A synergistic anti-bacterial and anti-adhesion activity of tea tree (Melaleuca alternifolia) and lemon eucalyptus tree (Eucalyptus cit ...

Oder, Martina; Piletić, Kaća; Fink, Rok; Marijanović, Zvonimir; Krištof, Romana; Bićanić, Lucija; Tomić Linšak, Dijana; Gobin, Ivana

Source / Izvornik: **Biofouling, 2024, 1 - 10**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.1080/08927014.2024.2310482

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:184:635006

Taylor & Francis
Taylor & Francis Group

Biofouling
The Journal of Bioadhesion and Biofilm Research

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/gbif20

A synergistic anti-bacterial and anti-adhesion activity of tea tree (*Melaleuca alternifolia*) and lemon eucalyptus tree (*Eucalyptus citriodora* Hook) essential oils on *Legionella pneumophila*

Martina Oder, Kaća Piletić, Rok Fink, Zvonimir Marijanović, Romana Krištof, Lucija Bićanić, Dijana Tomić Linšak & Ivana Gobin

To cite this article: Martina Oder, Kaća Piletić, Rok Fink, Zvonimir Marijanović, Romana Krištof, Lucija Bićanić, Dijana Tomić Linšak & Ivana Gobin (14 Feb 2024): A synergistic anti-bacterial and anti-adhesion activity of tea tree (*Melaleuca alternifolia*) and lemon eucalyptus tree (*Eucalyptus citriodora* Hook) essential oils on *Legionella pneumophila*, Biofouling, DOI: 10.1080/08927014.2024.2310482

To link to this article: https://doi.org/10.1080/08927014.2024.2310482

Published online: 14 Feb 2024.

BIOFOULING
https://doi.org/10.1080/08927014.2024.2310482

A synergistic anti-bacterial and anti-adhesion activity of tea tree (*Melaleuca alternifolia*) and lemon eucalyptus tree (*Eucalyptus citriodora* Hook) essential oils on *Legionella pneumophila*

Martina Oder[a], Kaća Piletić[b], Rok Fink[a], Zvonimir Marijanović[c], Romana Krištof[a], Lucija Bićanić[b], Dijana Tomić Linšak[d] and Ivana Gobin[b]

[a]Department of Sanitary Engineering, Faculty of Health Sciences, University of Ljubljana, Ljubljana, Slovenia; [b]Department of Microbiology and Parasitology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia; [c]Department of Food Technology and Biotechnology, Faculty of Chemistry and Technology, University of Split, Split, Croatia; [d]Department for Health Ecology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

ABSTRACT

Legionella pneumophila is a Gram-negative bacterial pathogen that colonizes natural and artificial water systems and has the ability to form a biofilm. The biofilm protects *L. pneumophila* from various environmental factors and makes it more resistant to chlorine-based disinfectants. This study investigated the anti-bacterial properties of tea tree (*Melaleuca alternifolia* (Maiden and Betche) Cheel) oil and lemon eucalyptus tree (*Eucalyptus citriodora* Hook) essential oils (EOs) and their synergistic, additive inhibitory and anti-adhesive effects against *L. pneumophila* biofilm formation on polystyrene. The minimum effective concentration (MEC) for tea tree is 12.8 mg ml^{-1} and for lemon eucalyptus tree EO 6.4 mg ml^{-1}. In the checkerboard assay, different combinations of these two EO show synergistic and additive anti-microbial activity. The minimum anti-adhesive concentration (MAC) for tea tree is 12.8 mg ml^{-1} and for lemon eucalyptus tree EO 6.4 mg ml^{-1}. A combination of 3.2 mg ml^{-1} tea tree EO and 0.8 mg ml^{-1} lemon eucalyptus tree EO showed the strongest anti-adhesive effect against *L. pneumophila* on polystyrene. The tested oils and their combination showed intriguing potential to inhibit *L. pneumophila* biofilm formation.

ARTICLE HISTORY
Received 11 July 2023
Accepted 22 January 2024

KEYWORDS
Anti-*Legionella* activity; anti-adhesion; checkerboard assay; essential oils

Introduction

Microorganisms inhabit all ecological niches on earth, and one of their environmental survival strategies is the formation of biofilms. Biofilms are communities of bacterial cells embedded in a matrix composed of exopolysaccharide substances (EPS). EPS serves as a protective layer for bacterial cells (Coughlan et al. 2016). Pathogenic bacteria that form biofilms and produce EPS, such as *Legionella pneumophila*, pose a significant public health problem because they can colonize water supply systems and cause various infectious diseases, such as outbreaks of Legionnaires' disease.

Humans usually become infected with *L. pneumophila* by inhaling contaminated mist or aerosols (Masaka et al. 2021). Outbreaks of *L. pneumophila* can be very serious when they spread in hospitals or nursing homes (Falkinham et al. 2015). The biofilm matrix is a protective shield that allows *L. pneumophila* to survive in the given environment and protects it from thermal, chemical, or physical treatments (Jjemba et al. 2015; Coughlan et al. 2016). *L. pneumophila* within biofilms may exhibit resistance to chlorine derivatives, which are commonly used to control pathogens in water (Cooper and Hanlon 2010; Berjeaud et al. 2016). In addition, *L. pneumophila* is known to live as a parasite in encystic amoebae, which may also increase its resistance to disinfectants and environmental conditions. When amoebae phagocytose bacterial cells, *L. pneumophila* can survive up to 50 ppm chlorine (Kilvington and Price 1990).

In recent years, several natural products have been tested for their biocidal properties against *L. pneumophila*. Essential oils (EOs) derived from a variety of plant materials are known for their anti-microbial activities. Some authors have had success in inhibiting *Legionella* growth with EOs of cinnamon (*Cinnamomum verum*), tea tree (*Melaleuca alternifolia*),

CONTACT Romana Krištof romana.kristof@zf.uni-lj.si

juniper (*Juniperus communis*), thyme (*Thymus vulgaris*), sage (*Salvia officinalis*), peppermint (*Mentha piperita*) and lemon (*Citrus limon*) (Berjeaud et al. 2016; Ceylan and Turasay 2017). Due to their anti-microbial activity, EOs are being investigated as disinfectants for small water systems such as spas or small plumbing systems. In addition to their anti-bacterial effect, Ceylan and Turasay (2017) also reported anti-biofilm activities of sage, thyme, peppermint and lemon EOs. EOs are rich in natural biocidal compounds known as terpenoids, which can exert a strong negative effect on *L. pneumophila* when used in synergy. Compounds such as citronellal, isopulegol, terpinen-4-ol, γ-terpinene, α-terpinene and α-terpineol exhibit anti-microbial activity by alkylating the amino group of proteins and DNA (Laird et al. 2014; Jerković et al. 2016; El-Sayed 2021). In addition, EOs are hydrophobic, so they can bind with bacterial cell wall structures such as lipids, which can disrupt the wall structure, alter membrane permeability, delocalize electrons and lead to cell death (Mondello et al. 2009).

The current study investigated the use of tea tree (*Melaleuca alternifolia*) and lemon eucalyptus (*Eucalyptus citriodora* Hook) EOs, derived from Australian plants, on *L. pneumophila* growth and inhibition of adhesion to polystyrene.

Materials and methods

Essential oils

The natural EOs of lemon eucalyptus tree (*Eucalyptus citriodora* Hook) (No. 3859892843168) and tea tree (*Melaleuca alternifolia* (Maiden and Betche) Cheel) (No. 3859892843113) used in this study were purchased from Dea Flores, Rijeka, Croatia. The EOs were obtained by hydrodistillation. Each EO was dissolved in dimethyl sulfoxide (DMSO) (Kemika, Zagreb, Croatia) to obtain a stock suspension (200 mg l^{-1}), which was stored in sterile glass vials at 4 °C in the dark before use. The final concentration of DMSO as solvent was 2.43%$_{(v\ v-1)}$ in the highest EO concentration, and no effect on *L. pneumophila* (DMSO control) growth was determined.

EO's characterization

To characterize the components of selected EO, gas chromatography coupled with mass spectrometry (GC-MS) analyses were performed using an Agilent Technologies model 8890 gas chromatograph and a model 5977E mass spectrometer (MSD) (both Palo Alto, CA, USA). Five µl of EO were diluted in 0.5 ml of pentane (Kemika, Zagreb, Croatia), and the injection volume of the samples was 1 µl. GC conditions were the same as previously described by Jerković et al. (2016). Briefly, ionization voltage 70 eV; ion source temperature 230 °C; transfer line temperature 280 °C; mass range 30–350 mass units. The oven temperature was set at 70 °C for 2 min, then increased from 70 to 200 °C (3 °C min^{-1}) and held at 200 °C for 18 min; the carrier gas was helium (1.0 ml min^{-1}); split ratio 1:50 (Jerković et al. 2016). Analytical replicates were measured twice. Identification of compounds was based on comparison of their retention indices (RI) determined relative to the retention times of n-alkanes (C9-C25) (49452-U, Supelco, Belleforte, PA, USA) with those reported in the literature (Adams 2006; El-Sayed 2021) and in the mass spectral libraries of Wiley 9 (Wiley, New York, NY, USA) and NIST 17 (D-Gaithersburg). The percent composition of the samples was calculated from the GC peak areas using the normalization method (without correction factors).

Bacteria cultivation

L. pneumophila serogroup 1, ATCC BAA-74 was used in this study. Bacteria were cultured on buffered charcoal yeast extract agar (BCYE agar) (Oxoid Ltd, Hampshire, UK) supplemented with a sterile additive consisting of ferric pyrophosphate (0.25 g l^{-1}), L-cysteine (0.4 g l^{-1}), and α-ketoglutarate (1 g l^{-1}) at 35 ± 2 °C for 3–5 days. Bacteria were stored at −80 °C in sterile tap water containing 10% glycerol.

For each experiment, *L. pneumophila* was cultured on BCYE agar at 35 ± 2 °C for three days and then sub-cultured in ACES [N-(2-acetamido)-2-aminoethanesulfonic acid]-buffered yeast extract (AYE) broth (Oxoid) for another day at 35 ± 2 °C. The bacterial suspension was centrifuged at 4,000 rpm for 10 min, the pellet was washed twice with sterile tap water, and the bacterial suspension was adjusted to a OD 600 nm of 1 (1×10^9 CFU ml^{-1}). Further dilutions were made and suspensions of approximately 10^6 CFU ml^{-1} in sterile tap water with 20%$_{(v\ v-1)}$ AYE broth were used for the experiments.

Tap water sample

Tap water from the Rijeka public water supply system was used for all experiments. The water was colourless and odourless with a low turbidity (2.6 NTU), a neutral to slightly alkaline pH (pH 7.9), a low conductivity (0.215 mS cm^{-1} at 20 °C), and moderate

total hardness (133 mg l^{-1}). For dechlorination and sterilization, tap water was sterilized by autoclaving at 121 °C for 15 min and stored at 4 °C until use.

Checkerboard synergy method

To test the potential interaction effect of tea tree (TT) and lemon eucalyptus tree oils on *L. pneumophila*, the checkerboard synergy method was used with some modifications (Chaftar et al. 2016). Briefly, working solutions to at least double the MIC concentration and serial twofold dilutions of each EO were prepared in sterile tap water containing 20%$_{(v\ v-1)}$ AYE broth. Lemon eucalyptus tree EO was serially diluted in the range between 0.2 and 12.8 mg ml^{-1}, while TT EO was diluted in the range between 0.8 and 12.8 mg ml^{-1}. An inoculum of *L. pneumophila* isolate (10^6 CFU ml^{-1}) was prepared in sterile tap water containing 20%$_{(v\ v-1)}$ AYE broth in wells with diluted combinations and individual dilutions of EOs. Positive (bacterial inoculum in sterile tap water with 20%$_{(v\ v-1)}$ AYE broth) and negative (sterile tap water with 20%$_{(v\ v-1)}$ AYE broth) growth controls were also prepared.

DMSO, sterility and growth control were also included in the tests. Plates were incubated for 24 h under aerobic conditions at 35 ± 2 °C, then dilutions from each well were inoculated in duplicate onto BCYE agar with supplement, and incubated at 35 ± 2 °C for five days. Fractional inhibitory concentration or fractional bactericidal concentration and fractional bactericidal concentration indices (FBCi) were determined as previously described by Bassolé and Juliani (2012) and White et al. (1996). Based on FBCi values, a combination of EOs was considered synergistic when FBCi was $\leq$0.5, additive when FBCi was $>$ 0.5 and $\leq$1.0, indifferent when FBCi was $>$ 1.0 and $\leq$4, and antagonistic when FBCi was $>$ 4.

Checkerboard synergy method for anti-adhesion testing

The checkerboard synergy method described previously was repeated under the same conditions and after 24 h, the supernatant containing non-adherent bacterial cells was removed, the microtitre plates were washed twice with sterile tap water containing 20%$_{(v\ v-1)}$ AYE broth and sonicated in a water bath (Bactosonic, Bandelin, Berlin, Germany) at 40 kHz for 1 min. *Legionella* were plated from each well by culturing on BCYE agar with supplements, in duplicate and incubated at 35 ± 2 °C or five days.

The minimum anti-adhesive concentration (MAC) was determined as the minimum dose that completely inhibited *Legionella* adhesion to polystyrene. As part of the study, various controls were implemented to ensure the accuracy and reliability of the results. These controls included testing for sterility, monitoring the growth of *L. pneumophila* in sterile tap water with 20%$_{(v\ v-1)}$ AYE broth, examining the effect of maximum DMSO concentration on growth and adhesion of *L. pneumophila* to polystyrene, and evaluating adhesion of *L. pneumophila* to polystyrene in sterile tap water with 20%$_{(v\ v-1)}$ AYE broth.

Anti-adhesion effect of selected concentration of tea tree and lemon eucalyptus tree EOs

The effect of different concentrations of tea tree EO (range 0.2–12.8 mg ml^{-1}) and lemon eucalyptus tree EO (range 0.8–12.8 mg ml^{-1}) and synergistic or additive combinations of these EOs on the adhesion of *L. pneumophila* to polystyrene was tested.

After 24 h of incubation, non-adherent bacteria were removed and microtitre plates were washed twice with sterile tap water containing 20%$_{(v\ v-1)}$ AYE broth and sonicated in a water bath (Bactosonic, Bandelin, Berlin, Germany) at 40 kHz for 1 min. *Legionella* were quantified by culturing on BCYE agar with supplements, in duplicate and incubated at 35 ± 2 °C for five days.

Transmission electron microscopy

The morphology of the bacteria exposed to the EOs was analysed. Briefly, 10 µl of the treated bacterial suspension (10^8 CFU ml^{-1}) was added to Formvar-coated copper grids (Agar Scientific Ltd, Stansted, UK) for 2 min. The excess liquid was wiped off the grids using Whatman No. 3 filter paper (pore size 6 mm). Bacteria remaining on the grids were stained with 1% phosphotungstic acid (PTA; Sigma-Aldrich Chemie, Taufkirchen, Germany) for 1 min, and the excess PTA was carefully removed with filter paper. The grids were then air dried for a few minutes. Bacteria were examined with a transmission electron microscope (JEM −2100 F, Jeol, Tokyo, Japan).

Statistical analysis

All experiments were repeated three times. Experimental data were expressed as means with standard deviations and analysed using R software, version 4.1.1. (Bell Laboratories, Murray Hill, NJ, USA). Normality was tested using the Shapiro–Wilk test

Table 1. Chemical composition of essential oil *Melaleuca alternifolia*.

No.	Compound name	RI_1[a]	RI_2[b]	Peak area Av.[c] (%)	Identification[d]
1.	α-Thujene	924	933	0.95	MS, RI_1
2.	α-Pinene	932	942	3.14	MS, RI_1
3.	Sabinene	969	981	0.73	MS, RI_1
4.	β-Pinene	974	985	0.46	MS, RI_1
5.	β-Myrcene	988	994	1.41	MS, RI_1
6.	α-Terpinene	1014	1023	10.93	MS, RI_1
7.	p-Cymene	1020	1030	2.37	MS, RI_1
8.	Limonene	1024	1035	1.35	MS, RI_1
9.	1,8-Cineole	1026	1039	2.27	MS, RI_1
10.	γ-Terpinene	1054	1065	21.61	MS, RI_1
11.	α-Terpinolene	1086	1092	3.68	MS, RI_1
12.	Terpinen-4-ol	1174	1182	40.17	MS, RI_1
13.	α-Terpineol	1186	1194	4.36	MS, RI_1
14.	Aromadendrene	1439	1449	1.12	MS, RI_1

Extraction method = hydrodistillation.
[a]RI_1, retention indices of reference compound from literature.
[b]RI_2, retention indices determined using n-alkanes (C9–C25) on the HP-5MS column.
[c]Av., average percentage.
[d]Identification methods: MS, comparison of the mass spectrum with those of computer mass libraries and Adams (2006); RI_1, comparison of calculated RI with those reported in the literature.

($p > 0.05$). One-way analysis of variance (ANOVA) and Duncan's test were used to determine significant differences at a significance level of $p < 0.05$.

Results

EOs characterization

The selected EO compounds were characterized before determining their anti-bacterial activity. The most important compound in tea tree EO was terpinen-4-ol (40.17%) and citronellal (64.91%), while in lemon eucalyptus tree EO they were citronellal (64.91%), isopulegol isomer (10.51%) and citronellol (6.86%). The results are presented in Tables 1 and 2.

Checkerboard synergy analysis

The minimum effective concentrations (MEC) of tea tree and lemon eucalyptus tree EOs were determined by the checkerboard method and were 12.8 and 6.4 mg ml^{-1}, respectively (Table 3).

Checkerboard synergy analysis revealed the lowest concentrations of the combinations of EO with a synergistic effect (Table 3): 3.2 mg ml^{-1} for TT EO and 0.2 mg ml^{-1} for LE EO, 3.2 mg ml^{-1} for TT EO and 0.4 mg ml^{-1} for LE EO, 1.6 mg ml^{-1} for TT EO and 0.8 mg ml^{-1} for LE EO and 1.6 mg ml^{-1} TT EO and 1.6 mg ml^{-1} for LE EO.

The lowest concentrations with an additive effect of combined EOs were 6.4 mg ml^{-1} for TT EO and 0.2 mg ml^{-1} for LE EO (Table 3). Therefore, the lowest concentration of TT EO which showed an inhibitory effect

Table 2. Chemical composition of essential oil *Eucalyptus citriodora*.

No.	Compound name	RI_1[a]	RI_2[b]	Peak area Av.[c] (%)	Identification[d]
1.	α-Pinene	932	940	0.32	MS, RI_1
2.	β-Pinene	974	981	0.92	MS, RI_1
3.	1,8-Cineole	1026	1039	0.98	MS, RI_1
4.	Linalool	1095	1103	0.39	MS, RI_1
5.	Isopulegol isomer	1145	1152	10.51	MS, –
6.	Citronellal	1148	1166	64.91	MS, RI_1
7.	Citronellol	1223	1240	6.86	MS, RI_1
8.	Citronellyl acetate	1350	1357	1.45	MS, RI_1
9.	trans-β-Caryophyllene	1417	1419	1.02	MS, RI_1

Extraction methods = hydrodistillation.
[a]RI_1, retention indices of reference compound from literature.
[b]RI_2, retention indices determined using n-alkanes (C9-C25) on the HP-5MS column.
[c]Av., average percentage.
[d]Identification methods: MS, comparison of the mass spectrum with those of computer mass libraries and Adams (2006); RI_1, comparison of calculated RI with those reported in the literature.

Table 3. The interaction of tea tree EO and lemon eucalyptus tree EO combinations against *L. pneumophila*.

MEC (BO) mg ml^{-1}		MEC (combination of EO) mg ml^{-1}		FIC (EO)		EO interaction	
TT EO (A)	LE EO (B)	MEC (AB)	MEC (BA)	FIC (A)	FIC (B)	FIC = FIC (A) + FIC(B)	
12.8	6.4	3.2	0.2	0.250	0.031	0.281	S
		6.4	0.2	0.500	0.031	0.531	A
		3.2	0.4	0.250	0.062	0.312	S
		6.4	0.4	0.500	0.062	0.562	A
		1.6	0.8	0.125	0.125	0.250	S
		3.2	0.8	0.250	0.125	0.375	S
		6.4	0.8	0.500	0.125	0.625	A
		1.6	1.6	0.125	0.250	0.375	S
		3.2	1.6	0.250	0.250	0.500	S
		6.4	1.6	0.500	0.250	0.750	A
		0.8	3.2	0.062	0.500	0.562	A
		1.6	3.2	0.125	0.500	0.625	A
		3.2	3.2	0.250	0.500	0.750	A
		6.4	3.2	0.500	0.500	1.000	I
		0.8	6.4	0.062	1.000	1.062	I
		1.6	6.4	0.125	1.000	1.125	I
		3.2	6.4	0.25	1.000	1.250	I

Abbreviations: TTEO, tea tree essential oil; LEEO, lemon eucalyptus tree essential oil; MEC, minimal effective concentration; FIC, fractional inhibitory concentration; FICi, fractional inhibitory concentration index; EO, essential oil; A, additive; S, synergy; I, indifferent.

in the combination was 1.6 mg ml^{-1}, corresponding to 1/8 MEC of each TT EO, while the lowest concentration of LE EO in the combination was 0.2 mg ml^{-1}, corresponding to 1/32 MEC of each LE EO.

The final concentration of DMSO ($2.43\%_{(v\ v-1)}$) as solvent had no inhibitory effect on *Legionella* growth.

Checkerboard synergy method for anti-adhesion testing

The results of the anti-adhesive effect of various combinations of individual EOs and their combinations against *Legionella* on polystyrene are shown in Figure 1.

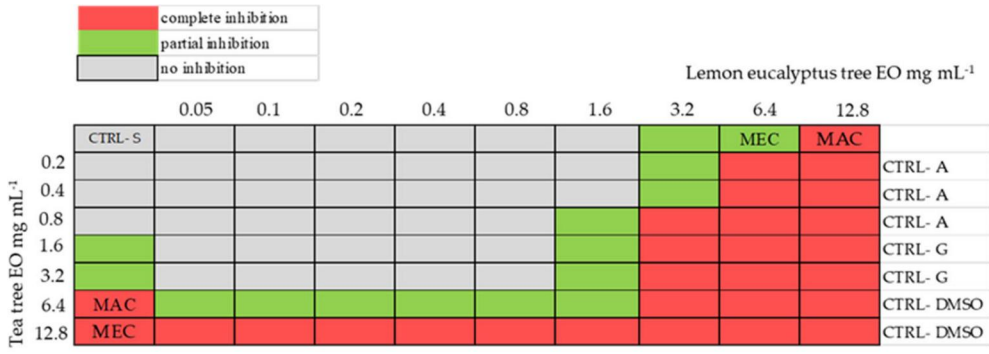

Figure 1. Checkerboard synergy method for the potential antiadhesion interaction of tea tree (TT) and lemon eucalyptus (LE) EOs on *L. pneumophila*. MEC (minimal effective concentration); MAC (minimal anti-adhesive concentration); sterility control (CTRL-S); DMSO control (DMSO-CTRL); growth control (CTRL-G); *L. pneumophila* adhesion control (CTRL-A).

The squares with complete adhesion inhibition were coloured in red. The minimum anti-adhesive concentration (MAC) for TT EO was 6.4 mg ml^{-1}, while for LE EO it was 12.8 mg ml^{-1}. Therefore, TT EO shows more pronounced anti-adhesive properties. The lowest concentrations of EO in combination that resulted in complete inhibition were 0.2 mg ml^{-1} for TT EO and 3.2 mg ml^{-1} for LE EO, 0.4 mg ml^{-1} for TT EO and 3.2 mg ml^{-1} for LE EO, and 0.8 mg ml^{-1} for TT EO and 1.6 mg ml^{-1} for LE EO.

Partial inhibition (coloured green) meant a significant reduction in the number of adherent bacteria when up to 20 colonies (10 µl drops) or up to 10^3 CFU ml^{-1} bacteria were detected. Partial inhibition for TT EO was observed at concentrations of 3.2 mg ml^{-1} (1/4 MEC) and 1.6 mg ml^{-1} (1/8 MEC), while for LE EO 6.4 mg ml^{-1} (MEC), 3.2 mg ml^{-1} (1/2 MEC), and 1.6 mg ml^{-1} (1/4 MEC). The lowest concentrations of each EO in combination that resulted in partial inhibition were 0.2 mg ml^{-1} TT EO and 1.6 mg ml^{-1} LE EO (Figure 1). When the number of bacteria present was above 2×10^3 CFU ml^{-1} the result was labelled as no inhibition of adhesion. Five EO combinations were found to have a synergistic effect, whereas only one combination (TT EO 1.6 mg ml^{-1} and LE EO 6.4 mg ml^{-1}) showed an additive effect. No inhibitory effect of the tested DMSO concentration on *L. pneumophila* growth and adhesion was detected.

Anti-adhesion effect of tea tree and lemon eucalyptus tree EOs

The selected concentrations of individual EOs and selected combined EO concentrations which had showed a synergistic effect were tested for their anti-adhesion activity against *L. pneumophila* (Figure 2). The results showed that increasing the LE EO concentration resulted in enhanced inhibition of *L. pneumophila* adhesion ($p < 0.05$). More detailed analysis showed that at a concentration of 0.8 mg ml^{-1} the inhibition of adhesion was not statistically significant compared to the control ($p > 0.05$). Increasing the concentration of LE EO resulted in statistically significant differences ($p < 0.05$) (Figure 2A).

The results of the anti-adhesion properties of TT EO showed a similar concentration dependence. Increasing the concentration of TT EO resulted in increased bacterial inhibition ($p < 0.05$). However, the lowest tested concentration of TT EO (0.2 mg ml^{-1}) was not statistically significantly different over the control (Figure 2B). The anti-adhesion potential of most tested EO combinations resulted in statistically significant ($p < 0.05$) differences in adhesion; the only combinations with no statistically significant difference ($p < 0.05$) (Figure 2C) were the combination of 1.6 mg ml^{-1} LE EO with 0.2 mg ml^{-1} TT EO and 1.6 mg ml^{-1} LE EO with 0.4 mg ml^{-1} TT EO.

Transmission electron microscopy

Transmission electron microscopy (TEM) analyses were performed to investigate the mechanisms of action of the EOs of tea tree and lemon eucalyptus tree. For the analysis, single EO (MEC concentrations) and the selected combination of EO (3.2 mg ml^{-1} TT EO + 1.6 mg ml^{-1} LE EO), which showed a synergistic effect, were tested (Figure 1).

In all treated samples, especially in those treated with the EO combination, severe damage and bacterial decay were observed. The bacterial cell wall was destroyed, and leakage of intracellular contents was evident. In addition, cytoplasmic condensation was observed in the treated cells. The destruction of the bacterial cells was more pronounced when using the combination EO (Figure 3).

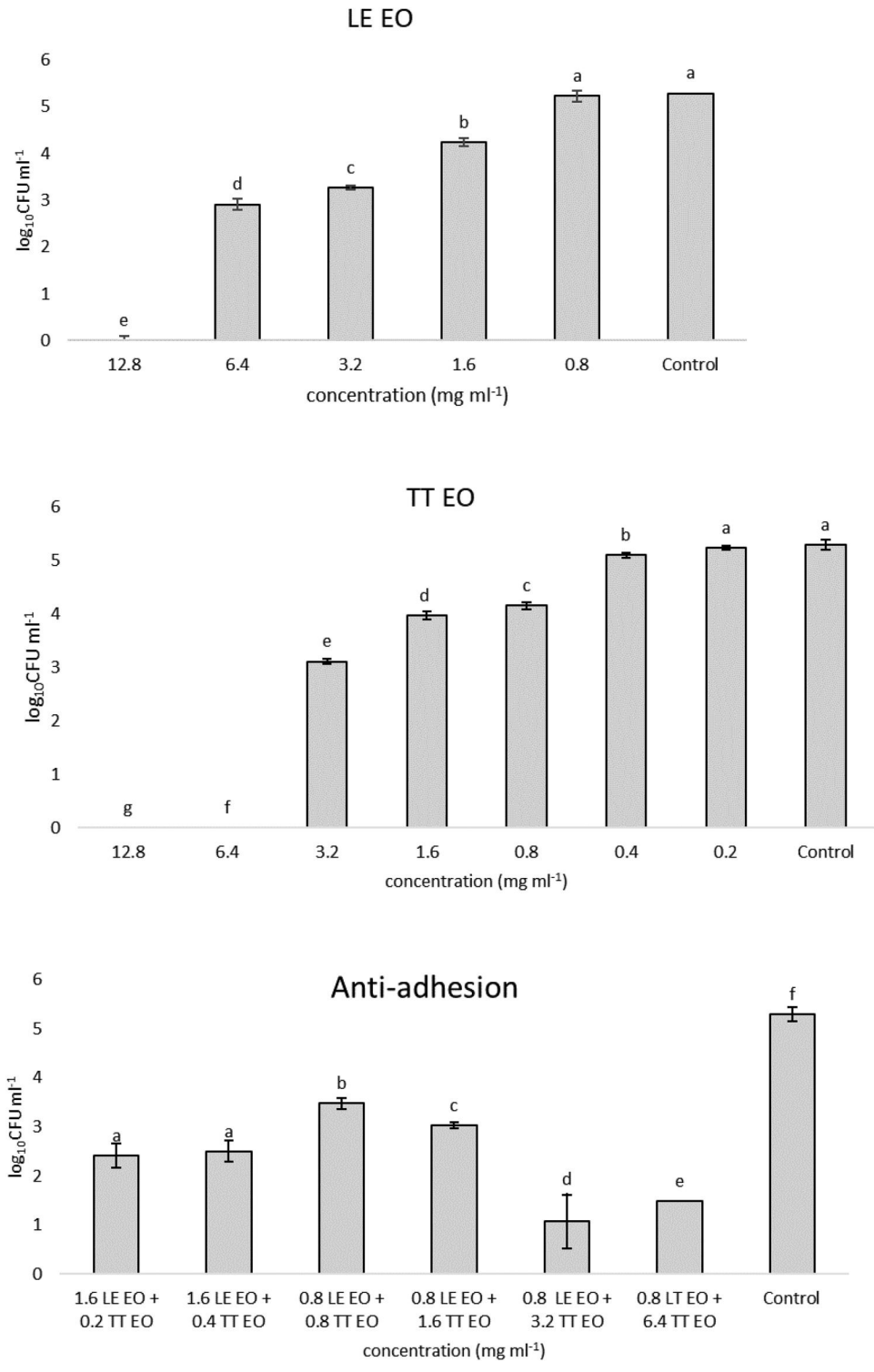

Figure 2. Effects of (A) LE EO, (B) TT EO and (C) a combination of LE EO and TT EO on the adhesion of *L. pneumophila*. The experiment was repeated three times in duplicate (six replicates in total), and the mean value with SD is shown. This means that $\log_{10}$ CFU m^{l-1} sharing a common letter (in A, a–e; in B, a–g; in C, a–f) are not significantly different at $p < 0.05$.

Discussion

L. pneumophila represents an important threat to public health and can cause enormous economic losses since it is difficult to eliminate the bacteria from the environment (Baker-Goering et al. 2021). *L. pneumophila* frequently colonizes water supply systems, and although it may exhibit resistance to chlorinated biocides, it appears to be sensitive to natural substances such as EOs (Berjeaud et al. 2016). This could represent an advantage in *L. pneumophila*

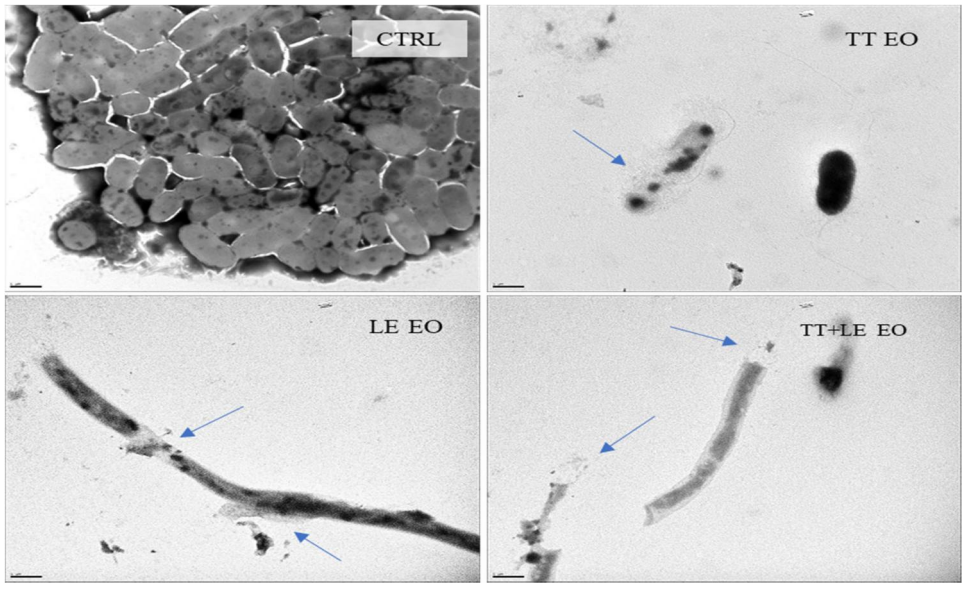

Figure 3. Morphological comparison of control *L. pneumophila* cells (CTRL) and *L. pneumophila* cells treated with the lemon eucalyptus tree (LE) EO, tea tree (TT) EO, and their combination (LT + ET) EO 24 h after treatment. Arrows indicate cellular leakage. Bar, 0.2 μm. Control – unexposed to EO; EO – essential oil.

management to prevent and control biofilm formation. Previous studies using different EOs from *Cinnamonum osmophloeum*, *Melaleuca alternifolia*, *Juniperus phoenicea* and *Thymus vulgaris* showed anti-bacterial activity on *L. pneumophila* planktonic cells (Chang et al. 2008; Mondello et al. 2009; Berjeaud et al. 2016; Chaftar et al. 2016). In the current study, the anti-bacterial properties, synergistic effect, and anti-adhesive (individual and synergistic) properties of tea tree (*Melaleuca alternifolia*) and lemon eucalyptus tree (*Eucalyptus citridora* Hook) EOs were examined. It was found that a lower MEC was required when using a combination of EOs than when using single EOs, indicating a strong synergistic effect of the combined EOs. Similarly, a study by Mondello et al. (2022) showed that a combination of terpinene and tea tree oil had a significant synergistic effect on *L. pneumophila*. The current study was conducted in sterile tap water with the addition of 20%$_{(v\ v-1)}$ AYE broth. The reason for adding the AYE broth was to reduce the potential stress on the bacteria during the tests and therefore provide more reliable results. In previous studies, it had been demonstrated that testing in sterile distilled water or tap water caused *Legionella* to switch to the formation of a resistant, potentially infectious, but non-culturable (VBNC) form (results not shown). Furthermore, the current study demonstrated that increasing the concentration of both EOs resulted in increased inhibition of *L. pneumophila*, with a slight increase in the inhibitory effect of tea tree EO. The current results are in agreement with the study of Mondello et al. (2009), in which the anti-bacterial activity of tea tree EO was determined against 22 strains of *L. pneumophila*, with all strains showing high sensitivity to tea tree EO. Similarly, citrus (orange (*Citrus sinensis*) and bergamot (*Citrus bergamia*) EOs (1:1 $_{(v\ v-1)}$) were tested against different strains of *Legionella* by Laird et al. (2014) who determined the antagonistic effect of their vapour phase and components (limonene, linalool, citral and β-pinene) against *L. pneumophila* in tap water and soil samples. EOs, due to their fatty origin, target lipids in the structure of the bacterial cell wall. They disrupt the wall structure and cause changes in membrane permeability. Since in tea tree EO, the main compounds detected by GC-MS were terpinen-4-ol, γ-terpinene, α-terpinene, α-terpineol and α-terpinolene it was assumed that those components are responsible for the anti-bacterial mode of action against *L. pneumophila*. For example, terpinen-4-ol is found in many plants, and its various bioactive properties have been demonstrated in previous studies (Kim et al. 2004; Bordini et al. 2018; Cordeiro et al. 2020).

In contrast, in lemon eucalyptus EO, the main compounds detected were citronellal, isopulegol isomer, citronellol, and citronellyl acetate. A study by Yang et al. (2023) reported that citronellal prevents EPS production in bacteria. The results of the current study show the composition of the tested lemon eucalyptus EO is similar to the previous characterization of EOs by Bossou et al. (2015). In their study,

Mulyaningsih et al. (2011) found that citronellal has lower anti-bacterial activity than citronellol, although citronellol is more reactive, as it has an aldehyde group that can cause alkylation of DNA. Nevertheless, citronellal and citronellol are terpenoids and show the same anti-bacterial activity as tea tree EO.

To explain the anti-bacterial mechanism of the EOs, TEM analyses were performed. Both EOs destroyed the cell wall of *L. pneumophila*, although the changes were more pronounced with the combined use of EOs (Figure 3). These results are consistent with previous studies described by Bhavaniramya et al. (2019) that also reported on bacterial cell wall defects. Furthermore, Chaftar et al. (2016) showed morphological changes in *Legionella* treated with *Thymus vulgaris* EO, where the cells appeared shorter, flatter, less homogeneous and less electron dense than in the untreated control, suggesting a loss of membrane integrity. Although these authors suggest that carvacrol leads to destabilization and damage of the cell membrane, when comparing their results with those presented here, it appears that other active compounds in EOs also have the same effect on the cell wall of *Legionella*.

Biofilm formation is an important characteristic of *Legionella*, and one way to control it is to prevent the bacterium from adhering to various materials. In this study, in addition to the inhibitory properties of EOs, the anti-adhesion properties of two EO mixtures were determined on polystyrene. A combination of EOs has shown to have statistically significant ($p < 0.05$) anti-adhesion potential compared to the application of single EOs. Ceylan and Turasay (2017) showed that lemon EO had the highest biofilm inhibition activity against *L. pneumophila* compared to sage, peppermint and thyme EOs. Inhibition of biofilm formation was higher for the tested EOs than destruction of already formed biofilms at the same concentrations. Similarly, Butucel et al. (2022) tested a combination of natural compounds against *L. pneumophila* and reported a reduction of biofilm formation and a substantial decreased in EPS production. Within the biofilm, bacteria can communicate *via* a unique intracellular communication system known as quorum sensing. Some studies, such as that of Brackman et al. (2008), have shown that EOs can decrease the DNA-binding activity of the quorum sensing response regulator LuxR, which may be one of the explanations for why EOs exhibit anti-bacterial activity on *L. pneumophila* in biofilms, while chlorine-containing disinfectants do not. There is little data in the literature on the anti-bacterial or anti-adhesive properties of individual tea tree and lemon eucalyptus tree oils and their combinations against *L. pneumophila*.

Therefore, the current study provides new and important findings on the anti-bacterial effect of EOs and the synergistic effect of combining different EOs on *L. pneumophila*. Since the main mechanism of the anti-bacterial effect of EOs is focused on the cell membrane, it can be assumed that the sensitivity to EOs is related to specific characteristics of the membrane, such as its thickness, phospholipid composition and fluidity. Disruption of quorum sensing within a biofilm may also be responsible for the anti-bacterial effect on the biofilm. However, the complete reasons for the sensitivity of *L. pneumophila* to EOs are still unknown and need further investigation. The current results suggest that EOs could be used as biocides to curb *L. pneumophila* biofilm formation.

Conclusion

In this study, the anti-bacterial properties, synergistic effects, and anti-adhesive properties of tea tree (*Melaleuca alternifolia*) and lemon eucalyptus tree (*Eucalyptus citriodora* Hook) essential oils (EOs) on *Legionella pneumophila* were investigated. The study was conducted in sterile tap water with the addition of 20% AYE broth to reduce bacterial stress and enhance reliability. A strong synergistic effect was discovered when combining the two EOs, requiring a lower MEC compared to individual EOs. Increasing the concentration of both EOs resulted in greater inhibition of *L. pneumophila*, with tea tree EO showing a slightly higher inhibitory effect. Transmission electron microscopy analyses revealed that both EOs destroyed the cell wall of *L. pneumophila*, with more pronounced effects when used in combination. The anti-bacterial mechanism involved destabilization and damage to the cell membrane, possibly attributed to active compounds in the EOs. The study also explored the anti-adhesive properties of the EOs on polystyrene, considering biofilm formation as an important characteristic of *Legionella*. Given the focus on the cell membrane as the main target of EO anti-bacterial effects, the study suggests that EOs could serve as biocides to control *L. pneumophila* biofilm formation, although further research is needed to fully understand the underlying mechanisms.

Disclosure statement

The authors report there are no competing interests to declare.

Funding

This work was supported by the Slovenian Research Agency under grant BI-HR/20-21-005 and by the University of Rijeka (under grant UNIRI-BIOMED-18-171).

References

Adams RP. 2006. Identification of essential oil components by gas chromatography/mass spectrometry. Carol Stream (IL): Allured Publishing Corporation.

Baker-Goering M, Roy K, Edens C, Collier S. 2021. Economic burden of Legionnaires' disease, United States, 2014. Emerg Infect Dis. 27:255–257. doi:10.3201/EID2701. 191198.

Bassolé IHN, Juliani HR. 2012. Essential oils in combination and their antimicrobial properties. Molecules. 17: 3989–4006. doi:10.3390/molecules17043989.

Berjeaud J-M, Chevalier S, Schlusselhuber M, Portier E, Loiseau C, Aucher W, Lesouhaitier O, Verdon J. 2016. *Legionella pneumophila*: the paradox of a highly sensitive opportunistic waterborne pathogen able to persist in the environment. Front Microbiol. 7:486. doi:10.3389/fmicb.2016.00486.

Bhavaniramya S, Vishnupriya S, Al-Aboody MS, Vijayakumar R, Baskaran D. 2019. Role of essential oils in food safety: antimicrobial and antioxidant applications. Grain Oil Sci Technol. 2:49–55. doi:10.1016/j.gaost.2019.03.001.

Bordini EAF, Tonon CC, Francisconi RS, Magalhães FAC, Huacho PMM, Bedran TL, Pratavieira S, Spolidorio LC, Spolidorio DP. 2018. Antimicrobial effects of terpinen-4-ol against oral pathogens and its capacity for the modulation of gene expression. Biofouling. 34:815–825. doi:10.1080/08927014.2018.1504926.

Bossou AD, Ahoussi E, Ruysbergh E, Adams A, Smagghe G, De Kimpe N, Avlessi F, Sohounhloue DCK, Mangelinckx S. 2015. Characterization of volatile compounds from three *Cymbopogon* species and *Eucalyptus citriodora* from Benin and their insecticidal activities against *Tribolium castaneum*. Ind Crops Prod. 76:306–317. doi:10.1016/j.indcrop.2015.06.031.

Brackman G, Defoirdt T, Miyamoto C, Bossier P, Van Calenbergh S, Nelis H, Coenye T. 2008. Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio spp*. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. BMC Microbiol. 8:149. doi:10.1186/1471-2180-8-149.

Butucel E, Balta I, McCleery D, Popescu CA, Iancu T, Pet I, Marcu A, Horablaga NM, Stef L, Corcionivoschi N. 2022. The effect Citrox BCL on *Legionella pneumophila* mechanisms of biofilm formation, oxidative stress and virulence. Antioxidants. 11:2186. doi:10.3390/ANTIOX11112186.

Ceylan O, Turasay B. 2017. Removing *Legionella pneumophila* and biofilms from water supply systems using plant essential oils. J Water Sanit Hyg Dev. 7:67–73. doi:10.2166/washdev.2017.140.

Chaftar N, Girardot M, Labanowski J, Ghrairi T, Hani K, Frère J, Imbert C. 2016. Comparative evaluation of the antimicrobial activity of 19 essential oils. In: Donelli G, editor. Advances in experimental medicine and biology. Cham, Switzerland: Springer International Publishing. Vol. 901; p. 1–15. doi:10.1007/5584_2015_5011.

Chang C-W, Chang W-L, Chang S-T, Cheng S-S. 2008. Antibacterial activities of plant essential oils against *Legionella pneumophila*. Water Res. 42:278–286. doi:10.1016/j.watres.2007.07.008.

Cooper IR, Hanlon GW. 2010. Resistance of *Legionella pneumophila* serotype 1 biofilms to chlorine-based disinfection. J Hosp Infect. 74:152–159. doi:10.1016/j.jhin.2009.07.005.

Cordeiro L, Figueiredo P, Souza H, Sousa A, Andrade-Júnior F, Medeiros D, Nóbrega J, Silva D, Martins E, Barbosa-Filho J, et al. 2020. Terpinen-4-ol as an antibacterial and antibiofilm agent against *Staphylococcus aureus*. Int J Mol Sci. 21:4531. doi:10.3390/IJMS21124531.

Coughlan LM, Cotter PD, Hill C, Alvarez-Ordóñez A. 2016. New weapons to fight old enemies: novel strategies for the (bio)control of bacterial biofilms in the food industry. Front Microbiol. 7:1641. doi:10.3389/fmicb.2016.01641.

El-Sayed AM. 2021. The Pherobase: database of pheromones and semiochemicals. [accessed 2021 Sep 28]. https://www.pherobase.com/.

Falkinham JO, Hilborn ED, Arduino MJ, Pruden A, Edwards MA. 2015. Epidemiology and ecology of opportunistic premise plumbing pathogens: *Legionella pneumophila*, *Mycobacterium avium*, and *Pseudomonas aeruginosa*. Environ Health Perspect. 123:749–758. doi:10.1289/ehp.1408692.

Jerković I, Marijanović Z, Kuś PM, Tuberoso CIG. 2016. Comprehensive study of Mediterranean (Croatian) propolis peculiarity: headspace, volatiles, anti-*Varroa*-treatment residue, phenolics, and antioxidant properties. Chem Biodivers. 13:210–218. doi:10.1002/cbdv.201500053.

Jjemba P, Johnson W, Bukhari Z, LeChevallier M. 2015. Occurrence and control of *Legionella* in recycled water systems. Pathogens. 4:470–502. doi:10.3390/pathogens4030470.

Kilvington S, Price J. 1990. Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. J Appl Bacteriol. 68:519–525. doi:10.1111/j.1365-2672.1990.tb02904.x.

Kim H-J, Chen F, Wu C, Wang X, Chung HY, Jin Z. 2004. Evaluation of antioxidant activity of Australian tea tree (*Melaleuca alternifolia*) oil and its components. J Agric Food Chem. 52:2849–2854. doi:10.1021/jf035377d.

Laird K, Kurzbach E, Score J, Tejpal J, Chi Tangyie G, Phillips C. 2014. Reduction of *Legionella* spp. in water and in soil by a citrus plant extract vapor. Appl Environ Microbiol. 80:6031–6036. doi:10.1128/AEM.01275-14.

Masaka E, Reed S, Davidson M, Oosthuizen J. 2021. Opportunistic premise plumbing pathogens. A potential health risk in water mist systems used as a cooling intervention. Pathogens. 10:462. doi:10.3390/pathogens10040462.

Mondello F, Fontana S, Scaturro M, Girolamo A, Colone M, Stringaro A, Di Vito M, Ricci ML. 2022. Terpinen-4-ol, the main bioactive component of tea tree oil, as an innovative antimicrobial agent against *Legionella*

pneumophila. Pathogens. 11:682. doi:10.3390/PATHOGENS11060682.

Mondello F, Girolamo A, Scaturro M, Ricci ML. 2009. Determination of *Legionella pneumophila* susceptibility to *Melaleuca alternifolia* Cheel (tea tree) oil by an improved broth micro-dilution method under vapour controlled conditions. J Microbiol Methods. 77:243–248. doi:10.1016/j.mimet.2009.02.012.

Mulyaningsih S, Sporer F, Reichling J, Wink M. 2011. Antibacterial activity of essential oils from *Eucalyptus* and of selected components against multidrug-resistant bacterial pathogens. Pharm Biol. 49:893–899. doi:10.3109/13880209.2011.553625.

White RL, Burgess DS, Manduru M, Bosso JA. 1996. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother. 40:1914–1918. doi:10.1128/AAC.40.8.1914.

Yang Z, He S, Wei Y, Li X, Shan A, Wang J. 2023. Antimicrobial peptides in combination with citronellal efficiently kills multidrug resistance bacteria. Phytomedicine. 120:155070. doi:10.1016/J.PHYMED.2023.155070.