ABSTRACT

Antibacterial and antibiofilm activities of sage (Salvia officinalis), thyme (Thymus vulgaris), peppermint (Mentha x piperita) and lemon (Citrus limonum) essential oils (EOs), supplied as commercial preparations, were investigated on Legionella pneumophila. Results showed that all EOs had antibacterial activity. Lemon oil demonstrated the best activity, with minimum inhibition concentration (MIC) and minimum bactericidal concentration values detected as 0.83–1.25 and 1.25–3.75 μL/mL, respectively. Antibiofilm activities of EOs were studied in two ways: (1) inhibition of biofilm formation at subMIC concentrations; and (2) eradication of preformed biofilms at MIC and subMIC concentrations. The highest biofilm inhibition values were 63.3, 45.5, 35.6 and 23%, and the highest eradication values were 34.2, 16.7, 39.4 and 31.9% for lemon oil, peppermint oil, sage oil and thyme oil, respectively. Lemon oil was detected to have the highest antibiofilm activity, as with antibacterial activity. The minimum antibiofilm activity was found for thyme oil. For all the EOs, the inhibition of biofilm formation was higher than the eradication of preformed biofilms at the same concentrations. The results suggested that EOs of lemon, peppermint, sage and thyme had the potential for being used in the removal of L. pneumophila from potable water systems, due to their antibacterial and antibiofilm activities.

Key words | antibiofilm activity, antimicrobial activity, essential oils, Legionella pneumophila

INTRODUCTION

Legionella pneumophila is widespread in aquatic environments (Fliermans et al. 1981), even in extreme conditions (Sheehan et al. 2005). In manmade water systems, proliferation of L. pneumophila can lead to legionella infection in humans. There are two important phenomena providing survival of L. pneumophila in manmade systems: biofilms (Abdel-Nour et al. 2015) and parasitism in protozoa (Richards et al. 2015). Biofilm formation is a key point in the spread and transmission to humans of L. pneumophila. Because of biofilms, L. pneumophila can persist in potable water systems and survive for a long time.

Biofilms are common in water supply systems, especially at dead points of old pipes. They can form a reservoir for pathogenic bacteria (Wingender & Fleming 2011). L. pneumophila can form monospecies biofilms (Tai et al. 2012), and attach to preformed multispecies biofilms (Stewart et al. 2012). Olson et al. (2002) reported that microorganisms in biofilms were more resistant to disinfectants according to planktonic forms. Additionally, it was reported that biofilm derived L. pneumophila had increased pathogenicity against macrophages (Khweek et al. 2015). Therefore, the prevention of biofilms occupies an important role in the elimination of L. pneumophila.

Applying chemicals to water supply systems may present risks for humans, the environment and the water pipes. Chlorination may result in the formation of trihalomethanes, chemical compounds known to be carcinogenic and toxic in high concentrations (Freese & Nozaic 2004). Consequently, searching for alternative products to
disinfect water systems is attracting more attention. Plant extracts have an important place in research for drug and antimicrobial substances (Fabricant & Farnsworth 2001), essential oils (EOs) have been the subject of many studies in terms of biological effects (Bakkali et al. 2008; Faleiro 2011). Studies on the antibacterial effects of natural products on *L. pneumophila* are limited in number. EOs of *Cinnamomum osmophloeum*, *Cryptomeria japonica* (Chang et al. 2008), *Chamaecyparis obtusa* (Hong et al. 2004) and *Melaleuca alternifolia* (Mondello et al. 2009) were shown to have antibacterial activities on *L. pneumophila*. Seeds of *Moringa oleifera* (Suarez et al. 2005), grapefruit seed extract (Furuhatata et al. 2003) and dried plant materials of 22 species (Furuhatata et al. 2006) were found to be bactericidal on *L. pneumophila*. Shimizu et al. (2009) studied anti-legionella effects of various fragrance ingredients and found oak moss and birch tar oil to have maximum activity. *Eucernia prunastri* (Oak moss) was also shown to have antibacterial (Nomura et al. 2012) and antbiofilm (Nomura et al. 2015) effects against *L. pneumophila*.

The antibacterial effects of the EOs used in this study have been documented. However, none of them has been studied for anti-legionella activity to our knowledge. This study was performed in order to determine the antibacterial and antbiofilm activities of the EOs, which may have benefit for widespread use in Turkey, against potable water isolates of *Legionella pneumophila*.

**METHODS**

*L. pneumophila* strains employed in this study were isolated from water systems of various buildings in Mugla, Turkey. Isolation was performed by culture with membrane filtration, acid treatment, and then inoculation to buffered charcoal yeast extract (BCYE) and Wadowsky and Yee medium (MWY) media. Eleven isolated strains were assigned numbers from L1 to L11. Four strains (L2, L5, L6, L10) were identified as serogroup (SG) 1 and the rest (L1, L3, L4, L7, L8, L9, L11) as SG 2-14, with latex agglutination test (Oxoid). Minimum inhibition concentration (MIC) and antbiofilm assays were performed in buffered yeast extract (BYE) broth, and reproduction of strains and minimum bactericidal concentration (MBC) assays were performed on BCYE agar. Both media include Legionella growth supplement (Oxoid). Sage oil (*Salvia officinalis*), thyme oil (*Thymus vulgaris*), peppermint oil (*Mentha x piperita*) and lemon oil (*Citrus limonum*) were employed in the study. EOs were supplied commercially (Biotama Inc) and they were chosen due to being pure and produced in Turkey.

Determination of MIC values was performed by broth dilution (EUCAST 2003) in glass tubes. Three to four day old *L. pneumophila* colonies were collected from BCYE agar, and diluted in BYE broth. EOs were diluted with dimethyl sulfoxide (DMSO) at 1:5 ratio, and then with BYE as required. One mL of *L. pneumophila* solution and 1 mL of various concentrations of EOs were dispersed to each test tube. Final concentrations in test tubes were 10⁶ CFU/mL for *L. pneumophila* and from 0.02 to 10 μL/mL for EOs. Tubes containing only BYE and BYE with *L. pneumophila* were employed as negative and positive controls, respectively. All tubes were incubated for 72 h at 37 °C, and evaluated according to the presence of bacterial growth compared to visual turbidity and optical density (OD) of control tubes in order to detect MICs. Ten μL of samples from tubes of greater or equal to MIC were spread on BCYE agar and incubated at 37 °C for 72 h to detect MBC.

Microtiter plate biofilm assay (Merritt et al. 2011) and the method of Mampel et al. (2006) were modified and performed in glass test tubes (100 × 160 mm). *L. pneumophila* colonies were collected from BCYE agar and diluted in BYE to give 0.2 OD at 600 nm. Suspension of 2 mL was dispersed into each test tube, and incubated at 37 °C for biofilm formation. Tubes containing only BYE were used as negative control. After 72 h incubation, planktonic cells and media were removed, and attached cells were fixed at 80 °C for 10 minutes. All tubes were stained with 2 mL of 0.3% crystal violet (CV) for 15 minutes, and rinsed with sterile distilled water three times. Tubes were left to dry upright at room temperature. Stained CV was dissolved in ethanol for 15 minutes and transferred to 12 × 92 mm polystyrene (PS) tubes for measuring OD in 570 nm. Biofilm formation capabilities of bacteria were evaluated as described by Stepanovic et al. (2000). Three standard deviations above mean OD of the negative
controls were defined as cut-off OD. Biofilm formation capabilities were scored as follows:

<table>
<thead>
<tr>
<th>Biofilm formation scores</th>
<th>Sample OD (ODS)</th>
<th>ODC ≤ ODS × 2</th>
<th>ODC ≤ ODS × 4</th>
<th>ODC ≤ ODS</th>
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<tr>
<td>No biofilm formation (−) score</td>
<td>cut off OD (ODC)</td>
<td>Weak biofilm</td>
<td>Moderate biofilm</td>
<td>Strong biofilm</td>
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<td>ODC &lt; ODS &lt; ODC × 2</td>
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<td>ODC &lt; ODS &lt; ODC × 4</td>
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The effects of sub-MIC concentrations of EOs on biofilm formation were verified as described by Nomura et al. (2013) with some modifications. *L. pneumophila* suspension was prepared as described previously and 1 mL was dispersed to each tube. EOs diluted with DMSO and BYE was dispersed (1 mL) to each tube to give concentrations below MIC. Tubes containing only BYE and BYE with *L. pneumophila* were employed as negative and positive controls, respectively. Staining and measuring the formed biofilm was performed as 'biofilm formation', and the biofilm inhibition rate was calculated by the formula:

\[
\text{Inhibition R(\%)} = \frac{100 \times (O_D^{\text{Positive Cont}} - O_D^{\text{Negative Cont}})}{O_D^{\text{Positive Cont}} - O_D^{\text{Negative Cont}}}
\]

Biofilm formation was performed as described previously. After 72 h, 0.1 mL of each EOs dilution was added to the sample and negative control tubes to give concentrations of MIC and below MIC. BYE was added as positive control. Incubation was continued for 24 h. Removal of planktonic cells, fixation, staining of attached cells, dissolving of CV and measuring the OD values was performed as before. Biofilm eradication rate was calculated by the formula:

\[
\text{Eradication R(\%)} = \frac{100 \times (O_D^{\text{Positive Cont}} - O_D^{\text{Negative Cont}})}{O_D^{\text{Positive Cont}} - O_D^{\text{Negative Cont}}}
\]

Tables and charts were prepared using MS Excel. Statistical significance of results was calculated using Student’s t-test function in MS Excel. P values less than 0.05 and 0.01 were accepted to have statistical significance and are indicated in the charts.

**RESULTS AND DISCUSSION**

Biofilm formation capabilities of eleven *L. pneumophila* isolates in filter sterilized tap water (STW) and BYE medium at 37°C–72 h are described in Figure 1. Most biofilm forming strains were identified as L7, L8 and L11 in STW, and L8, L10 and L7 in BYE, respectively. While no biofilm formation was detected for L1 in STW, other isolates were observed to have weak (+) biofilm formation. On the other hand, it was found that five of the isolates formed weak (+) biofilms and the other six formed...
moderate (++) biofilms in BYE. Biofilm forming ratios (OD 570 nm/cut off) in BYE were calculated as approximately two-fold higher than ratios in STW (Figure 1). According to the results, six of the L. pneumophila isolates, L8, L10, L7, L4, L3 and L5, were detected as the best biofilm producers and were chosen to be employed in antibiofilm studies.

All EOs were detected to have antibacterial activity against L. pneumophila isolates (Table 1). Lemon, thyme and peppermint oils were found to have similar MIC values (0.83–2.50 μL/mL). The minimum anti-legionella activity was detected for sage oil (MICs 13.3–20 μL/mL). No significant difference was detected between susceptibilities of L. pneumophila isolates to EOs.

EOs used in the study were determined to inhibit Legionella biofilm formation (Table 1). The highest biofilm inhibition was detected for the lemon oil at a concentration of MIC/2 (63.3 ± 4.8%). It was found that EOs inhibited the biofilm formation at all subMIC concentrations studied.

Minimum and maximum inhibition rates of six L. pneumophila isolates are shown in Table 1. According to these results, the highest biofilm inhibition was identified for lemon oil and then, successively, peppermint oil, sage oil and thyme oil. Average inhibition and eradication rates of six L. pneumophila isolates are given in Figures 2 and 3. Values of sage oil and thyme oil were very close at concentrations of MIC/8 and MIC/16 (Figure 2). Biofilm inhibition was detected even in minimum concentrations of EOs. It is an important finding for future research on the disinfection of large scale water supply systems with EOs.

The addition of EOs to media containing 72 h biofilms of L. pneumophila resulted in a reduction of existing biofilms at MIC and subMIC concentrations. The results showed that all EOs had biofilm eradication activity at concentrations of MIC and MIC/2, but statistically significant biofilm reduction was not detected at concentrations of MIC/8 and below (Table 1, Figure 2). The highest biofilm eradication was identified in sage oil at a concentration of

<table>
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<th>Antibacterial and antibiofilm activity results of EOs</th>
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<tr>
<td><strong>Table 1</strong></td>
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<td><strong>Sage oil</strong></td>
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<td><strong>MIC (μL/mL)</strong></td>
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<td><strong>MBC (μL/mL)</strong></td>
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<td><strong>Biofilm inhibition (%)</strong></td>
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Results are given in table as minimum/maximum values. ND, Not Detected, *P < 0.05, **P < 0.01.
MIC with a rate of 39.4 ± 6.5%. However, sage oil was detected to have minimum antibacterial and average biofilm inhibition activity. No correlation was found between antibiofilm and antibacterial activities of EOs. Eradication rates of EOs were detected very close to each other according to average values shown in Figure 3. It is shown in Figure 3 that maximum biofilm eradicating activity at a concentration of MIC was detected in sage oil and then, successively, lemon oil, thyme oil and peppermint oil, while at a concentration of MIC/2 values of EOs were found to be similar, except for peppermint oil. All eradication ratios were close to zero at a concentration of MIC/4.

It was understood from the results that activities of inhibition of biofilm formation and eradicating of preformed biofilms were quite different. At the concentration of MIC/2, the difference between the rates of inhibition and eradication rates was 1/2 for thyme oil, 1/3 for sage oil, 1/6 for lemon oil and 1/10 for peppermint oil (Figures 2 and 3).
Antibacterial and antibiofilm activities of sage oil (Mitic-Culafic et al. 2003), thyme oil (Thosar et al. 2013; Al-Shuneigat et al. 2014; Khan et al. 2014; Sadiki et al. 2014), lemon oil (Upadhyay et al. 2010; Oliveira et al. 2014) and peppermint oil (Sandasi et al. 2009, 2011; Sokovic et al. 2009; Saharkhiz et al. 2012; Thosar et al. 2013; Ceylan et al. 2014) were reported against different bacteria. Reviewing the literature results showed that reported antibacterial activities of lemon, thyme and peppermint oils were similar with their anti-legionella activity in our study. However, the anti-legionella activity of sage oil was lower than its antibacterial activity reported for other microorganisms. There are no reports in literature on antibacterial activities of EOs used in this study against L. pneumophila. However, dried plant extracts of thyme, sage and peppermint was shown to have anti-legionella activity by Furuhat et al. (2000). A few natural products such as tea tree oil, Melaleuca alternifolia (Mondello et al. 2009), oak moss (Nomura et al. 2012) and birch tar oil (Shimizu et al. 2009) were reported to have anti-legionella activity. Only one antibiofilm study on L. pneumophila in literature was performed by Nomura et al. (2013), with Eremia prunastri (oak moss) and its derivatives. In that study, it was reported that absolute oak moss had no biofilm inhibition activity but dipeptide and phenol derivatives inhibited the L. pneumophila biofilm up to 82–83% at a concentration of MIC/2, which was higher than our results.

CONCLUSIONS

According to literature and our results, EOs used in this study have antibacterial and antibiofilm activities against L. pneumophila and other microorganisms. Using the EOs, or substances obtained from EOs for disinfection of potable water systems, will provide inactivation of many other microorganisms as well as L. pneumophila.

EOs may be used in water supply systems for disinfection and inhibition of L. pneumophila biofilms. The results show that inhibiting the biofilm formation of L. pneumophila is easier than eradication of existing biofilms, so the routine use of EOs may be recommended to prevent settling of L. pneumophila in water systems. Spa, whirlpool and bath tubs are also related to the transmission of Legionellosis, and EOs may be used in these equipments due to their cosmetic attributes besides anti-legionella activity. Consequently, further studies must concentrate on the common use of EOs in water supply systems, including hot water equipment and on comparing their advantages to oxidizing disinfectants.

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