Rhodomertus tomentosa (Aiton) Hassk. ethanol extract and rhodomertone: a potential strategy for the treatment of biofilm-forming staphylococci

Jongkon Saising,^{1,2} Metta Ongsakul¹
and Supayang Piyawan Voravuthikunchai^{1,2}

Correspondence
Supayang Piyawan
Voravuthikunchai
supayang.v@psu.ac.th

¹Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

²Natural Products Research Center, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

The anti-staphylococcal activity of an ethanol extract of *Rhodomertus tomentosa* and its pure compound, rhodomertone, as well as their effects on staphylococcal biofilm formation and biofilm-grown cells were assessed. MIC and minimal bactericidal concentration values of the ethanol extract and rhodomertone against planktonic cultures and biofilms of five clinical strains each of *Staphylococcus aureus* and *Staphylococcus epidermidis*, and American Type Culture Collection (ATCC) strains of both species, were 32–512 and 0.25–2 µg ml⁻¹, respectively. Results from time–kill studies indicated that rhodomertone at a concentration of 4× MIC could reduce the number of *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 35984 cells by 99.9 % within 3 and 13 h, respectively. The ability of rhodomertone and the ethanol extract to prevent biofilm formation and kill mature biofilms was assessed: both demonstrated better activity than vancomycin at inhibiting staphylococcal biofilm formation. In addition, the viability of 24 h and 5-day staphylococcal biofilm-grown cells decreased after treatment with the ethanol extract and rhodomertone. The ability to reduce biofilm formation and kill mature biofilms occurred in a dose-dependent manner. Scanning electron microscopy clearly confirmed that treatment with rhodomertone at 16× MIC could reduce 24 h biofilm formation and the numbers of staphylococci, whilst at 64× MIC this compound destroyed the organisms in the 5-day established biofilm. These results suggest that rhodomertone has the potential for further drug development for the treatment of biofilm-forming staphylococcal infections.

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INTRODUCTION

Staphylococci are widely involved in minor and severe infections. Currently, these organisms are resistant to many commonly used antibiotics, and treatment of staphylococcal infections is becoming difficult due to the increase in antibiotic resistance of these organisms. Importantly, biofilm formation is often associated with antibiotic resistance in these organisms, and antimicrobial resistance in biofilm-forming cells is increased compared with that in free-living cells (Cerca *et al.*, 2005; Nishimura *et al.*, 2006; Frank *et al.*, 2007). Biofilm formation is one of the most important staphylococcal virulence factors. The formation of biofilm involves two stages: (i) initial adherence of bacterial cells to the host-cell surface or other materials; and (ii) the formation of a multilayered cell cluster surrounded by extracellular polysaccharide matrix (Götz, 2002). The production of extracellular polysaccharide matrix has been

suggested to prevent the access of antibiotics to the bacterial cells embedded in the biofilm (Mah & O'Toole, 2001). Consequently, effective agents are necessary to control biofilm-producing staphylococci.

Medicinal plants have been studied extensively as alternative antimicrobial therapeutic agents. An ethanol extract of *Rhodomertus tomentosa* leaves as well as rhodomertone, a pure compound in the acylphloroglucinol class isolated from the leaves of this medicinal plant species, have shown profound antibacterial activity against a wide range of Gram-positive pathogenic bacteria with low MIC values, comparable to available antibiotics including vancomycin (Saising *et al.*, 2008; Limsuwan *et al.*, 2009; Voravuthikunchai *et al.*, 2010). Natural compounds from certain plants such as carvacrol, thymol and salvipisone have also been demonstrated to exert effects on staphylococcal biofilm (Kuzma *et al.*, 2007; Nostro *et al.*, 2007; Wang *et al.*, 2009).

The aim of this study was to investigate further the ability of an ethanol extract of *R. tomentosa* leaves and pure

Abbreviation: MBC, minimal bactericidal concentration.

Table 1. MIC and MBC values ($\mu\text{g ml}^{-1}$) of *R. tomentosa* ethanol extract, rhodomyrtone and vancomycin against *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from acne lesions

Isolate	Ethanol extract		Rhodomyrtone		Vancomycin	
	MIC	MBC	MIC	MBC	MIC	MBC
<i>Staphylococcus aureus</i>						
NPRC 310	32	128	0.5	0.5	1.0	1.0
NPRC 313	32	128	0.5	1.0	0.5	1.0
NPRC 326	128	256	1.0	2.0	1.0	4.0
NPRC 334	32	64	0.5	2.0	1.0	4.0
NPRC 361	32	128	1.0	2.0	1.0	2.0
ATCC 25923	32	64	0.5	1.0	0.5	1.0
<i>Staphylococcus epidermidis</i>						
NPRC 511	32	64	1.0	2.0	2.0	4.0
NPRC 522	32	64	1.0	2.0	1.0	2.0
NPRC 535	128	128	0.25	0.25	1.0	1.0
NPRC 576	128	512	0.25	1.0	1.0	2.0
NPRC 585	128	128	1.0	1.0	2.0	2.0
ATCC 35984	256	512	0.5	0.5	1.0	1.0

rhodomyrtone to eradicate staphylococcal biofilms, as well as to study their effects on the biofilm-grown bacterial cells.

METHODS

Bacteria. *Staphylococcus aureus* ($n=5$) and *Staphylococcus epidermidis* ($n=5$) were isolated from acne lesions and identified on the basis of colony characteristics, Gram staining, and catalase and coagulase tests. The cultures were stored at 4°C until use. The biofilm-forming strains *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 35984 were included as controls.

Antimicrobial agents. Leaves of *R. tomentosa* (Aiton) Hassk. were dried and ground, and the powder was extracted with 95% ethanol. The ethanol was evaporated using a rotary evaporator and the extract was resuspended in 10% DMSO (Merck) before use. Rhodomyrtone was isolated according to a method described previously and dissolved in 10% DMSO (Saising *et al.*, 2008). Vancomycin (Fluka) and DMSO were included as controls.

Determination of MICs and minimal bactericidal concentrations (MBCs). The MICs of antimicrobial agents were determined using a modified broth microdilution method (CLSI, 2006). Aliquots (20 μl) of the ethanol extract and rhodomyrtone were diluted in a 96-well microtitre plate to final concentrations of 1024–0.5 and 32–0.06 $\mu\text{g ml}^{-1}$, respectively, and 80 μl Mueller–Hinton broth (MHB; Difco) was added to each well. One hundred microlitres of bacterial suspension (10^6 c.f.u. ml^{-1}) was added to each well and incubated at 35°C for 16–18 h. The MIC was read as the lowest concentration that completely inhibited bacterial growth. The MBC was assessed as the extract that gave significant MIC values by streaking the culture on trypticase soy agar (Difco). Experiments were carried out in triplicate.

Time-kill study. *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 35984 were used as representative strains in time-kill kinetic experiments. An inoculum (10^6 c.f.u. ml^{-1}) of the growing cultures was added to trypticase soy broth (TSB; Difco) supplemented with rhodomyrtone at concentrations of $0.5\times$, $1\times$, $2\times$ and $4\times$ MIC and incubated at 35°C . Viable counts were determined by a drop plate method. Briefly, samples were collected at various time intervals and

Fig. 1. Time-kill curves of *Staphylococcus aureus* ATCC 25923 (a) and *Staphylococcus epidermidis* ATCC 35984 (b) in the presence of rhodomyrtone at various concentrations. The limit of detection was 100 c.f.u. ml^{-1} . $\circ$, Control; $\bullet$, $0.5\times$ MIC; $\blacksquare$, MIC; $\blacktriangle$, $2\times$ MIC; $\blacklozenge$, $4\times$ MIC.

serially diluted tenfold. Each dilution was dropped onto a trypticase soy agar plate. The experiment was carried out in duplicate and the results were presented as mean log numbers of organisms $\pm$ SEM.

Effect of *R. tomentosa* ethanol extract, rhodomyrton and vancomycin on biofilm formation. An experiment was performed according to the protocol of Karaolis *et al.* (2005) with slight modifications. Briefly, 100 μ l of an overnight culture grown in TSB was diluted to 10^6 c.f.u. ml $^{-1}$ and transferred to a 96-well microtitre plate containing 20 μ l test antibacterial agents at subinhibitory concentrations and 80 μ l TSB. After incubation at 37 °C for 24 h, the wells were washed twice with PBS, air-dried and stained with 200 μ l 0.1 % crystal violet solution (Merck) for 30 min. The plate was washed with water and air-dried. Stained biofilms were dissolved in 200 μ l DMSO and the A_{595} was measured using a microplate reader (BioTek). The relative percentage of biofilm formation was defined as: (mean A_{595} of treated well/mean A_{595} of control well) $\times$ 100.

Effect of *R. tomentosa* ethanol extract, rhodomyrton and vancomycin on established biofilms. Established biofilms were grown as described by Kuźma *et al.* (2007). Two hundred microlitres of growing culture (10^6 c.f.u. ml $^{-1}$) was transferred to a 96-well microtitre plate and incubated at 37 °C for 24 h and 5 days. For 5-day biofilms, planktonic cells were removed and fresh TSB was added daily. After incubation, the medium was removed and the wells were rinsed twice with PBS. TSB (180 μ l) and 20 μ l of the test agents at different concentrations were added. After incubation at 37 °C for 24 h, the medium was removed and replaced with 200 μ l PBS

supplemented with 10 μ l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg ml $^{-1}$; Sigma) and further incubated at 37 °C for 2 h. The insoluble purple formazan was obtained by cleavage of MTT by the dehydrogenase enzyme of living staphylococcal cells. The formazan crystals were dissolved in DMSO and the A_{595} was determined. The viability percentage of biofilm was defined as: (mean A_{595} of treated well/mean A_{595} of control well) $\times$ 100.

Scanning electron microscopy. *Staphylococcus epidermidis* ATCC 35984 was used to study the appearance of established biofilm on polystyrene discs. After incubation for 24 h and 5 days, the discs were washed three times with PBS. Rhodomyrton in TSB was added and the discs were incubated at 37 °C for 24 h. The discs were washed three times with PBS, fixed in 2 % glutaraldehyde (Electron Microscopy Sciences) in PBS for 2 h, washed with PBS and then post-fixed in 4 % osmium tetroxide (Electron Microscopy Sciences) in PBS for 1 h. After washing with distilled water, the specimens were dehydrated in a graded ethanol series (50–100 %), mounted on aluminium stubs, allowed to dry and then coated with gold. The samples were examined under a scanning electron microscope (Quanta 400 FEG; FEI).

Table 2. Effect of *R. tomentosa* ethanol extract, rhodomyrton and vancomycin on biofilm formation of *Staphylococcus aureus*

Values with different superscripts differ significantly (Duncan's test; $P < 0.05$). Biofilm formation was measured relative to a positive control with no treatment (100 %).

Isolate	Agent*	Biofilm formation (%)		
		0.5 $\times$ MIC	0.25 $\times$ MIC	0.125 $\times$ MIC
NPRC 310	E	96.8 $\pm$ 5.6 ^a	98.2 $\pm$ 5.1 ^a	100.8 $\pm$ 3.7 ^a
	R	90.9 $\pm$ 4.2 ^a	91.8 $\pm$ 4.2 ^a	105.7 $\pm$ 4.2 ^a
	V	107.4 $\pm$ 13.3 ^a	109.1 $\pm$ 6.3 ^a	109.0 $\pm$ 13.9 ^a
NPRC 313	E	119.8 $\pm$ 6.8 ^f	119.9 $\pm$ 6.3 ^f	115.4 $\pm$ 6.9 ^{ef}
	R	56.9 $\pm$ 8.6 ^a	99.2 $\pm$ 6.3 ^{de}	93.2 $\pm$ 3.1 ^{cd}
	V	69.0 $\pm$ 9.1 ^{ab}	75.1 $\pm$ 9.8 ^{abc}	85.9 $\pm$ 10.3 ^{bd}
NPRC 326	E	67.6 $\pm$ 8.0 ^{ab}	63.4 $\pm$ 3.6 ^a	68.9 $\pm$ 3.4 ^{abc}
	R	83.6 $\pm$ 4.7 ^{cd}	89.0 $\pm$ 4.4 ^d	86.6 $\pm$ 3.1 ^d
	V	80.3 $\pm$ 1.7 ^{bcd}	91.9 $\pm$ 6.8 ^d	95.8 $\pm$ 7.2 ^d
NPRC 334	E	77.5 $\pm$ 7.9 ^a	75.6 $\pm$ 7.6 ^a	91.9 $\pm$ 11.3 ^{ab}
	R	76.3 $\pm$ 10.7 ^a	119.5 $\pm$ 0.8 ^d	114.2 $\pm$ 7.1 ^{cd}
	V	95.0 $\pm$ 10.0 ^{abc}	110.1 $\pm$ 9.5 ^{bcd}	119.5 $\pm$ 7.7 ^d
NPRC 361	E	90.6 $\pm$ 3.8 ^b	91.7 $\pm$ 1.4 ^b	93.7 $\pm$ 4.4 ^b
	R	59.9 $\pm$ 0.6 ^a	97.5 $\pm$ 4.0 ^b	112.3 $\pm$ 8.0 ^{cd}
	V	91.0 $\pm$ 1.0 ^b	104.1 $\pm$ 8.8 ^{bc}	124.4 $\pm$ 6.4 ^d
ATCC 25923	E	82.7 $\pm$ 4.1 ^{ab}	93.0 $\pm$ 1.7 ^{bc}	104.5 $\pm$ 1.2 ^{bc}
	R	80.5 $\pm$ 1.4 ^{ab}	108.8 $\pm$ 1.0 ^c	101.7 $\pm$ 2.5 ^{bc}
	V	62.5 $\pm$ 7.5 ^a	82.9 $\pm$ 8.3 ^{ab}	80.6 $\pm$ 9.0 ^{ab}

*E, Ethanol extract; R, rhodomyrton; V, vancomycin.

Table 3. Effect of *R. tomentosa* ethanol extract, rhodomyrton and vancomycin on biofilm formation of *Staphylococcus epidermidis*

Values with different superscripts differ significantly (Duncan's test; $P < 0.05$). Biofilm formation was measured relative to a positive control with no treatment (100 %).

Isolate	Agent*	Biofilm formation (%)		
		0.5 $\times$ MIC	0.25 $\times$ MIC	0.125 $\times$ MIC
NPRC 511	E	85.6 $\pm$ 4.6 ^a	90.4 $\pm$ 7.2 ^a	94.8 $\pm$ 8.6 ^a
	R	85.8 $\pm$ 11.2 ^a	85.0 $\pm$ 1.9 ^a	92.3 $\pm$ 6.2 ^a
	V	91.8 $\pm$ 3.2 ^a	83.9 $\pm$ 1.6 ^a	93.2 $\pm$ 2.3 ^a
NPRC 522	E	44.9 $\pm$ 2.5 ^a	53.4 $\pm$ 5.3 ^a	76.3 $\pm$ 2.6 ^b
	R	48.3 $\pm$ 7.1 ^a	87.9 $\pm$ 8.7 ^{bc}	92.1 $\pm$ 6.9 ^c
	V	95.1 $\pm$ 3.2 ^c	99.7 $\pm$ 0.5 ^c	98.3 $\pm$ 2.5 ^c
NPRC 535	E	46.0 $\pm$ 0.5 ^a	56.4 $\pm$ 1.3 ^b	57.5 $\pm$ 0.8 ^{bc}
	R	67.2 $\pm$ 4.6 ^c	95.4 $\pm$ 3.1 ^e	88.6 $\pm$ 0.9 ^{de}
	V	86.4 $\pm$ 4.2 ^{de}	87.6 $\pm$ 5.3 ^{de}	82.2 $\pm$ 4.5 ^d
NPRC 576	E	31.8 $\pm$ 5.7 ^a	42.3 $\pm$ 3.9 ^{ab}	52.2 $\pm$ 1.8 ^b
	R	68.2 $\pm$ 4.9 ^c	67.3 $\pm$ 6.1 ^c	67.6 $\pm$ 2.6 ^c
	V	101.8 $\pm$ 5.2 ^d	109.5 $\pm$ 3.3 ^d	104.8 $\pm$ 4.4 ^d
NPRC 585	E	24.3 $\pm$ 5.4 ^a	34.8 $\pm$ 3.1 ^b	62.2 $\pm$ 3.2 ^c
	R	82.3 $\pm$ 1.3 ^d	105.7 $\pm$ 1.6 ^e	109.0 $\pm$ 2.8 ^e
	V	102.4 $\pm$ 4.9 ^e	108.5 $\pm$ 0.4 ^e	101.8 $\pm$ 3.9 ^e
ATCC 35984	E	43.7 $\pm$ 8.1 ^a	103.1 $\pm$ 6.4 ^{bc}	102.3 $\pm$ 5.9 ^{bc}
	R	85.5 $\pm$ 0.7 ^b	96.0 $\pm$ 5.6 ^{bc}	103.3 $\pm$ 6.2 ^{bc}
	V	102.4 $\pm$ 3.2 ^{bc}	104.2 $\pm$ 3.3 ^c	104.4 $\pm$ 1.6 ^c

*E, Ethanol extract; R, rhodomyrton; V, vancomycin.

Statistical analysis. Statistical analysis was performed using analysis of variance. Comparisons between means were carried out according to Duncan's test. Differences were considered significant at $P < 0.05$.

RESULTS

MICs and MBCs

The MIC and MBC values of the *R. tomentosa* ethanol extract against the clinical isolates ranged from 32 to 512 $\mu\text{g ml}^{-1}$. Interestingly, the MIC and MBC values of rhodomyrtone ranged from 0.25 to 2.0 $\mu\text{g ml}^{-1}$, which were similar to those of vancomycin (0.5–4.0 $\mu\text{g ml}^{-1}$) (Table 1).

Time-kill study

The number of viable cells of *Staphylococcus aureus* ATCC 25923 after exposure to rhodomyrtone at a concentration of $4 \times \text{MIC}$ decreased by at least 3 logs within 3 h (Fig. 1a), whilst it took almost 13 h to reduce the numbers of

Staphylococcus epidermidis ATCC 35984 to the same level (Fig. 1b).

Effect of *R. tomentosa* ethanol extract, rhodomyrtone and vancomycin on biofilm formation

Treatment with the *R. tomentosa* ethanol extract and rhodomyrtone at $0.5 \times$ and $0.25 \times$ MIC was found to be effective in reducing biofilm formation in most of the *Staphylococcus aureus* isolates. At $0.5 \times$ MIC, rhodomyrtone could reduce biofilm production in all isolates, whilst the ethanol extract and vancomycin reduced biofilm formation in most but not all of the isolates at this concentration (Table 2). For *Staphylococcus epidermidis*, the ethanol extract at $0.5 \times$ and $0.25 \times$ MIC clearly demonstrated a better ability to reduce biofilm formation than rhodomyrtone and vancomycin. At $0.5 \times$ MIC, ethanol extract and rhodomyrtone reduced biofilm formation in all six isolates, whilst at $0.25 \times$ MIC, they each inhibited biofilm formation in five of the six isolates. Vancomycin reduced biofilm formation in three of the six isolates at $0.5 \times$ and $0.25 \times$ MIC (Table 3).

Fig. 2. Effect of *R. tomentosa* ethanol extract (open bars), rhodomyrtone (shaded bars) and vancomycin (black bars) on *Staphylococcus aureus* viability for 24 h biofilm of NPRC 361 (a), 24 h biofilm of ATCC 25923 (b), 5-day biofilm of NPRC 361 (c) and 5-day biofilm of ATCC 25923 (d).

The impact of the compounds on biofilm formation was found to be dose-dependent.

Effect of *R. tomentosa* ethanol extract, rhodomyrton and vancomycin on established biofilms

Ethanol extract, rhodomyrton and vancomycin exhibited antibiofilm activity on established biofilms in a dose-dependent manner. The results demonstrated that rhodomyrton and the ethanol extract were more efficient than vancomycin in killing organisms in an established biofilm. Rhodomyrton at 16–64 $\times$ MIC demonstrated better efficiency than the ethanol extract and vancomycin at reducing the bacterial viability of 24 h and 5-day biofilms of *Staphylococcus aureus* ($P < 0.05$) (Fig. 2). For *Staphylococcus epidermidis*, significant differences in cell viability were observed among different test antibacterial agents at 64 $\times$ MIC ($P < 0.05$). At 8 $\times$ MIC, rhodomyrton exhibited a better activity than the ethanol extract and vancomycin in reducing cell viability of 24 h biofilms ($P < 0.05$) (Fig. 3). The survival of 5-day biofilms was 58.5 % in the presence of rhodomyrton at 64 $\times$ MIC. Vancomycin demonstrated no effect on

5-day biofilm established organisms. It should be noted that the compounds demonstrated better activity in killing the organisms in 24 h biofilms than those in 5-day biofilms.

Scanning electron microscopy

The structure of 24 h established biofilms is shown in Fig. 4. Treatment of the organisms with rhodomyrton at 16 $\times$ MIC damaged the biofilm structure (Fig. 4b) and the bacterial population. Well-organized biofilm layers were still observed, although some bacterial cells were destroyed after treatment with vancomycin at 16 $\times$ MIC (Fig. 4c). For 5-day biofilms, proliferating bacteria covered the whole surface with a large amount of biofilm and polysaccharide matrix after treatment with 1 % DMSO (Fig. 4d) and vancomycin at 64 $\times$ MIC (Fig. 4f). In contrast, treatment with rhodomyrton at 64 $\times$ MIC demonstrated destruction of the established staphylococcal biofilm (Fig. 4e).

DISCUSSION

The *R. tomentosa* ethanol extract and rhodomyrton both possessed strong activity against *Staphylococcus aureus* and

Fig. 3. Effect of *R. tomentosa* ethanol extract (open bars), rhodomyrton (shaded bars) and vancomycin (black bars) on *Staphylococcus epidermidis* viability for 24 h biofilm of NPRC 522 (a), 24 h biofilm of ATCC 35984 (b), 5-day biofilm of NPRC 522 (c) and 5-day biofilm of ATCC 35984 (d).

Fig. 4. Scanning electron micrographs of *Staphylococcus epidermidis* ATCC 35984 biofilm after exposure to the test antibacterial agents. (a) Control; (b) 24 h biofilm exposed to 16× MIC rhodomyrtone; (c) 24 h biofilm exposed to 16× MIC vancomycin; (d) 5-day biofilm control (exposed to DMSO); (e) 5-day biofilm exposed to 64× MIC rhodomyrtone; (f) 5-day biofilm exposed to 64× MIC vancomycin.

Staphylococcus epidermidis. In addition to direct antibacterial activity, antibacterial agents that are able to prevent or interfere with biofilm formation and are active against biofilm-forming organisms would be of considerable

advantage in treatment of infections. The *R. tomentosa* ethanol extract was able to reduce biofilm formation more readily than rhodomyrtone and vancomycin. Limsuwan & Voravuthikunchai (2008) previously demonstrated the

antibiofilm property of *R. tomentosa* ethanol extract against *Streptococcus pyogenes*. Active components in this plant such as tannins (Liu *et al.*, 1998) and triterpenoids (Hui *et al.*, 1975) possibly affect biofilm formation. The mechanisms of the antimicrobial action of tannins may be related to the ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins (Cowan, 1999). Protein levels during initial biofilm development were found to decrease fivefold after treatment with carvacrol, a compound in the terpenoid group (Knowles *et al.*, 2005).

Studies of plant natural products on biofilm eradication have been reported. Their actions on biofilm may involve the inhibition of cell accumulation, interrupting the biofilm structure, destruction of extracellular matrix, dissemination through the biofilm extracellular matrix and subsequent damage due to their powerful antimicrobial activity (Nostro *et al.*, 2007; Kwieciński *et al.*, 2009; Marino *et al.*, 2010).

The ethanol extract and rhodomyrtone demonstrated a better ability than vancomycin to reduce the viability of biofilm-grown cells. Although planktonic bacterial cells were rapidly exposed to vancomycin, bacterial cells inside biofilm were only exposed slowly to vancomycin because of its reduced rate of penetration, resulting in gradual exposure of the bacterial cells to low concentrations (Jefferson *et al.*, 2005). A low molecular mass (442.6 g mol^{-1}) has been reported for rhodomyrtone (Salni *et al.*, 2002). Thus, it can be speculated that this compound could easily penetrate the biofilm matrix with a high accumulation rate, resulting in efficient elimination of the bacterial cells.

Although rhodomyrtone exhibited potential activity with a very low MIC value, the mechanisms of action of this compound are still unclear. Proteomic studies on cellular proteins in methicillin-resistant *Staphylococcus aureus* treated with rhodomyrtone have suggested changes in proteins related to cell-wall biosynthesis and cell division, protein degradation, stress response and oxidative stress, cell-surface antigen and virulence factors, and various metabolic pathways (Sianglum *et al.*, 2011). In addition, glyceraldehyde-3-phosphate dehydrogenase, CAMP factor and streptococcal pyrogenic exotoxin C were reduced in rhodomyrtone-treated *Streptococcus pyogenes* cells (Limsuwan *et al.*, 2011).

In conclusion, *R. tomentosa* ethanol extract and rhodomyrtone possessed strong antibacterial activity. Moreover, rhodomyrtone had significant effects on both biofilm formation and the survival of the organisms in the established biofilms. These results support the application of rhodomyrtone as an alternative agent against biofilm-producing staphylococcal infections.

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REFERENCES

- Cerca, N., Martins, S., Cerca, F., Jefferson, K. K., Pier, G. B., Oliveira, R. & Azeredo, J. (2005). Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. *J Antimicrob Chemother* **56**, 331–336.
- CLSI (2006). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 7th edn; Approved Standard. M7-A7. Wayne, PA: Clinical and Laboratory Standards Institute.
- Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clin Microbiol Rev* **12**, 564–582.
- Frank, K. L., Reichert, E. J., Piper, K. E. & Patel, R. (2007). In vitro effects of antimicrobial agents on planktonic and biofilm forms of *Staphylococcus lugdunensis* clinical isolates. *Antimicrob Agents Chemother* **51**, 888–895.
- Götz, F. (2002). *Staphylococcus* and biofilms. *Mol Microbiol* **43**, 1367–1378.
- Hui, W.-H., Li, M.-M. & Luk, K. (1975). Triterpenoids and steroids from *Rhodomyrtus tomentosa*. *Phytochemistry* **14**, 833–834.
- Jefferson, K. K., Goldmann, D. A. & Pier, G. B. (2005). Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother* **49**, 2467–2473.
- Karaolis, D. K., Rashid, M. H., Chythanya, R., Luo, W., Hyodo, M. & Hayakawa, Y. (2005). c-di-GMP (3'-5'-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell–cell interactions and biofilm formation. *Antimicrob Agents Chemother* **49**, 1029–1038.
- Knowles, J. R., Roller, S., Murray, D. B. & Naidu, A. S. (2005). Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. *Appl Environ Microbiol* **71**, 797–803.
- Kuźma, L., Rózsalski, M., Walencka, E., Rózsalska, B. & Wysokińska, H. (2007). Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L.: salvipisone as a potential anti-biofilm agent active against antibiotic resistant staphylococci. *Phytomedicine* **14**, 31–35.
- Kwieciński, J., Eick, S. & Wójcik, K. (2009). Effects of tea tree (*Melaleuca alternifolia*) oil on *Staphylococcus aureus* in biofilms and stationary growth phase. *Int J Antimicrob Agents* **33**, 343–347.
- Limsuwan, S. & Voravuthikunchai, S. P. (2008). *Boesenbergia pandurata* (Roxb.) Schltr., *Eleutherine americana* Merr. and *Rhodomyrtus tomentosa* (Aiton) Hassk. as antibiofilm producing and antiquorum sensing in *Streptococcus pyogenes*. *FEMS Immunol Med Microbiol* **53**, 429–436.
- Limsuwan, S., Trip, E. N., Kouwen, T. R., Piersma, S., Hiranrat, A., Mahabusarakam, W., Voravuthikunchai, S. P., van Dijk, J. M. & Kayser, O. (2009). Rhodomyrtone: a new candidate as natural antibacterial drug from *Rhodomyrtus tomentosa*. *Phytomedicine* **16**, 645–651.
- Limsuwan, S., Hesselting-Meinders, A., Voravuthikunchai, S. P., van Dijk, J. M. & Kayser, O. (2011). Potential antibiotic and anti-infective effects of rhodomyrtone from *Rhodomyrtus tomentosa* (Aiton) Hassk. on *Streptococcus pyogenes* as revealed by proteomics. *Phytomedicine* **18**, 934–940.
- Liu, Y., Hou, A., Ji, C. & Wu, Y. (1998). Isolation and structure of hydrolysable tannins from *Rhodomyrtus tomentosa*. *Tianran Chanwu Yanjiu Yu Kaifa* **10**, 14–19.
- Mah, T.-F. C. & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**, 34–39.
- Marino, A., Bellingeri, V., Nostro, A., Miceli, N., Taviano, M. F., Güvenç, A. & Bisignano, G. (2010). In vitro effect of branch extracts

of *Juniperus* species from Turkey on *Staphylococcus aureus* biofilm. *FEMS Immunol Med Microbiol* **59**, 470–476.

Nishimura, S., Tsurumoto, T., Yonekura, A., Adachi, K. & Shindo, H. (2006). Antimicrobial susceptibility of *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms isolated from infected total hip arthroplasty cases. *J Orthop Sci* **11**, 46–50.

Nostro, A., Sudano Roccaro, A., Bisignano, G., Marino, A., Cannatelli, M. A., Pizzimenti, F. C., Cioni, P. L., Procopio, F. & Blanco, A. R. (2007). Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *J Med Microbiol* **56**, 519–523.

Saising, J., Hiranrat, A., Mahabusarakam, W., Ongsakul, M. & Voravuthikunchai, S. P. (2008). Rhodomyrtone from *Rhodomyrtus tomentosa* (Aiton) Hassk. as a natural antibiotic for staphylococcal cutaneous infections. *J Health Sci* **54**, 589–595.

Salni, D., Sargent, M. V., Skelton, B. W., Soediro, I., Sutisna, M., White, A. H. & Yulinah, E. (2002). Rhodomyrtone, an antibiotic from *Rhodomyrtus tomentosa*. *Aust J Chem* **55**, 229–232.

Sianglum, W., Srimanote, P., Wonglumsom, W., Kittiniyom, K. & Voravuthikunchai, S. P. (2011). Proteome analyses of cellular proteins in methicillin-resistant *Staphylococcus aureus* treated with rhodomyrtone, a novel antibiotic candidate. *PLoS ONE* **6**, e16628.

Voravuthikunchai, S. P., Dolah, S. & Charernjiratrakul, W. (2010). Control of *Bacillus cereus* in foods by *Rhodomyrtus tomentosa* (Ait.) Hassk. leaf extract and its purified compound. *J Food Prot* **73**, 1907–1912.

Wang, X., Yao, X., Zhu, Z., Tang, T., Dai, K., Sadovskaya, I., Flahaut, S. & Jabbouri, S. (2009). Effect of berberine on *Staphylococcus epidermidis* biofilm formation. *Int J Antimicrob Agents* **34**, 60–66.