Antimicrobial activities of leptospermone isolated from *Leptospermum scoparium* seeds and structure–activity relationships of its derivatives against foodborne bacteria

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Abstract This study was carried out to determine the antimicrobial activities of leptospermone isolated from *Leptospermum scoparium* and its derivatives against six foodborne bacteria (*Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus intermedius* and *Staphylococcus aureus*), with a view to developing safer antimicrobial agents. The essential oil of *L. scoparium* seeds possessed potent antimicrobial activity against six bacterial strains. The antimicrobial compound of *L. scoparium* was isolated by chromatographic analyses and identified as leptospermone. To investigate the structure–activity relationships, the antimicrobial activities of leptospermone and its derivatives (2-acetyl-1,3-cyclohexanedione, 1,3-cyclohexanedione, 1,2,3-cyclohexanetrione-1,3-dioxime, 5,5-dimethyl-1,3-cyclohexanedione and 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione) were examined against six foodborne bacteria. Based on the MIC values, leptospermone (MIC 23.6–69.7 µg/mL), 1,2,3-cyclohexanetrione-1,3-dioxime (MIC 43.9–88.5 µg/mL) and 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione (MIC 43.9–88.5 µg/mL) exhibited antimicrobial activities against the six foodborne bacteria. These results indicated that leptospermone and its derivatives could potentially be developed as natural food preservatives, rather than using hazardous synthetic preservatives.

Keywords Antimicrobial activity · Foodborne bacteria · Leptospermone · Structure–activity relationship · *Leptospermum scoparium*

Introduction

Foodborne diseases are one of the most important public health problems, which globally threatens millions of people (Kim et al., 2016; Law et al., 2015). According to a study from Scallan et al. (2011), 9.4 million cases of foodborne diseases, 1351 deaths and 55,961 hospitalizations caused by foodborne pathogens have been reported annually in the United States. *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* were among the most prevalent foodborne bacteria involved in foodborne disease outbreaks (Bhargava et al., 2015; Law et al., 2015). Generally, foodborne disease can be attributed to consumption of food or water polluted with pathogens or their toxins (Law et al., 2015). To prevent foodborne disease outbreaks, artificial food additives and preservatives are used. However, the use of several food processing and preservation methods have raised concerns about failures to control foodborne bacteria, because of the increase in antibiotic resistance of foodborne pathogens (Gyawali and Ibrahim, 2014). In addition, consumers are concerned about the safety of foods with artificial preservatives (Smith-Palmer et al., 1998; Wilcock et al., 2004). Increasing consumer perception of the potential side-effects of artificial preservatives on health has increased interest in new types of natural additives or preservatives, such as naturally derived chemicals, plant extracts and their essential oils (Gyawali and Ibrahim, 2014; Wilcock et al., 2004).

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Essential oils are secondary metabolites that are generally extracted from various parts of plants. These products are used to control a wide spectrum of insect pests, viruses, fungi, and other pathogens, because many essential oils are known to exhibit antimicrobial, antioxidant and insecticidal activities (Cho et al., 2016; Kim and Lee, 2016; Sanyacharemkul et al., 2016). Since ancient times, essential oils have been used worldwide as food additives to prolong the shelf-life of foods and to prevent food spoilage (Shan et al., 2007). *Leptospermum scoparium* (Myrtaceae), commonly called the Manuka myrtle, is an indigenous ‘tea-tree’ to eastern Australia and New Zealand (Christoph et al., 2000; Douglas et al., 2004). *L. scoparium* oil is commercially used as an antimicrobial agent and in natural remedies for infections and other diseases throughout the world (Christoph et al., 2000; Lis-Balchin et al., 2000; Song et al., 2013). *L. scoparium* oil contains separate chemotypes such as monoterpenes, sesquiterpenes and triketones (Perry et al., 1997). Especially, triketones are known to have antimicrobial properties in the essential oil of one chemotype of *L. scoparium* (Lis-Balchin et al., 2000; Porter and Wilkins, 1999). This study was conducted to identify the active components of *L. scoparium* oil and to examine the antimicrobial activities of *L. scoparium* oil, leptospermone, and its derivatives against six foodborne bacteria.

Materials and methods

Chemicals

1,2,3-Cyclohexanetrione-1,3-dioxime, 1,3-cyclohexanedione, 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione and 5,5-dimethyl-1,3-cyclohexanedione were supplied from Sigma (St. Louis, MO, USA).

Isolation and identification

Essential oil of *L. scoparium* seeds was supplied from Tairawhiti Pharmaceuticals Co. Ltd., and originally produced in New Zealand. Essential oil (12 g) was purified by column chromatography (diameter, 8 × 93 cm; 70–230 mesh), using silica gel (620 g) from Merck (Rahway, NJ, USA) and eluted with hexane:ethyl acetate (10:0 to 0:10, v/v). Each fraction was loaded on a thin layer chromatography (TLC) plate to identify similar fraction patterns. Four fractions (LS1 to LS4) were obtained and then bio-assayed at a dose of 5.0 mg/disc. Active fractions (LS2, 6.3 g) were separated on a silica gel column using solvent gradients composed of hexane:ethyl acetate (5:1, v/v). Bioassay-guided fractionation (LS22, 4.1 g) was performed and then the fractions were further isolated by

prep. HPLC (LC908C-W60, recycling prep. HPLC, JAI Co., LTD., Tokyo, Japan). In this step, the fraction was uploaded on a Jai gel GS Series column (GS 310 column 50 cm plus GS 310 column 50 cm), with a mobile phase composed of methanol (100%, v/v) at a flow rate of 2.8 mL/min. This procedure produced 5 fractions (LS221–225), which were each bioassayed. Active fraction (LS223, 1.4 g) was then additionally separated using a Jaigel W Series Column (W-252 50 cm plus W-253 50 cm, inside diameter 20.0 mm, JAI Co., Ltd., Tokyo, Japan), using chloroform (100%, v/v) delivered at a rate of 4.5 mL/min. Finally, active compound (LS2231, 0.9 g) was isolated as a single peak (Fig. 1). The structure of LS2231 was determined using NMR spectroscopy. The ^1H and ^{13}C NMR spectroscopy were measured in CDCl_3 using a JNM-LA 400F7 spectrometer (JEOL Co., Tokyo, Japan) at 150 and 600 MHz. Additionally, DEPT, COSY and HMQC experiments were carried out.

Bacterial strains and preparation of cultures

Three Gram-positive bacteria: *Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus intermedius* ATCC 29663, and three Gram-negative bacteria: *Salmonella typhimurium* IFO 14193, *Shigella flexneri* ATCC 29903 and *Shigella sonnei* ATCC 25931 were prepared by the Korean Culture Center of Microorganisms (Seoul, South Korea). Bacterial strains were grown in nutrient broth (NB; Difco, USA) and incubated overnight at 37 °C, excluding *S. aureus*, which was grown in Tryptic Soy broth (TSB; Difco, USA).

Agar diffusion method

To assess the antimicrobial activity of *L. scoparium* seed oil, leptospermone and its derivatives were tested against six foodborne bacteria, by using an agar diffusion method. The foodborne bacteria were maintained in NB at 37 °C for 24 h, except for *S. aureus*, which was grown in TSB, to yield roughly 1.0×10^7 colony-forming units (CFU)/mL, that corresponded to the turbidity of the McFarland turbidity standard. Tested bacterial suspensions (0.1 mL with 1.0×10^7 CFU/mL) were spread on Muller Hinton agar (MHA; Difco, USA) plates using a sterile glass spreader. The concentration tested were 20, 10, 5, 2, 1, 0.5, 0.25 and 0.125 mg/disc. Appropriate amount of each sample was dissolved to methanol as a solvent (40 μL) to obtain desired concentrations. The sample (40 μL) was injected on sterilized paper discs (8 mm in diameter, Advantec Co., Tokyo, Japan). The negative control was prepared using the same solvent. After drying in a fume hood for 20 min, the loaded paper discs were placed in the center of each MHA plate. These plates were maintained aerobically and

Fig. 1 Procedure of leptospermone isolation from the essential oil of *L. scoparium* seeds

incubated at 37 °C for 24 h. Triplicate sets of plates were prepared for each experiment.

Minimum inhibitory concentration (MIC)

A two-fold serial dilution assay was used to determine the MIC values of *L. scoparium* seed oil, leptospermone and its derivatives. Each sample (10 mg) was dissolved in methanol (10 mL) and then was consecutively diluted using the same solvent, ranging from 100 to 1 µg/mL. Each dilution (50 µL) was prepared in a 96-well microtiter plate which contained 100 µL of Mueller–Hinton Broth (MHB). A 50 µL bacterial suspension (10^7 CFU/mL) was inoculated into each well. After incubation at 37 °C for 24 h, MIC values of six foodborne bacteria in microdilution wells were confirmed through turbidity readings at 600 nm.

Results and discussion

The antimicrobial activity of the essential oil extracted from *L. scoparium* seeds was determined using an agar diffusion method (Table 1). Based on the diameter of inhibition zone values, the essential oil of *L. scoparium* seeds had excellent antimicrobial activity against six foodborne bacteria, *S. typhimurium*, *S. flexneri*, *S. sonnei*, *S. aureus*, *S. intermedius* and *L. monocytogenes*, at concentrations of 10–2 mg/disc. The negative control did not exhibit antimicrobial activity against all the tested bacteria. According to previous studies, the natural products derived from *L. scoparium* have an antimicrobial activity against a variety of bacterial species (Henriques et al., 2010; Lis-Balchin et al., 2000) and have virucidal activity against *Herpes* virus (Reichling et al., 2005).

Due to the notable antimicrobial activity of *L. scoparium* oil, active component from *L. scoparium* oil was purified by various analytical techniques such as silica gel column chromatography, TLC, and prep. HPLC.

Table 1 Antimicrobial activities of essential oil of *L. scoparium* seeds against foodborne bacteria

Sample	Conc. (mg/disc)	Bacterial species					
		Gram positive bacteria			Gram negative bacteria		
		<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. intermedius</i>	<i>S. typhimurium</i>	<i>S. flexneri</i>	<i>S. sonnei</i>
<i>L. scoparium</i> oil	20	++++ ^a	++++	++++	++++	+++	+++
	10	++++	++++	++++	++++	+++	+++
	5	+++	++	++	+++	++	++
	2	+	+	+	++	+	+
Negative control (only solvent)	20	–	–	–	–	–	–

Cultured on Mueller–Hinton agar at 37 °C for 24 h in an incubator

^aDiameter of inhibition zone > 30 mm; +++++; 21–30 mm, ++++; 16–20 mm, ++; 10–15 mm, +; and < 10 mm, –

Table 2 ¹H NMR, ¹³C NMR, and DEPT spectral data^a of LS2231

Carbon	Partial structure	δ _c (ppm)	δ _H (ppm)
1	C	209.9	
2	C	209.9	
3	C	203.6	
4	C	199.5	
5	CH	109.5	3.63 (1H, d ^b , <i>J</i> = 7.2 Hz)
6	C	56.9	
7	C	52.4	
8	CH ₂	47.2	2.88 (2H, d, <i>J</i> = 6.6 Hz)
9	CH	26.9	2.17 (1H, m, <i>J</i> = 26.2 Hz)
10	CH ₃	26.1	0.92 (3H, m, <i>J</i> = 92.4 Hz)
11	CH ₃	24.2	0.92 (3H, m, <i>J</i> = 92.4 Hz)
12	CH ₃	23.8	1.25 (3H, d, <i>J</i> = 49.8 Hz)
13	CH ₃	22.6	1.25 (3H, d, <i>J</i> = 49.8 Hz)
14	CH ₃	16.9	1.34 (3H, m, <i>J</i> = 18.0 Hz)
15	CH ₃	11.8	1.34 (3H, m, <i>J</i> = 18.0 Hz)

^a¹H NMR (600 MHz), ¹³C NMR and DEPT (150 MHz), TMS, δ ppm, *J* in Hz

^b*d* doublet, *m* multiplet

Identification of the active component (LS2231) was achieved by spectroscopic analyses, such as EI-MS, ¹³C NMR and ¹H NMR (Table 2). LS2231 was characterized as leptospermone (Fig. 2(A)). Leptospermone (6-isovaleryl-2,2,4,4-tetramethyl-1,3,5-cyclohexanetrione) has an oily liquid with a characteristic light-yellow appearance (C₁₅H₂₂O₄, molecular weight 266.0): EI-MS (70 eV) *m/z* M⁺ 266, 251, 238, 223, 209, 196, 181, 163, 150, 139, 126, 111, 96, 81, 69, 57, 41; ¹H NMR (CD₃OD, 600 MHz) δ 3.63 (1H, d, *J* = 7.2 Hz), 2.88 (2H, d, *J* = 6.6 Hz), 2.17 (1H, m, *J* = 26.2 Hz), 1.34 (3H, m, *J* = 18.0 Hz), 1.34 (3H, m, *J* = 18.0 Hz), 1.25 (3H, d, *J* = 49.8 Hz), 1.25 (3H, d, *J* = 49.8 Hz), 0.92 (3H, m, *J* = 92.4 Hz), 0.92 (3H, m,

Fig. 2 Structures of leptospermone and its derivatives (A) leptospermone; (B) 1,3-cyclohexanedione; (C) 5,5-dimethyl-1,3-cyclohexanedione; (D) 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione; (E) 1,2,3-Cyclohexanetrione-1,3-dioxime

$J = 92.4$ Hz); ^{13}C NMR (CDCl_3 , 150 MHz) δ 209.9 (C), 209.9 (C), 203.6 (C), 199.5 (C), 109.5 (CH), 56.9 (C), 52.4 (C), 47.2 (CH_2), 26.9 (CH), 26.1 (CH_3), 24.2 (CH_3), 23.8 (CH_3), 22.6 (CH_3), 16.9 (CH_3), 11.8 (CH_3). The analytical data of leptospermone was consistent with those of previously reported triketones (Kashman et al., 1974). Triketones were found mainly in the essential oils of various species of Myrtaceae, including Eucalyptus, Leptospermum, and others (Hellyer, 1968; Porter and Wilkins, 1999).

The antimicrobial activity of leptospermone isolated from *L. scoparium* seeds was evaluated by an agar diffusion method against six foodborne bacteria (Table 3). To establish the structure–activity relationships of leptospermone and its derivatives, 1,3-cyclohexanedione, 1,2,3-cyclohexanetrione-1,3-dioxime, 2-acetyl-1,3-cyclohexanedione, 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione and 5,5-dimethyl-1,3-cyclohexanedione were selected as derivatives (Fig. 2). Leptospermone had excellent antimicrobial activity against six foodborne bacteria at 2.0–0.125 mg/disc. At a concentration of 2.0 mg/disc, 1,2,3-cyclohexanetrione-1,3-dioxime had strong antimicrobial activity against *L. monocytogenes* and *S. typhimurium* and moderate antimicrobial activity against *S. aureus*, *S. flexneri*, *S. intermedius* and *S. sonnei*. In addition, 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione showed strong antimicrobial activity against *S. aureus* and

moderate activity against the other five foodborne bacteria. However, 1,3-cyclohexanedione and 5,5-dimethyl-1,3-cyclohexanedione did not exhibit antimicrobial activities against six foodborne bacteria at 2.0 mg/disc. The MIC values of leptospermone and its derivatives were comparable to the positive control (tetracycline) for six foodborne bacteria using a broth-dilution method (Table 4). Leptospermone possessed the strongest inhibitory activity (MIC 23.63–69.7 $\mu\text{g/mL}$) against six foodborne bacteria. 1,2,3-Cyclohexanetrione-1,3-dioxime and 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione possessed inhibitory activities at 43.9–88.5 $\mu\text{g/mL}$ and 48.1–75.8 $\mu\text{g/mL}$, respectively. However, 1,3-cyclohexanedione and 5,5-dimethyl-1,3-cyclohexanedione did not show inhibitory activities against six foodborne bacteria. The MIC values of tetracycline were determined at 5.85–25.4 $\mu\text{g/mL}$. The antimicrobial activity of leptospermone, 1,2,3-cyclohexanetrione-1,3-dioxime and 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione was less than that of tetracycline against five food-borne bacteria. Although leptospermone and its derivatives presented higher MIC values than tetracycline, it was possible that leptospermone and its derivatives (1,2,3-cyclohexanetrione-1,3-dioxime and 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione) had potential as substitutes for more problematic, commercial antibiotics. Especially, earlier studies (van Klink et al., 2005) showed

Table 3 Antimicrobial activities of leptospermone and its derivatives against foodborne bacteria

Compound	Conc. (mg/disc)	Bacterial species					
		Gram positive bacteria			Gram negative bacteria		
		<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. intermedius</i>	<i>S. typhimurium</i>	<i>S. flexneri</i>	<i>S. sonnei</i>
Leptospermone	2.0	+++ ^a	++++	++++	++++	+++	+++
	1.0	+++	+++	++++	++++	+++	+++
	0.5	++	++	++	+++	++	++
	0.25	++	+	+	++	+	+
	0.125	+	+	+	+	–	–
1,3-Cyclohexanedione	2.0	–	–	–	–	–	–
5,5-Dimethyl-1,3-cyclohexanedione	2.0	–	–	–	–	–	–
2,2,4,4,6,6-Hexamethyl-1,3,5-cyclohexanetrione	2.0	++	+++	++	++	++	++
	1.0	+	++	++	+	+	+
	0.5	–	+	+	–	+	+
	0.25	–	+	–	–	–	–
1,2,3-Cyclohexanetrione-1,3-dioxime	2.0	+++	++	++	+++	++	++
	1.0	++	+	+	+++	+	+
	0.5	+	+	–	++	+	–
	0.25	–	–	–	+	–	–

Cultured on Mueller–Hinton agar at 37 °C for 24 h in an incubator

^aDiameter of inhibition zone > 30 mm, ++++ ; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and < 10 mm, –

Table 4 Minimum inhibition concentration (MIC) values of leptospermone and its derivatives against foodborne bacteria

Compound	MIC ($\mu\text{g/mL}$) ^a					
	Gram positive bacteria			Gram negative bacteria		
	<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. intermedius</i>	<i>S. typhimurium</i>	<i>S. flexneri</i>	<i>S. sonnei</i>
Leptospermone	41.4	53.5	58.1	23.6	65.3	69.7
1,3-Cyclohexanedione	100 <	100 <	100 <	100 <	100 <	100 <
5,5-Dimethyl-1,3-cyclohexanedione	100 <	100 <	100 <	100 <	100 <	100 <
2,2,4,4,6,6-Hexamethyl-1,3,5-cyclohexanetrione	71.4	48.1	66.4	68.3	69.9	75.8
1,2,3-Cyclohexanetrione-1,3-dioxime	65.3	81.7	79.8	43.9	73.4	88.5
Tetracycline ^b	20.2	5.85	19.8	25.4	26.1	25.2

Cultured on Mueller–Hinton broth at 37 °C for 24 h in an incubator

^aMIC values < 100 $\mu\text{g/mL}$

^bTetracycline was served as positive control

that leptospermone possessed antimicrobial activities against methicillin-resistant *Staphylococcus aureus* and methicillin-resistant *S. pseudintermedius*).

Considering the chemical structure of leptospermone and its derivatives, the difference was the number of ketone groups. Leptospermone, which has a cyclic triketone conjugated with carbons in an acyl side chain had the most antimicrobial activity against six foodborne bacteria. In addition, 1,2,3-cyclohexanetrione-1,3-dioxime and 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione, containing hydroxylamine or methyl functional group on cyclic triketone, also had strong antimicrobial activities against six foodborne bacteria. However, 1,3-cyclohexanedione and 5,5-dimethyl-1,3-cyclohexanedione, containing cyclic diketone, observed no antimicrobial activities against six foodborne bacteria. Therefore, the higher antimicrobial activity of triketone group (leptospermone, 1,2,3-cyclohexanetrione-1,3-dioxime and 2,2,4,4,6,6-hexamethyl-1,3,5-cyclohexanetrione) than diketone group (1,3-cyclohexanedione and 5,5-dimethyl-1,3-cyclohexanedione) could be related to this property. These results indicated that the good antimicrobial activity of *L. scoparium* oil could also be associated with the presence of cyclic triketones, as described by Porter and Wilkins (1999) and Christoph et al. (2000). Kashman et al. (1974) also suggested that the core part of the cyclic triketone, which contains a syncarpic acid residue exhibited antimicrobial activity against Gram-positive bacteria. Moreover, cyclic triketones may disrupt the bacteria cytoplasmic membrane because of the function of the hydrophobic properties (van Klink et al., 2005). Earlier *L. scoparium* studies reported that the triketone group (leptospermone and 2,2,4,4,6,6-Hexamethyl-1,3,5-cyclohexanetrione) possessed potent acaricidal activities against *Dermatophagoides pteronyssinus*, *D. farinae*, and *Tyrophagus putrescentiae*, but the

diketone group (1,3-cyclohexanedione and 5,5-Dimethyl-1,3-cyclohexanedione) had no acaricidal activity (van Klink et al., 1999). In addition, the essential oil of *Eucalyptus nitens*, rich in triketones, showed repellent and larvicidal activities against *Aedes aegypti* and *A. albopictus* (Alvarez Costa et al., 2017). The highly antimicrobial compound of *L. scoparium*, which is grown widely in most areas of New Zealand, gives us a chance to develop natural food preservatives rather than hazardous synthetic preservatives. Furthermore, the findings of the correlation between triketones and antimicrobial activity would also serve as a guide for using triketones as natural antimicrobial agents. Our studies are just a first step in releasing the complex mechanisms of six foodborne bacteria by leptospermone isolated from *L. scoparium* seeds and its derivatives. The pharmacological safety and efficacy of leptospermone isolated from *L. scoparium* seeds and its derivatives makes it a potential compound for treatment and prevention foodborne diseases. Nonetheless, leptospermone and its derivatives has not yet been studied the relative bioavailability. Further study is required to evaluate the bioavailability of leptospermone isolated from *L. scoparium* seeds and its derivatives.

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