviation of three separate observations ($^*p < 0.05$ compared to the control sample).

Effects on the uptake of *L. pneumophila* into *A. castellanii*

In the present study, the inhibitory effects of oakmoss and its components **5**, **10**, **12** and **14** on the uptake of *L. pneumophila* into *A. castellanii* were examined at a concentration of 0.25×MIC. Compounds **5** and **12** are didepside and isochromen derivatives, respectively, and they exhibited high amoebicidal activity. Compounds **10** and **14** are also didepside and isochromen derivatives, respectively, but they did not show any amoebicidal activity. After pre-treatment of *L. pneumophila* with sub-MIC concentrations of OMAT and compound **5**, the uptake of *L. pneumophila* into *A. castellanii* were significantly inhibited ($p < 0.05$) (Fig. 1). The mean value of the uptake into *A. casrellanii* observed for the untreated control ($2.1\times10^4\pm9.5\times10^3$ CFU/mL) was reduced to $4.5\times10^1\pm5.5\times10^1$ CFU/mL (OMAT), and $2.7\times10^1\pm1.8\times10^1$ CFU/mL (compound **5**). These activities were equivalent to that of the reference ABPC ($3.1\times10^1\pm3.2\times10^1$ CFU/mL), which is known to reduce the uptake of *L. pneumophila* into *A. casrellanii* (Lück et al., 1998). Compound **10** and **14** also showed inhibitory activity against the uptake of *L. pneumophila*, even their activities were lower than that of OMAT and compound **5**; the mean value of the uptake into *A. casrellanii* observed for the untreated control ($2.1\times10^4\pm9.5\times10^3$ CFU/mL) was reduced to $1.3\times10^3\pm1.7\times10^3$ CFU/mL (compound **10**), and $1.6\times10^3\pm2.6\times10^3$ CFU/mL

(compound **14**).

In contrast, compound **12**, which showed high amoebicidal activity and as well bactericidal activity against *L. pneumophila* (Nomura et al., 2012), did not show inhibitory effect on the uptake of *L. pneumophila* into *A. castellanii*. Compound **14** exhibited a weak inhibitory effect on the uptake. Though the pretreatment of bacterial cells with OMAT and compound **5** affected the uptake of *L. pneumophila* as described above, pretreatment of *A. castellanii* cells or the simultaneous presence of these compounds in the medium where *A. castellanii* and *L. pneumophila* cells were in contact with each other did not affect the uptake of *L. pneumophila*.

DISCUSSION

We reported the antibacterial activity and anti-biofilm forming activity of oakmoss and its components against *L. pneumophila* (Nomura et al., 2012 and 2013). In the present study, we examined their amoebicidal activity and the inhibitory effect on the uptake of *L. pneumophila* into *A. castellanii*. Since the high amoebicidal activity of CHG against *A. castellanii* trophozoites has been reported (Borazjani et al., 2000), we used CHG as a reference compound.

Six phenol derivatives, one didepside derivative and two isochromen derivatives exhibited amoebicidal activity, in particular, the IC_{50} values of compounds **5** and **12** were almost equivalent to that of CHG. Among the phenol derivatives, compounds **4**, **6**, **8** and **11** share a common structure of 6-methylbenzoic acid. The differences in the effects of these compounds on *A. castellanii* may be attributed to the type of the ester group and methoxyl group on position 4. It is reported that the lipophilic nature of the molecule affect antibacterial and cytotoxic activity (Gomes et al., 2003 and 2006).

Three (compounds **9**, **10** and **16**) out of four didepside derivatives were inactive against *A. castellanii*. The chemical structures of these didepside derivatives are closely related to each other. The differences in the observed effects of these compounds on *A. castellanii* may have arisen from the number and/or position of the substitution of the hydroxyl group with a methoxyl group, resulting in different physicochemical characteristics and hence, different interactions with the *A. castellanii* cell membrane.

This might be also true in the case of isochromen derivatives; the chemical structures of two active (compounds **12** and **20**) and two inactive (compounds **14** and **17**) compounds are closely related except for the number and/or position of the substitution of the hydroxyl group with a methoxyl group. Oakmoss is a natural fragrance ingredient comprising more than 20 identified compounds. These compounds might confer the amoebicidal activity on oakmoss itself in an additive or a synergistic manner.

Lück et al. (1998) reported that the pre-treatment of *L. pneumophila* with sub-MIC levels of antibiotics such as ABPC, imipenem, ciprofloxacin, erythromycin and rifampicin reduced the uptake of the bacteria into *A. castellanii*. In their report, it is described that ABPC reduced the uptake of the bacteria into *A. castellanii* to 40 %, however, our investigation revealed that ABPC reduced it to approximately 0.15 %. The higher reduction of the uptake observed in our investigation than that reported by Lück et al. might be caused by the use of a different strain of *A. castellanii* and also by the different culture used for uptake inhibition tests (Lück et al. used *N*-(2-acetamido)-2-aminoethanesulfonic acid buffered yeast extract medium and we used the Ac buffer). In addition, we observed that the mean uptake of the untreated control into amoebae amounted to $2.1 \times 10^4 \pm 9.5 \times 10^3$ CFU/mL which was approximately 50 times higher than that reported by Lück et al. Although the precise reason is not known, these different experimental conditions might lead to the different results of uptake reduction by ABPC. Beta-lactum antibiotics (ABPC and imipenem) inhibit D-alanin transpeptidase resulting in disruption of murein synthesis and subsequent structural disturbance of the outer membrane. Lück et al. (1998) speculated that the outer membrane and/or lipopolysaccharide (LPS) structures that may play a role in the adhesion and/or uptake of legionellae could be affected by the sub-MIC level of ABPC and imipenem.

The pre-treatment of *L. pneumophila* with compound **5** in our investigation led to significant inhibition of the uptake of the bacteria into *A. castellanii* to the same extent with that of ABPC. Since compound **5** is not an antibiotic, its inhibitory mechanism may differ from that of ABPC. Although the exact inhibitory mechanism is not known at present, compound **5** may interact with the outer membrane and/or LPS of *L. pneumophila* because of its amphiphilic characteristic. The O-specific polysaccharide constituting the LPS of *L. pneumophila* JCM 7571 (ATCC 33152) (serogroup O1) is a homopolymer of 5-acetamidino-7-acetamido-8-*O*-acetyl-3, 5, 7, 9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid (legionaminic acid) (Knirel et al. 1994) and is highly hydrophobic. Compound **5** might interact with this hydrophobic part of the LPS resulting in alteration of the cell surface and/or outer membrane structures or physicochemical properties that may play a role in the adhesion and/or uptake of *L. pneumophila*. For such interaction, the valance of the hydrophobicity/hydrophilicity of compounds maybe important because the difference in the structure between compound **5** (active in uptake

inhibition) and compound **10** (inactive in uptake inhibition) is only one substitution of a hydroxyl group; one of two hydroxyl groups residing in compound **5** is substituted with a methyl group in compound **10**. Isochromem derivatives compound **12** and compound **14** possess high bactericidal activity (Nomura et al., 2012) but they are not active against the uptake of *L. pneumophila* into *A. castellanii*. The bactericidal mechanism(s) of these compounds might not be effective against the uptake of *L. pneumophila* into *A. castellanii*.

In the present study, we used the representative strains *L. pneumophila* JCM 7571 (ATCC 33152) and *A. castellanii* ATCC 30234; therefore, it still remains to be clarified whether the amoebicidal activities and inhibitory activities against the uptake of *L. pneumophila* of OMAT and its components are strain specific or not. Further investigation should be done using other strains of *L. pneumophila* and *A. castellanii* to make this clear.

Amoebae are the hosts of *Legionella* spp. in natural environments (Greub and Raoult, 2004; Molmeret et al., 2005; Rowbotham, 1980; Winiecka-Krusnell and Linder, 2001). In addition, *L. pneumophila* co-cultured with amoeba is less sensitive to typical chlorine disinfectants (Barker et al., 1992; Dupuy et al., 2011; García et al., 2007; Kim et al., 2002) and *L. pneumophila* multiplied in amoebae possess greater resistance to disinfectants and antibacterial agents than *L. pneumophila* from cultures in broth (Bandyopadhyay et al., 2004; Barker et al., 1995). *L. pneumophila* in biofilms is also resistant to stressful environmental conditions and the actions of antibacterial agents (Cooper and Hanlon, 2010; Kim et al., 2002; Wright et al., 1991). *L. pneumophila* multiplies in *A. castellanii* cells and is widely distributed in water environments through the formation of biofilm. Therefore, it is very important to inhibit each stage of the *L. pneumophila* life cycle. In addition, Barker et al. (1992 and 1995) have reported that *L. pneumophila* grown in *A. polyphage* was less sensitive than *L. pneumophila* from cultures in broth to disinfectants and antibiotics because these bacteria undergo phenotype modifications upon intracellular growth.

The oakmoss and its components show anti-*Legionella* activity, including antibacterial activity against *Legionella* spp. (Nomura et al., 2012), anti-biofilm forming activity and bactericidal activity against *L. pneumophila* in the biofilm (Nomura et al., 2013). In addition, as described above, the oakmoss components exhibited amoebicidal activity and inhibitory effect on the uptake of *L. peumophila* into *A. castellanii*. It is therefore suggested that a combination of these oakmoss components may act as a new type of disinfectant to inhibit the growth of both *Legionella* spp. and *A. castellanii*. Controlling both *L. peumophila* and *A. castellanii* should lead to better prevention of a Legionellosis outbreak.

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