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Antibacterial activities of plant essential oils against *Legionella pneumophila*

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ABSTRACT

The objective of this study was to determine the antimicrobial activity of essential oils (EOs) extracted from *Cinnamomum osmophloeum* leaves and different tissues of *Cryptomeria japonica* against pathogenic *Legionella pneumophila* at 42 °C. Ten kinds of EOs were extracted by water distillation and their chemical constituents were quantified by gas chromatography–mass spectroscopy (GC–MS). The results showed that cinnamon leaf EO possessed stronger anti-*L. pneumophila* activity than *C. japonica* EO. In particular, the highest bactericidal effect was noted in contact with *C. osmophloeum* leaf EO of cinnamaldehyde type (characterized by its major constituent of cinnamaldehyde accounting for 91.3% of EO), regardless of contacted cell concentration (2 and 4 log CFU ml⁻¹) or exposure time (10 and 60 min). Cinnamaldehyde is responsible for anti-*L. pneumophila* activity based on the results of antimicrobial testing and statistical analysis. Stepwise regression analyses show that EO concentration is the most significant factor affecting the bioactivity of EO. It is concluded that *C. osmophloeum* leaf oil of cinnamaldehyde type and its major constituent, cinnamaldehyde, possess strong anti-*L. pneumophila* activities, and have the great potential to be used as an antibacterial agent to control legionellosis associated with hot tubs and spa facilities widely used in homes and resorts.

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1. Introduction

Legionella pneumophila is the pathogen responsible for severe pneumonia known as Legionnaires' disease (LD) and non-pneumonic Pontiac fever, collectively referred to as legionellosis (Fields, 1997). In addition to rivers and lakes (Fliermans et al., 1981), this bacterium may grow in hot spring water (Furuhata et al., 2004) and hot water outlets of hospitals, hotels and private apartments (Leoni et al., 2005). Contamination of *L. pneumophila* in hot tubs and spa facilities used in homes, resorts and exhibition shows has been associated with severe LD outbreaks (Den Boer et al., 2002; NIID, 2003) and many sporadic cases of legionellosis (Johnson et al., 1994; Miller et al., 1993; Thomas et al., 1993; Tolentino et al., 1996;

Watson et al., 2004), and even caused human deaths (Johnson et al., 1994). This indicates that use of spas and hot tubs presents a potential risk of legionellae exposure and may contribute to severe health effects (Den Boer et al., 2002; Johnson et al., 1994; NIID, 2003). It is estimated that approximately 5 million public and private hot tubs, spas and whirlpools are used in the United States (CDC, 2004).

With increasing awareness of legionellosis associated with spas and bathtubs, regular clean-up and disinfection of these becomes essential. In addition to superheating and chlorine disinfections, antimicrobial agents extracted from a variety of plants have recently been evaluated for their antibacterial properties against *Legionella* (Friis-Moller et al., 2002; Furuhat et al., 2003; Morita et al., 2004a,b). The application of plant

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extracts as a bactericidal agent in hot springs and public baths was suggested (Furuhata et al., 2003). Addition of plant extracts in bathtubs and spas may avoid the problems of accident scald and trihalomethane exposure concerned with superheating and chlorination, respectively. Besides, it is suitable for the prevention of legionellosis in resorts and independent residences without centralized water heating systems. However, up to now, there is only one type of plant extract (grapefruit seed extract) that has been tested on *Legionella* under warm water situation (Furuhata et al., 2003).

A variety of essential oils (EOs), the natural mixtures of volatile metabolites extracted from plants, show their biocidal effects on bacteria, fungi, viruses, protozoa, insects and plants (Kalemba and Kunicka 2003). *Cinnamomum osmophloeum* Kaneh. (Lauraceae) is an endemic tree in the natural hardwood forests of Taiwan at elevations between 400 and 1500 m. The Japanese cedar, *Cryptomeria japonica* D. Don, on the other hand, is an important plantation tree of Taiwan with high values as a building material. The EO extracted from *C. osmophloeum* and *C. japonica* demonstrates inhibitory effect against bacteria (Chang et al., 2001), fungi (Morita et al., 1997; Wang et al., 2005) and other organisms such as mites (Chen et al., 2002). In particular, *C. osmophloeum* leaf EO was reported to inhibit the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella* sp. and *Vibrio parahaemolyticus* (Chang et al., 2001). However, there are no reports regarding the antibacterial activity of *C. osmophloeum* and *C. japonica* EO against *L. pneumophila*. In light of the existence of *Legionella* exposure in hot tubs and of the antimicrobial activity of EO, it is interesting to study the bioactivity of various EOs obtained from *C. osmophloeum* and *C. japonica* on *L. pneumophila*. In this study, six EOs from *C. osmophloeum* leaves containing various chemical polymorphisms and four EOs from different tissues of *C. japonica* were extracted by water distillation. The chemical constituents and contents of extracted EOs were analyzed by gas chromatography-mass spectrometry (GC-MS). The antibacterial activity of EOs and one of the major chemical components, cinnamaldehyde, was further investigated against *L. pneumophila* at 42 °C for a contact period of 10–60 min. This test water temperature represents the averaged hot bathing temperature (Bornstein et al., 1989) at which *L. pneumophila* has been isolated from the bath water during a LD investigation (Mashiba et al., 1993).

2. Materials and methods

2.1. Bacterial strains and media

L. pneumophila serogroup 1 ATCC 33152 was inoculated into buffered charcoal yeast extract agar supplemented with α -ketoglutarate (BCYE α agar) (Sigma Chemical Co., St. Louis, MO, USA), and incubated at 37 °C in an atmosphere of 5% CO₂ (Chang et al., 2007; Greub and Raoult, 2003) for 2 days. Forming colonies were removed and serial dilutions with sterile ultrapure water (Milli-Q Gradient, Millipore, Billerica, MA, USA) were performed to obtain a concentration of 6 log CFU ml⁻¹.

2.2. Essential oils

Mature leaves of *C. osmophloeum* of six provenances were collected from the Da-Pin-Ting of Taiwan Sugar Farm (Table 1A), the Lien Hua-Chin Research Center (Table 1B–E) in Nantou County of central Taiwan and the Haw-Lin Experimental Forest (Table 1F) in Taipei County of northern Taiwan. The leaves, twigs, bark and wood of *C. japonica* (Table 2G–J) were obtained from 43 aged Japanese cedars grown in the Experimental Forest of National Taiwan University in Nantou County of central Taiwan. The species were identified by Mr. Yen-Ray Hsui of the Taiwan Forestry Research Institute and the voucher specimens were deposited at the Laboratory of Wood Chemistry, School of Forestry and Resource Conservation, National Taiwan University. EOs were extracted from these botanical samples (200 g each) by water distillation for 6 h, and stored in airtight containers until analysis of oil compositions by GC-MS.

2.3. GC-MS analysis

The compositions of EOs from these different tissues of Japanese cedar and from *C. osmophloeum* leaf of six provenances were analyzed on a Finnigan Trace GC-Polaris Q mass instrument (Finnigan-Spectronex, Thermo, MA, USA), equipped with a fused silica column (30 m $\times$ 0.25 mm i.d.), and coated with a DB-5MS (df = 0.25 μ m). Mass spectra were recorded over the 50–650 amu range at 1 scans⁻¹, with an ionization energy of 70 eV and an ion source temperature of 200 and 230 °C. Helium was the carrier gas at a flow rate of 1 ml min⁻¹. For Japanese cedar EO, the injector temperature was maintained at 270 °C. The oven temperatures were held at 50 °C for 2 min, then programmed to increase from 50 to 250 °C at a rate of 5 °C min⁻¹ and held for 5 min. The samples (1.0 μ l, 1/100, v v⁻¹, in ethyl acetate) were injected neat with a 1:60 split ratio. *C. osmophloeum* leaf EOs were held at 80 °C for 1 min, raised to 200 °C at a rate of 4 °C min⁻¹ and held for 5 min; 250 °C injector temperature; carrier helium at a flow rate of 1.0 ml min⁻¹; 1:10 split ratio. Diluted samples (1.0 μ l) were injected manually in the split mode. Identification of individual compartments was done using the Wiley/NBS Registry of Mass Spectral Database and NIST MS Search, literature (Adams, 2001) and several authentic reference compounds. The quantity of compounds was obtained by integrating the peak area of the spectrograms.

2.4. Antibacterial test

Ten EOs were dissolved in 95% (v v⁻¹) ethanol and diluted with phosphate-buffered saline (PBS) to 200, 500, 1000 and 2000 μ g ml⁻¹. Vials containing 500 μ l of tested EOs were then placed in a water bath (Sheldon Manufacturing, Cornelius, OR, USA) preheated at 42 °C. Vials were inoculated with 25 μ l of *L. pneumophila* culture to give a final inoculum of 4 log CFU ml⁻¹. The negative control was prepared with 42 °C-heated PBS to which only *L. pneumophila* was added in the absence of EO. After 10 and 60 min of exposure at 42 °C in vials with 50 rpm shaking, 0.1 ml of each EO sample and negative control was plated on BCYE α agar and incubated at 37 °C for 5 days in an atmosphere of 5% CO₂. The antibacterial

Table 1 – Main components and their relative content (%) of six essential oils (A–F) from *C. osmophloeum* leaf

No.	Compounds	RT ^a	KI ^b	Relative content, %					
				A	B	C	D	E	F
1	(+)-Limonene	5.28	1034	– ^c	–	7.31	–	–	–
2	Linalool	6.75	1100	–	–	–	95.41	–	10.73
3	Camphor	7.99	1151	–	–	53.74	–	–	–
4	Benzenepropanal	8.34	1160	3.18	1.50	–	–	–	–
5	2-Methylbenzofuran	8.77	1179	–	–	–	–	8.27	–
6	α -Terpineol	9.18	1193	–	–	2.43	0.38	–	5.26
7	4-Allylanisole	9.36	1199	1.42	0.67	–	0.61	0.92	–
8	<i>Trans</i> -cinnamaldehyde	11.38	1271	91.32	35.09	0.84	1.40	3.15	2.82
9	<i>L</i> -Bornyl acetate	11.93	1288	0.59	0.69	22.09	–	0.94	–
10	Geranyl acetate	14.74	1383	–	–	0.93	–	4.01	0.73
11	Cinnamyl acetate	16.58	1445	0.44	56.44	0.58	1.15	60.77	0.61
12	Spathulenol	20.59	1581	–	–	–	–	0.99	20.38
	Others			3.05	4.51	12.08	1.05	17.65	24.77
Identified components, %				100	98.9	100	100	96.7	65.3
Monterpene hydrocarbons, %				0	0	11.1	0	0	0
Oxygenated monoterpenes, %				0.9	0.7	80.6	96.2	5	26.9
Sesquiterpene hydrocarbons, %				0.2	0.7	0	0.6	3.5	7.3
Oxygenated sesquiterpenes, %				0.5	0.5	4.5	0	8.1	25.4
Other, %				98.4	97	3.8	3.2	80.1	5.7
Oil yield, %				1.11	1.64	0.82	4.69	1.59	0.55

^a Retention time (min).^b Kovats retention index relative to *n*-alkanes (C₉–C₂₁) on DB-5MS column.^c Not detected.**Table 2 – Main components and their relative content (%) of four essential oils (G–J) from various tissues of *C. japonica***

No.	Compounds	RT ^a	KI ^b	Relative content, %			
				G, bark	H, twig	I, leaf	J, wood
1	γ -Cadinene	23.34	1523	0.66	2.62	0.06	22.07
2	δ -Cadinene	23.40	1525	4.65	10.41	0.55	12.12
3	Elemol	24.11	1555	1.03	6.80	20.48	6.27
4	γ -Eudesmol	26.00	1637	0.65	6.05	7.34	1.92
5	α -Eudesmol	26.52	1660	1.73	10.87	6.20	4.01
6	<i>ent</i> -Kaur-16-ene	34.31	2044	2.19	0.11	20.23	0.09
7	Kauran-16-ol	37.27	2208	10.22	3.37	– ^c	0.53
8	Isopimarol	38.40	2302	4.29	8.98	–	1.77
9	Ferruginol	39.30	2334	14.70	4.68	–	1.52
	Others			28.68	22.91	41.34	29.3
Identified components, %				68.8	76.8	96.2	79.6
Monterpene hydrocarbons, %				3.6	–	24.3	–
Oxygenated monoterpenes, %				0.9	–	8.7	–
Sesquiterpene hydrocarbons, %				18.4	22.7	0.8	48.8
Oxygenated sesquiterpenes, %				11.3	43.3	40.3	27.5
Diterpene hydrocarbons, %				8.7	0.6	22.1	0.3
Oxygenated diterpenes, %				25.9	10.2	0.0	3.0
Oil yield, %				0.03	0.05	1.42	0.38

^a Retention time (min).^b Kovats retention index relative to *n*-alkanes (C₉–C₂₄) on DB-5MS column.^c Not detected.

activity of EO was measured as minimal bactericidal concentrations 100 (MBC₁₀₀), which was defined as the concentration of the tested EO that inactivated at least 99.9%

L. pneumophila and resulted in no growth of *L. pneumophila* on BCYE α agar. All of the experiments were performed in triplicate and each experiment was repeated twice.

For positive controls, the susceptibility of *L. pneumophila* to erythromycin (potency $\geq 850 \text{ mg g}^{-1}$, Sigma-Aldrich, St. Louis, MO, USA) and rifampicin (95% purity, Sigma-Aldrich) was determined following the method suggested by the British Society for Antimicrobial Chemotherapy (BSAC, 2004), with modification of the use of BCYE α agar instead of buffered yeast extract agar containing 5% water-lysed horse blood. The minimum inhibitory concentrations (MICs) of erythromycin and rifampicin were always observed within the ranges of 0.06–0.5 and 0.004–0.06 mg l^{-1} , respectively, recommended for *L. pneumophila* (BSAC, 2004), which indicates that our test strain appropriately responded to antibacterial agents. In addition, the MICs were also found within the acceptable ranges when performing the susceptibility testing on *Staphylococcus aureus* ATCC 29213, a quality control strain suggested by the National Committee for Clinical Laboratory Standards (NCCLS, 1993) (data not shown).

Based on the results of the antibacterial test, four of the most effective EOs were selected to further determine their MBC values in response to different cell concentrations. Four to seven concentrations of EO were prepared at 100–1000 $\mu\text{g ml}^{-1}$ in PBS. *L. pneumophila* cells were then adjusted to 2 and 4 log CFU ml^{-1} in PBS, respectively, and subjected to EO for 10 and 60 min at 42 °C. Plating was then performed on BCYE α agar and visible colonies were enumerated after incubation for 5 days. Positive and negative controls were also conducted as described previously. The percentage reduction in CFUs, defined as the inactivation rate of *L. pneumophila* with EO exposure, was determined as follows: $100 \times [(Ca - Cb)/Ca]$, where Ca: CFUs of negative control at 42 °C and Cb: CFUs of EO-treated sample at 42 °C. In addition to four selected EOs, the percentage reduction in CFUs was also determined for cinnamaldehyde (98% purity, Sigma-Aldrich) at 150–750 $\mu\text{g ml}^{-1}$ in PBS. Cell culturability at 42 °C relative to that at room temperature in the absence of EO was further determined as a percentage. All of the experiments were repeated four times.

The maximum concentration of ethanol in PBS was 1% (v/v). Our preliminary trials revealed that the culturability of *L. pneumophila* in PBS containing 1% ethanol was only reduced by 1.5% relative to that of cells in PBS without addition of ethanol (data not shown), indicating that 1% ethanol had no significant adverse effect on *L. pneumophila*. In addition, the beneficial effect of agar addition as an emulsifying agent in EO antibacterial activity (Burt et al., 2005) was also examined. It was observed that there was no improvement in bioactivity of EO against *L. pneumophila* in PBS containing 0.05% agar. Therefore, no agar was added in the present study.

2.5. Statistical analysis

The inactivation rates of *L. pneumophila* against concentrations of EO were plotted, and the best-fit model was constructed by Sigmaplot software (SPSS Inc., Chicago, IL, USA) at $R^2 \geq 0.99$. The values of MBC₂₀, MBC₅₀ and MBC₈₀, which represented the minimum concentration of tested EO or the constituent that killed 20%, 50% and 80% of *L. pneumophila*, respectively, were determined from the best-fit model. In addition, the univariate analysis of variance was conducted by SPSS software (SPSS Inc.) to examine the effects

of EO type, EO concentration, concentration of *L. pneumophila*, cell culturability and contact time on the inactivation rate of *L. pneumophila*. There were totally 289 samples for analysis. Pairwise comparisons between any of the two kinds of EO were further performed to examine whether the mean difference of inactivation rate was statistically significant. Forward stepwise regression analysis was also performed to determine the factors significantly affecting the inactivation of *L. pneumophila* for each of the EOs, and to determine the role of cinnamaldehyde using 115 inactivation data collected from cinnamaldehyde and cinnamaldehyde-containing EOs. Statistical significance was referred to as $P < 0.05$ for all analyses.

3. Results

3.1. Chemical compositions of EO

As shown in Table 1, the yields of six EO (A–F) from *C. osmophloeum* leaf ranged between 0.55% and 4.69% (wt/dry wt). The highest oil yield was found in EO D (4.69%), followed by EO B (1.64%), E (1.59%), A (1.11%), C (0.82%) and F (0.55%). Totally, there were 39 compounds identified from these six leaf oils, accounting for 65.3–100% of EO. The three major components and their relative contents of each of the six EOs were *trans*-cinnamaldehyde (91.32%), benzenepropanal (3.18%) and 4-allylanisole (1.42%) for A, cinnamyl acetate (56.44%), *trans*-cinnamaldehyde (35.09%) and benzenepropanal (1.50%) for B, camphor (53.74%), L-bornyl acetate (22.09%) and (+)-limonene (7.31%) for C, linalool (95.41%), *trans*-cinnamaldehyde (1.40%) and cinnamyl acetate (1.15%) for D, cinnamyl acetate (60.77%), 2-methylbenzofuran (8.27%) and geranyl acetate (4.01%) for E, and spathulenol (20.38%), linalool (10.73%) and α -terpineol (5.26%) for F. Based on the identified major components and the classification by Hu et al. (1985), EOs A–E were classified as cinnamaldehyde type, cinnamaldehyde/cinnamyl acetate type, camphor type, linalool type and cinnamyl acetate type, respectively. However, EO F was classified as a mixed type due to lack of a predominant compound.

The oil yields extracted from the bark, twig, leaf and wood of *C. japonica* (G, H, I and J, respectively) were between 0.03% and 1.42% (Table 2). Totally, 69 compounds were identified from these four *C. japonica* EOs, constituting 68.8–96.2% of EO. The three major identified components and their relative contents of G, H, I and J were, respectively, ferruginol (14.7%), kauran-16-ol (10.22%) and δ -cadinene (4.65%), α -eudesmol (10.87%), δ -cadinene (10.41%) and isopimarol (8.98%), elemol (20.48%), *ent*-kaur-16-ene (20.23%) and γ -eudesmol (7.34%), and γ -cadinene (22.07%), δ -cadinene (12.12%) and elemol (6.27%). Unlike *C. osmophloeum* leaf EO, no obviously dominant compound was identified in any of the *C. japonica* EOs.

3.2. Anti-*L. pneumophila* activity of EO

MBC₁₀₀ values of EO are presented in Table 3. *C. osmophloeum* leaf EO possessed much stronger anti-*L. pneumophila* activity than *C. japonica* EO. In particular, four types of cinnamon leaf EOs (A–D), i.e., cinnamaldehyde type, cinnamaldehyde/

cinnamyl acetate type, camphor type and linalool type, demonstrated the best bactericidal properties against *L. pneumophila*. The culturability of *L. pneumophila* was completely inhibited by these four EOs at 1000 µg ml⁻¹ for as short as a 10-min contact. Thus, these four EOs were further tested in contact with *L. pneumophila* at 2 and 4 log CFU ml⁻¹.

After exposure to any one of the four EOs at 750 µg ml⁻¹ for 10 min, the culturability of 2 log CFU ml⁻¹ of *L. pneumophila* was inhibited completely. However, as shown in Table 4, the minimal concentration of EO to inactivate 80% *L. pneumophila* was found to be 436 µg ml⁻¹ for EO A, followed by EO B (523 µg ml⁻¹), C (598 µg ml⁻¹) and D (644 µg ml⁻¹). An increase in exposure time from 10 to 60 min resulted in an evident and consistent reduction of MBC₂₀, MBC₅₀ and MBC₈₀ for each of the EOs. MBC₁₀₀ of EO A was also decreased to 500 µg ml⁻¹ at the 60-min contact, while that of the other three EOs remained at 750 µg ml⁻¹ (data not shown). EO A was the most active EO as shown by the lowest MBC₈₀ (224 µg ml⁻¹), followed by EO C (245 µg ml⁻¹), B (451 µg ml⁻¹) and D (580 µg ml⁻¹). The least effective EO against *L. pneumophila* was EO D, of which MBC₂₀, MBC₅₀ and MBC₈₀ were always the highest regardless of contact time.

When cell concentration was increased to 4 log CFU ml⁻¹, at the 10-min contact, EO A still exhibited the greatest bactericidal effect, followed by EO C, D and B in terms of MBC₂₀, MBC₅₀ and MBC₈₀. When extending the exposure time from 10 to 60 min, the MBC₂₀, MBC₅₀ and MBC₈₀ of both EO A and B consistently decreased; however, this was not the case for EO C and D. After contacting with 4 log CFU ml⁻¹ of cells for 60 min, EO A was still the best in the aspect of *Legionella* inactivation with the smallest values of MBC₂₀, MBC₅₀ and MBC₈₀. The second effective EO was B, followed by C and D.

Results of univariate analysis of variance showed that factors of bacterial concentration, EO type, EO concentration and exposure time had statistically significant effects on

inactivation of *L. pneumophila* (all *P*'s < 0.001), whereas cell culturability (determined as a percentage of the colony count at 42 °C relative to that at room temperature) had not (*P* = 0.78). Pairwise comparison of inactivation rates demonstrated that EO A possessed inactivation ability higher than EO B, C and D (all *P*'s < 0.005), and EO B and C were also higher than EO D (*P* = 0.015 and < 0.001, respectively). No statistical difference was detected between EO B and C. The average difference of inactivation rate between EO A and EO B, C and D was 15.4%, 11.3% and 24.5%, respectively.

The results of multiple regression models for EO A, B, C and D showed that four models significantly predict the inactivation rate of *L. pneumophila* (*P* < 0.001 for all four EO models), with *R*² values ranging between 0.57 and 0.72 (Table 5). EO concentration was consistently the most significant factor positively affecting every EO's inactivation on *L. pneumophila* (*P* < 0.001 for all four EOs). For EO A and B, the exposure time was the second significant factor (*P* = 0.008 and 0.001, respectively), whereas both cell concentration and culturability were not statistically significant. For EO C and D, however, concentration of *L. pneumophila* adversely affected their inactivation, shown as the second significant predictor (*P* ≤ 0.001). In addition to concentrations of EO and *L. pneumophila*, inactivation ability of EO C was also significantly associated with exposure time, which was not the case in EO D.

3.3. Anti-*L. pneumophila* activity of cinnamaldehyde

According to Table 4 and results of statistical analyses, EO A and B exhibited the highest and moderate anti-*Legionella* activity, respectively. Because both EO A and B contained cinnamaldehyde accounting for 91.32% and 35.09%, respectively (Table 1), we hypothesized that anti-*L. pneumophila* activity of these two cinnamon leaf oils could be attributed to the presence of cinnamaldehyde. Thus, we further examined the anti-*L. pneumophila* activity of cinnamaldehyde and compared the result with that of EO A and B. Fig. 1 shows that, at exposure for 10 min at 350 µg ml⁻¹, the percentage reduction in CFUs of *L. pneumophila* caused by cinnamaldehyde was more than twice that induced by EO A, and EO B showed no CFU reduction at all. Comparable CFU reduction was found between cinnamaldehyde and EO A at 500 and 750 µg ml⁻¹. At the 60-min contact, *L. pneumophila* cells were also inactivated more significantly by cinnamaldehyde than EO A at 150, 250 and 500 µg ml⁻¹, while EO B contained the lowest antibacterial ability at all tested EO concentrations. Stepwise regression analysis of 115 inactivation data revealed that percentage of cinnamaldehyde, concentration of antibacterial agent and exposure time were significant determinants of *L. pneumophila* inactivation (all *P*'s < 0.001).

Table 3 – MBC₁₀₀ values of *C. osmophloeum* and *C. japonica* essential oils against *L. pneumophila* at 4 log CFU ml⁻¹ after 10- and 60-min contact

Essential oils	MBC ₁₀₀ , µg ml ⁻¹	
	10 min	60 min
<i>C. osmophloeum</i>		
A	1000	1000
B	1000	1000
C	1000	1000
D	1000	1000
E	> 2000	2000
F	> 2000	2000
<i>C. japonica</i>		
G	> 2000	> 2000
H	> 2000	> 2000
I	> 2000	2000
J	> 2000	> 2000

Note: MBC₁₀₀ was the minimal bactericidal concentration of essential oil that inactivated at least 99.9% of the bacteria and resulted in no growth of *L. pneumophila* on BCYEα agar. The essential oils were tested at 200, 500, 1000 and 2000 µg ml⁻¹.

4. Discussion

The major purpose of this study was to evaluate the antibacterial properties of selected EOs against *Legionella* and their potential usefulness in recreational water. Previous investigation of two legionellosis outbreaks that occurred with spa and hot spring water indicates that *Legionella*

Table 4 – MBC values of four essential oils against *L. pneumophila* at two cell concentrations for 10- and 60-min contact at 42 °C

Cell, log CFU ml ⁻¹	Contact time, min	Essential oils	MBC, µg ml ⁻¹		
			MBC ₂₀	MBC ₅₀	MBC ₈₀
2	10	A	292	358	436
		B	454	486	523
		C	227	436	598
		D	587	612	644
	60	A	171	189	224
		B	278	336	451
		C	95	142	245
		D	303	430	580
4	10	A	329	381	447
		B	555	636	716
		C	459	550	684
		D	498	577	688
	60	A	158	208	311
		B	312	396	512
		C	557	639	730
		D	627	682	753

Note: MBC₂₀, MBC₅₀ and MBC₈₀, determined from the best-fit inactivation model against EO concentrations, represented the minimal concentration of EO that inactivated 20%, 50% and 80% of *L. pneumophila*, respectively. The essential oils were tested at 100–1000 µg ml⁻¹ (n = 4 for each concentration).

Table 5 – Multiple linear regression model of individual EO with inactivation rate as dependent variable

EO	Predictors	β	T	P value	Model		
					F	P value	Adjusted R ²
A	EO conc.	0.76	10.5	0.000	56.0	0.000	0.57
	Time	0.2	2.7	0.008			
B	EO conc.	0.85	12.6	0.000	81.0	0.000	0.71
	Time	0.23	3.4	0.001			
C	EO conc.	0.88	13.2	0.000	60.9	0.000	0.72
	Cell conc.	−0.4	−6.0	0.000			
	Time	0.3	4.6	0.000			
D	EO conc.	0.81	10.8	0.000	60.4	0.000	0.65
	Cell conc.	−0.26	−3.5	0.001			

Note: The multiple linear regression model was performed with forward selection. Concentrations of EO (EO conc.) and *L. pneumophila* (Cell conc.), exposure time (Time) and cell culturability were initially included in the model. β = standardized regression coefficient; T = t statistic, used to evaluate the predictor; F = F statistic, used to evaluate the model; adjusted R² = variance in inactivation rate accounted for by the predictors.

organisms reached 3–4 log CFU ml⁻¹ (NIID, 2003), while 0–3 log CFU ml⁻¹ could be detected at no occurrence of outbreaks with *L. pneumophila* serogroup 1 predominating (Bornstein et al., 1989; Furuhashi et al., 2004; Verissimo et al., 1991). Therefore, *L. pneumophila* serogroup 1 at concentrations

≤ 4 log CFU ml⁻¹ was examined for cellular response to the studied EOs.

Our results showed that *C. osmophloeum* leaf EO inactivated *L. pneumophila* better than *C. japonica* EO. Although the antimicrobial activity of cinnamon oil on various bacteria

Fig. 1 – Susceptibility of *L. pneumophila* of 4 log CFU ml⁻¹ to antibacterial agents at 42 °C for 10-min (A) and 60-min (B) contact. The percentage reduction in CFUs, defined as the inactivation rate of *L. pneumophila* with exposure to antibacterial agents, is expressed as mean with an error bar representing a standard deviation. Antibacterial agents are cinnamaldehyde (▨, 98% purity), EO A (▤, cinnamaldehyde: 91.32%) and EO B (□, cinnamaldehyde: 35.09%, cinnamyl acetate: 56.44%). *No measurable inactivation effect for EO B.

has been reported (Chang et al., 2001; Hill et al., 1997; Kim et al., 2004; Ouattara et al., 1997; Smith-Palmer et al., 1998), to the best of our knowledge, this is the first study to demonstrate the bactericidal effect of cinnamon leaf oil against *Legionella* species. Among the six cinnamon leaf oils studied, four EOs (i.e., A–D) had the same MBC₁₀₀ of 1000 µg ml⁻¹, which was less than that for the other two, EOs E and F. This accords with our previous results that *C. osmophloeum* leaf EO (similar to EO A) of cinnamaldehyde type had stronger ability than that (similar to EO F) of mixed type against nine strains of bacteria (Chang et al., 2001).

The same MBC₁₀₀ value found in EOs A, B, C and D (Table 3) seems to suggest that these four cinnamon leaf EOs possessed equivalent anti-*L. pneumophila* property. However, as we further investigated their bactericidal abilities by a number of concentrations beyond the twofold serial dilution principle and displayed the results by MBC indicators corresponding to various killing levels, we successfully distinguished their anti-*L. pneumophila* properties. Regardless of concentration of *L. pneumophila* and contact time tested, *C. osmophloeum* leaf EO (EO A) of cinnamaldehyde type was always the best among the four EOs, shown by its MBC₈₀ values and by the statistical results of pairwise comparison analyses. In contrast with EO A, EO D, a linalool-type cinnamon oil, displayed the least ability against *L. pneumophila* with statistically significant difference. Linalool was the major component constituting up to 95.41% of EO D. Chang et al. (2001) reported that linalool showed no inhibitory effect against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* at 1000 µg ml⁻¹, whereas cinnamaldehyde (the major constituent of EO A) inhibited the growth of these four bacteria at levels of 250–1000 µg ml⁻¹. A recent study by Cheng et al.

(2006) also revealed that the antifungal activity of cinnamaldehyde-type *C. osmophloeum* leaf EO was higher than that of linalool-type cinnamon oil. These findings by Chang et al. and Cheng et al. on considerable difference in antimicrobial ability between linalool and cinnamaldehyde or between their respective EO imply the possibility that low antibacterial property of EO D against *L. pneumophila* might be attributed to its major constituent, linalool. Further studies for this possibility are warranted.

Being the most effective EO, the major constituent of EO A, cinnamaldehyde, was therefore examined for its bioactivity against *L. pneumophila*. The highest bactericidal effect was observed with cinnamaldehyde of 98% purity, followed by EO A containing 91.32% cinnamaldehyde and EO B with 35.09% cinnamaldehyde, indicating that the antibacterial activity was greater in the agents containing larger amounts of cinnamaldehyde. Additionally, the percentage of cinnamaldehyde in antibacterial agents was the statistically significant determinant positively affecting the inactivation rate of *L. pneumophila* ($P < 0.001$). On the other hand, benzenepropenal, the second highest constituent of EO A (3.18%, Table 1), showed no antibacterial effect at 1000 µg ml⁻¹ against 4 log CFU ml⁻¹ of *L. pneumophila* (data not shown). These results strongly suggest that cinnamaldehyde definitely relates to the anti-*L. pneumophila* property of *C. osmophloeum* leaf oil. Cinnamaldehyde has been identified as an effective antibacterial against a variety of foodborne pathogens (Burt, 2004; Kim et al., 2004), and its bactericidal ability was further demonstrated by the evidence of a decline in viable counts of *E. coli* O157:H7 with an increase in contact time and cinnamaldehyde concentration (Kim et al., 2004). We also observed a similar phenomenon when challenging

L. pneumophila with EO A or cinnamaldehyde, indicating that both EO A and cinnamaldehyde against this particular waterborne pathogen are also bactericidal.

The antimicrobial mechanism of EO A or cinnamaldehyde on *L. pneumophila* is not clear. However, previous investigations on other Gram-negative bacteria have shown that cinnamaldehyde may severely damage and destroy the surface structure of *E. coli* O157:H7 (Kim et al., 2004), inhibit amino acid decarboxylase activity of *Enterobacter aerogenes* (Wendakoon and Sakaguchi 1995) and decrease cellular glutathione levels in *E. coli* and *S. typhimurium* (Haines and Shankel 1990). The aldehyde characteristic with a conjugated double bond and a long CH chain outside the carbon ring is thought to contribute to the bioactivity of cinnamaldehyde primarily based on the results from cinnamaldehyde congeners (Chang et al., 2001; Wang et al., 2005). The carbonyl group of cinnamaldehyde reacting with cellular proteins by hydrogen bonding, ionic interactions and hydrophobic interactions is also suggested to be responsible for the antibacterial action (Wendakoon and Sakaguchi, 1995).

Cinnamaldehyde is recognized as a safe food additive and is widely used as a flavoring agent. It is also permitted by the US Environmental Protection Agency as the active ingredient of regulating pesticides due to the fact that cinnamaldehyde shows no unreasonable adverse effects to humans and environments and quick degradation (EPA, 2000). In particular, neither irritation nor sensitization was observed in the skin of human subjects exposed to a solution of <3% and <1% cinnamaldehyde, respectively (Cocchiara et al., 2005). According to Table 3, *C. osmophloeum* leaf EO A completely inhibited *L. pneumophila* at 1000 µg ml⁻¹, which is 10 times lower than the minimum concentration (i.e., 1%) that may cause adverse dermal effects by cinnamaldehyde. Significant anti-*Legionella* activity at relatively low concentrations of cinnamaldehyde indicates that *C. osmophloeum* leaf EO of cinnamaldehyde type possesses the potential to be used in recreational water against *L. pneumophila*, not only because of its bactericidal property but also because of its low human toxicity, quick degradation in environment and inexhaustible leaf source. In addition, the present study showed that the ability of this EO against *L. pneumophila* was mainly determined by EO concentration and contact time, and was not significantly affected by tested cell concentrations representing the possible levels detected during the regular period (Bornstein et al., 1989; Furuhashi et al., 2004; Verissimo et al., 1991) or at LD outbreaks (NIID, 2003). The lack of a cellular concentration effect on anti-*L. pneumophila* properties of EO A provides another advantage in its practical application since the concentration of *L. pneumophila* in recreational water is usually unknown.

5. Conclusion

This study demonstrated that *C. osmophloeum* leaf oil of cinnamaldehyde type and its major constituent, cinnamaldehyde, possess remarkable anti-*L. pneumophila* activities, and have great potential to be used for control of *Legionella* growth in recreational hot water.

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