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Antimicrobial activity of leptospermone and its derivatives against human intestinal bacteria

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ABSTRACT

The anaerobic growth-inhibiting activity of the essential oil of *Leptospermum scoparium* seeds against intestinal bacteria was evaluated *in vitro*. The essential oil at 5.0 mg/disc strongly (+++) inhibited the growth of *Clostridium difficile* and *Clostridium perfringens*, but did not inhibit the growth of *Bifidobacterium breve*, *Bifidobacterium longum*, *Escherichia coli*, or *Lactobacillus casei*. Spectroscopic analyses revealed that the active component of the essential oil was leptospermone. Leptospermone strongly inhibited the growth of *C. difficile* and *C. perfringens* at 1.0 mg/disc and moderately inhibited their growth at 0.5 mg/disc. However, leptospermone did not inhibit the growth of *B. breve*, *B. longum*, and *L. casei*. When compared with the leptospermone derivatives, 1,2,3-cyclohexanetrione-1,3-dioxime exerted strong inhibition against *C. perfringens* and moderate inhibition against *C. difficile* at 0.5 mg/disc, whereas the other derivatives exerted weak or no growth inhibition against all bacteria tested. Taken together, these results indicate that cyclohexanetrione is required for triketone derivatives to inhibit the growth of *C. difficile* and *C. perfringens*. These findings indicate that *L. scoparium* seed-derived materials and 1,2,3-cyclohexanetrione-1,3-dioxime are naturally occurring antimicrobial agents that could be useful in the development of new agents for the specific control of *C. difficile* and *C. perfringens*.

1. Introduction

Intestinal bacteria play various roles in the gastrointestinal tract and human health including protecting the host against infection, performing physical functions, and immunopotentiation (Hentges, 1983; Tannock, 2002). Intestinal bacteria are classified as beneficial or harmful based on the effects they exert on human health. Beneficial bacteria are continuously propagated to maintain ecological equilibrium that may be altered by diet, medication, stress, ageing and various other environmental factors (Rasic, 1983). Conversely, harmful intestinal bacteria such as *Clostridium* spp. and *Escherichia coli* produce toxic and carcinogenic substances and are primarily associated with colon cancer, colitis, and diarrohea (Hentges, 1983). Gastrointestinal diseases resulting from harmful intestinal bacteria and their toxins are a public health concern. Therefore, many studies have been conducted to evaluate methods of maintaining the balance of intestinal bacteria using compounds that promote the growth of beneficial bacteria or inhibit the growth of harmful bacteria (Ahn et al., 1990; Kim et al., 2006; Lim, Jeon, Jeong, Lee, & Lee, 2007). Recently, many studies have been conducted to evaluate the intake of probiotics containing lactic-acid producing bacteria (Rasic, 1983; Sulliva & Nord, 2002). However, these studies have all been limited by the inability of the organism fed to the host to colonise the colon and become part of the microbial community of the gut (Lim et al., 2007). Due to the problems associated with this increased use of antibiotics (resistance, impacts on non-target organisms), there is an imperative need for the development of safer treatment options. As a result, there is heightened interest in the use of bifidus factors and natural growth inhibitors against harmful intestinal bacteria (Ahn et al., 1990; Kim et al., 2006; Lim et al., 2007). Many of these products offer great potential when compared to traditional antibiotics due to their widespread availability, lack of toxicity and harmful adverse effects and their ability to be easily degraded in the environment (Vagionas et al., 2007).

To date, studies designed to identify solutions to antibiotic resistance and residual toxicity have focused on the characterisation of antimicrobial compounds from a variety of medicinal plants. *Leptospermum scoparium* has long been used by the Maori tribes of New Zealand, for the treatment of fevers and pain (Lauten et al., 2005; Tonks et al., 2003; Wyatt et al., 2005). In addition, *Leptospermum* honey has been reported to exhibit antimicrobial activity against *Staphylococcus aureus* and *Helicobacter pylori* (Al Somal, Coley, Molan, & Hancock, 1994; Cooper, Molan, & Harding, 2002; Lee, Churey, & Worobo, 2008). The attention that *Leptospermum* has received is partially due their production of triketones. This is because the derivatives of triketones have many applica-

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tions. Nitisinone and sethoxydim have been used for the treatment of tyrosinaemia type I disease and control grasses, respectively (Burton et al., 1987; Linstedt, Holme, Lock, Hialmarson, & Stranvik, 1992). Furthermore, it has already been reported and confirmed that triketone derivatives extracted from *L. scoparium* seeds exhibit acaricidal activity against *Dermatophagoides farinae*, *D. pteronyssinus*, and *Tyrophagus putrescentiae* (Jeong, 2008). However, relatively little work has been conducted to evaluate the antimicrobial activities of these materials. Therefore, this study was conducted to isolate and identify the antimicrobial compound(s) responsible for the antimicrobial activity of *L. scoparium* oil. Moreover, we evaluated the antimicrobial activities of the leptospermone derivatives against human intestinal bacteria.

2. Materials and methods

2.1. Chemicals

2-Acetyl-1,3-cyclohexanedione, 1,3-cyclohexanedione, 1,2,3-cyclohexanetrione-1,3-dioxime, and 5,5-dimethyl-1,3-cyclohexanedione were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade. Chloroform and methanol (HPLC grade) were used as the mobile phase for HPLC purification.

2.2. Bacteria strains and culture conditions

Six intestinal bacteria, namely *B. breve* ATCC 15700, *B. longum* ATCC 15707, *Clostridium difficile* ATCC 9689, *C. perfringens* ATCC 13124, *E. coli* ATCC 11775, and *Lactobacillus casei* ATCC 393, which were isolated from human faeces were used in this study. Stock cultures of these strains were stored routinely on Eggerth–Gagnon (EG) liver extract-Field's slants at −80 °C, and then subcultured on EG agar (Eiken Chemical, Tokyo, Japan) when required. The plates were then incubated for 2 days at 37 °C in an anaerobic chamber (Coy Lab., Grass Lake, MI, USA) with an atmosphere comprised of 80% N_2, 15% CO_2, and 5% H_2. The bacteria were subsequently grown in EG broth (pH 6.8).

2.3. Isolation and identification

Essential oil of *L. scoparium* seeds was purchased from Tairawhiti Pharmaceuticals Co. Ltd., Gisborne, New Zeeland. Essential oil (11 g) was fractionated on a silica gel column (Merck 70–230 mesh; 620 g; inside diameter, 8 × 93 cm, Rahway, NJ, USA) and then consecutively eluted utilising a gradient step of hexane/ethyl acetate (7:1 to 1:9, vol/vol). In this step, the five fractions (LS1–LS3) were obtained and bioassayed at a concentration of 5 mg/disc. The bioactive fraction (LS2, 5.75 g) was consecutively rechromatographed on a silica gel column using hexane/ethyl acetate (4:1, vol/vol). The five fractions (LS21–LS25) were obtained and bioassayed at a concentration of 5 mg/disc. The bioactive fraction (LS23, 3.72 g) was then purified by Prep. HPLC (LC908C-W60, recycling preparative HPLC, Japan Analytical Industry Co., Ltd., Tokyo, Japan). To accomplish this, the fraction was applied to a Jaigel GS 310 column (GS 310 50 cm plus GS 310 50 cm, inside diameter 21.5 mm, long 1000 mm; Japan Analytical Industry Co., Ltd., Tokyo, Japan), after which methanol (100%, vol/vol) was applied at a flow rate of 3.8 ml/min and the active fraction was detected at 300 nm. This procedure generated four fractions (LS231–LS235), which were each examined for their biological activity. The active fraction (LS233, 1.4 g) was then further separated on a Jaigel W Series Column (W-253 50 cm plus W-252 50 cm, inside diameter 20.0 mm, long 1000 mm; Japan Analytical Industry Co., Ltd., Tokyo, Japan) using chloroform (100%, vol/vol) at a flow rate 2.8 ml/min for HPLC, with the active fraction being detected at 300 nm. Finally, a potent active compound (LS2333, 915 mg) was isolated. The structure of the active component was then determined by spectroscopic analyses. To accomplish this, the ^1H and ^{13}C NMR spectra of the compound were recorded in deuterio chloroform using a JNM-LA 400F7 spectrometer (JEOL, Tokyo, Japan) at 600 and 150 MHz (TMS as an internal standard), respectively, with the chemical shifts (δ) being given in parts per million. Unambiguous UV spectra were obtained in chloroform using a UV spectrometer (Jasco V-550, JASCO, Tokyo, Japan).

2.4. Antimicrobial activity

The paper disc agar diffusion method was used to assess the antimicrobial activity of leptospermone isolated from *L. scoparium* and commercial derivatives such as 2-acetyl-1,3-cyclohexanedione, 1,3-cyclohexanedione, 1,2,3-cyclohexanetrione-1,3-dioxime, and 5,5-dimethyl-1,3-cyclohexanedione. To assay the effects of the samples with regard to their inhibition of the growth of the evaluated bacteria, the suspension (10^6 cfu/ml) of test bacteria was inoculated in 1 ml of sterilized physiological saline. A 0.1 ml aliquot of these bacterial suspensions was then seeded on EG agar. The desired dose of the sample was then dissolved in 100 μl of methanol, which was subsequently applied to a paper disc (Advantee 8 mm-diameter and 1 mm thickness) using a Drummond glass microcapillary tube. The disc was then placed under a fume hood (25 °C) for 1 h to allow the solvent to evaporate, after which it was placed on the surface of the agar plates that had been inoculated with the test bacteria. All plates were then anaerobically incubated for 2 days at 37 °C. In addition, the bacteria were treated with control discs that received 0.1 ml of methanol. The control samples exerted no adverse effects against the tested organisms. All tests were conducted in triplicate and the antimicrobial activity was determined by assigning one of the following values based on the estimated size (diameter) of the zone of inhibition produced by the samples: potent response (++++), zone diameter >30 mm; strong response (+++), zone diameter 21–30 mm; moderate response (++), zone diameter 16–20 mm; weak response (+), zone diameter 10–15 mm; and little or no response (−), zone diameter <10 mm (Lim et al., 2007).

3. Results and discussion

The antimicrobial activities of the essential oil of *L. scoparium* seeds against six intestinal bacteria were assayed using the paper disc agar diffusion method. During routine screening, the essential oil at 5.0 mg/disc exhibited potent inhibiting activity against *C. difficile* and *C. perfringens*, but showed no growth-inhibitory response against *B. breve*, *B. logum*, *E. coli*, or *L. casei* (Table 1). When the essential oil was tested against *C. difficile* and *C. perfringens*, it produced a strong (+++) inhibitory effect at 5–10 mg/disc and moderate (++) inhibition at 2.0 mg/disc (Table 1).

Due to the potent activity of the essential oil, the active component was isolated by silica gel column chromatography and HPLC. Bioassay-guided fractionation of the essential oil enabled the active constituent to be identified by spectroscopic analyses, such as ^{13}C NMR and ^1H NMR. The active component was identified as leptospermone (Fig. 1, (1)). Specifically, leptospermone (6-isovaleryl-2,2,4,4-tetramethyl-1,3,5-cyclohexanetrione) was obtained as a pale yellow oily liquid with the following characteristics: EI-MS (70 eV) *m/z* (percent relative intensity) M^+ 266, 251, 238, 223, 209, 196, 181, 163, 150, 139, 126, 111, 96, 81, 69, 57; ^1H NMR (CD_3OD, 600 MHz) δ *3.62* ∼ *3.63* (1H, d, *J* = 7.2 Hz), 2.87 ∼ 2.288 (2H, d, *J* = 7.2 Hz), 2.15∼2.20 (1H, m, *J* = 27 Hz), 1.44∼1.45 (3H, d, *J* = 5.4 Hz), 1.37∼1.37 (3H, d, *J* = 5.4 Hz), 0.91∼0.99 (3H, m,

Table 1
Antimicrobial activities of essential oil of *L. scoparium* seeds against intestinal bacteria, as determined by the paper disc agar diffusion method[a].

Sample	Dosage (mg/disc)	Bacterial strain[b]					
		B. breve	*B. longum*	*C. difficile*	*C. perfringens*	*E. coli*	*L. casei*
Oil	10	–[c]	–	+++	+++	–	–
	5	–	–	+++	+++	–	–
	2	–	–	++	++	–	–

[a] Cultured on Eggerth–Gagnon agar at 37 °C for 2 days in an atmosphere comprised of 80% N_2, 15% CO_2, and 5% H_2.
[b] Concentration of each bacteria was approximately 106 (cfu)/ml.
[c] Inhibitory zone diameter >30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, –.

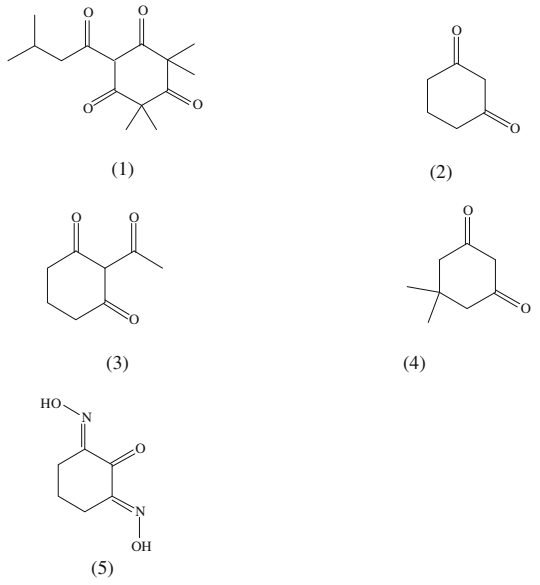

Fig. 1. Structures of leptospermone and its derivatives. (1) Leptospermone (915 mg). (2) 1,3-Cyclohexanedione. (3) 2-Acetyl-1,3-cyclohexanedione. (4) 5,5-Dimethyl-1,3-cyclohexanedione. (5) 1,2,3-cyclohexanetrione-1,3-dioxime.

J = 52.8 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 209.9, 199.4, 109.4, 56.9, 47.1, 23.8, 22.5, 20.9, 19.0, 16.8 (Fig. 1). The spectroscopic data generated by the analysis of leptospermone in this study matched those of previously reported triketones (van Klink, Brophy, Perry, & Weavers, 1999).

The antimicrobial activity of leptospermone and its derivatives (triketone) against six intestinal bacteria was examined (Table 2). When tested against *C. difficile* and *C. perfringens*, leptospermone produced strong inhibitory effect at 1.0 mg/disc and moderate inhibitory effect at 0.5 mg/disc. However, leptospermone showed no growth inhibition against *B. breve*, *B. longum*, *E. coli*, and *L. casei*. When the effects of other triketone derivatives were evaluated, 1,3-cyclohexanedione exerted moderate or weak growth inhibition against the five intestinal bacteria evaluated. In addition, 2-acetyl-1,3-cyclohexanedione showed weak inhibition against *B. longum*, while 5,5-dimethyl-1,3-cyclohexanedione exhibited moderate inhibition at 5.0 mg/disc. However, treatment with 1,2,3-cyclohexanetrione-1,3-dioxime at a concentration of either 5.0 or 2.0 mg/disc exerted strong inhibition against *B. breve*, *B. longum*, *C. difficile*, *C. perfringens* and *E. coli*, without exerting an inhibition effect on the growth of *L. casei*. In addition, 1,2,3-cyclohexanetrione-1,3-dioxime exerted moderate inhibition against *C. perfringens* at a dose 0.25 mg/disc, whereas treatment with the same concentration of this compound did not inhibit the growth of beneficial intestinal bacteria. Based on these results, low concentrations of leptospermon and 1,2,3-cyclohexanetrione-1,3-dioxime appear to exert strong growth inhibition against *C. perfringens* without adversely effecting beneficial bacteria. Recently, many studies have been conducted to evaluate natural growth promoters of bifidobacteria or inhibitors of harmful intestinal bacteria (Ahn et al., 1990; Lim et al., 2007). The use of medicinal plants as growth modulators has been of interest because plants are one of the richest sources of bioactive chemicals (Kim et al., 2006; Lim et al., 2007). For example, Lim et al. (2007) evaluated 5-hydroxy-1,4-naphthoquinone isolated from *Caesalpinia sappan* to determine if it inhibited the

Table 2
Antimicrobial activities of leptospermone and its derivatives against intestinal bacteria, as determined by the paper disc agar diffusion method[a].

Sample	Dosage (mg/disc)	Bacterial strain[b]					
		B. breve	*B. longum*	*C. difficile*	*C. perfringens*	*E. coli*	*L. casei*
Leptospermone	5.0	–[c]	–	++++	++++	–	–
	2.0	–	–	+++	++++	–	–
	1.0	–	–	+++	+++	–	–
	0.5	–	–	++	++	–	–
	0.25	–	–	+	++	–	–
1,3-Cyclohexanedione	5.0	+	++	+	+	+	–
	2.0	–	+	–	–	–	–
2-Acetyl-1,3-cyclohexanedione	5.0	–	+	–	–	–	–
	2.0	–	–	–	–	–	–
5,5-Dimethyl-1,3-cyclohexanedione	5.0	–	++	–	–	–	–
	2.0	–	+	–	–	–	–
1,2,3-Cyclohexanetrione-1,3-dioxime	5.0	+++	+++	+++	++++	+++	–
	2.0	+++	++	+++	++++	+++	–
	1.0	++	++	++	++++	++	–
	0.5	+	–	++	+++	–	–
	0.25	–	–	–	++	–	–

[a] Cultured on Eggerth–Gagnon agar at 37 °C for 2 days in an atmosphere comprised of 80% N_2, 15% CO_2, and 5% H_2.
[b] Concentration of each bacteria was approximately 106 (cfu)/ml.
[c] Inhibitory zone diameter >30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, –.

growth of harmful intestinal bacteria, *C. perfringens* (Lim et al., 2007).

Leptospermum scoparium, which is a shrub native to New Zealand, is traditionally used for the treatment of various diseases. Until recently, phytochemical analysis and biological screening of *L. scoparium* has focused on its essential oil (Wyatt et al., 2005). The results of these analyses have demonstrated that the essential oil of *L. scoparium* seeds have antibacterial, antifungal, and spasmolytic properties (Lis-Balchin, Hart, & Deans, 2000; Perry et al., 1997; Rasic, 1983; Wyatt et al., 2005). However, relatively little work has been conducted to evaluate the antimicrobial activities of active components isolated from *L. scoparium* seeds and their derivatives against intestinal bacteria. In this study, the growth-inhibitory activity of leptospermone isolated from *L. scoparium* seeds confirmed that it effectively inhibits harmful intestinal bacteria. Leptospermone is one of triketone chemotypes that possess multiple hydroxyl ketones on a six-membered ring (cyclohexane). In this study, the structural relationships of leptospermone and its triketone derivatives against six intestinal bacteria were evaluated by comparing the size zone of inhibition that each compound produced. Leptospermone and 1,2,3-cyclohexanetrion-1,3,-dioxime, which contains cyclohexane conjugated trihydroxyl ketones, were found to inhibit the growth of *C. difficile* and *C. perfringens*; however, 1,3-cyclohexandione, 2-acetyl-1,3,-cyclohexanedione, and 5,5-dimethyl-1,3,-cyclohexanedione exerted either a weak or no inhibitory response. These findings indicate that the cyclohexane containing the 1,3-dihydroxyl ketones is responsible for the antibacterial effects of *L. scoparium*.

In conclusion, the results presented here indicate that the essential oil of *L. scoparium* seeds and triketone derivatives can be useful for the inhibition against *C. difficile* and *C. perfringens*. In addition, our findings demonstrated that leptospermone and 1,2,3-cyclohexanetrion-1,3-dioxime were responsible for these effects. However, additional studies should be conducted to evaluate safety issues associated with the use of *L. scoparium* oil, leptospermone and 1,2,3-cyclohexanetrion-1,3-dioxime to treat humans. Furthermore, future studies should be conducted to evaluate the pharmacological actions of these compounds and to develop formulations to improve their antimicrobial potency and stability.

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