Antibacterial mechanisms of rhodomyrtone against important hospital-acquired antibiotic-resistant pathogenic bacteria

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The antibacterial mechanisms of rhodomyrtone, a member of the acylphloroglucinols isolated from Rhodomyrtus tomentosa leaves, against important hospital-acquired antibiotic-resistant pathogenic bacteria were assessed. The results indicated that rhodomyrtone exhibited pronounced antibacterial activity against key antibiotic-resistant pathogens including epidemic meticillin-resistant Staphylococcus aureus (EMRSA), vancomycin-intermediate S. aureus and vancomycin-resistant enterococcal strains. The strains EMRSA-16, Enterococcus faecalis ATCC 29212 and VRE-3 demonstrated a significant decrease in survival ability after treatment with rhodomyrtone at 1× (0.5 μg ml⁻¹), 2×, 4× and 8× MIC for 24 h. Moreover, the compound was observed in the cytoplasmic fraction of rhodomyrtone-treated S. aureus, and only a very fine band of the compound was seen following separation of the cell-wall and cell-membrane fractions of the treated cells. In addition, exposure of S. aureus to rhodomyrtone at 4×, 2× and 1× MIC for 24 h produced no significant effect on the bacterial cell membrane and cell lysis, suggesting that neither of these is the main target of rhodomyrtone action in these organisms. Stepwise isolation of the bacterial cells with increasing resistance to rhodomyrtone was not induced in either S. aureus or EMRSA-16 after 45 passages on Luria–Bertani agar supplemented with rhodomyrtone. In addition, in vitro toxicity of rhodomyrtone at 128× MIC on human erythrocytes was not observed. These results provide evidence to support therapeutic challenges of rhodomyrtone against Gram-positive pathogens.

INTRODUCTION

Infectious diseases are a major cause of death worldwide, and particularly in developing countries (Bilal et al., 2009; Brusselaers et al., 2011). An important reason is that the number of multidrug-resistant bacteria has been increasing as a result of the indiscriminate use of available antibiotics throughout the world (Aligholi et al., 2008; Sievert et al., 2008; Lim et al., 2009; De Kraker et al., 2011). Moreover, in some countries, there is some limitation in the use of synthetic antibiotics because of high cost (Walsh & Amyes, 2004; Alder, 2005). Many studies are therefore trying to discover new antimicrobial agents and find alternative ways to reduce and prevent the problem of antibiotic resistance in bacteria. A number of bioactive plant extracts have had success in serving as a guidepost for new antibacterial drug discovery (Bibi et al., 2011; Njume et al., 2011). Most of the bioactive metabolites in medicinal plants probably evolved as chemical agents against pathogenic microbes or predators in the environment. Moreover, antimicrobial agents obtained in this way are more naturally biologically friendly (Walsh, 2003; Koehn & Carter, 2005; Izzo & Ernst, 2009). Thus, such natural products may display new mechanisms of action towards some of the newer antimicrobial targets (Gibbons, 2008). Rhodomyrtus tomentosa (Aiton) Hassk. is a flowering plant in the family Myrtaceae. The plant has significant value in traditional medicine and is used to treat diarrhoea (Ong & Nordiana, 1999), gynaecopathy (Wei, 2006a) and urinary tract infections (Wei, 2006b), and as an antiseptic wash for wounds (Geetha et al., 2010b). In addition, the antioxidant and gastroprotective properties of the different extracts of R. tomentosa leaves have been studied (Geetha et al., 2010a, b). Extensive work has revealed that both R. tomentosa extract and rhodomyrtone, a member of the acylphloroglucinols
isolated from this plant, exhibit pronounced antibacterial activity against a wide range of Gram-positive bacteria (Limsuwan et al., 2009, 2011; Voravuthikunchai et al., 2010; Saising et al., 2011). Therefore, the objective of this study was to investigate further the antibacterial mechanisms of rhodomyrtone against important pathogenic Gram-positive bacteria.

**METHODS**

**Antimicrobial agents and chemicals.** The following commercially available compounds were purchased from indicated manufacturers: Luria–Bertani (LB) agar and broth from Difco, Mueller–Hinton (MH) agar and broth from Oxoid, DMSO and vancomycin from Sigma–Aldrich, and ethanol and NaCl from Fisher Scientific. All standard chemicals were of analytical grade.

**Rhodomyrtone purification.** Rhodomyrtone was isolated as described elsewhere by our group (Hiranrat & Mahabusarakam, 2008; Limsuwan et al., 2009). The purity of the compound was confirmed by NMR and MS (Salni et al., 2002; Mohamed & Ibrahim, 2007).

**Bacterial strains and determination of MIC and minimum bactericidal concentration (MBC).** Twelve clinical bacterial isolates were taken from the collection of the Laboratory of Microbiology, School of Pharmacy, University College London, UK (Table 1). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as reference strains.

A modified broth microdilution method was performed, as outlined by the Clinical and Laboratory Standards Institute (CLSI, 2009). Rhodomyrtone was dissolved in 10 % DMSO and diluted twofold to give final concentrations ranging from 32 to 0.0625 μg ml⁻¹. All bacterial strains were cultured on MH agar and incubated at 37 °C overnight. One hundred millilitres of the culture was suspended in 50 ml MH broth and incubated at 37 °C for 3–5 h with aeration. The bacterial suspensions were adjusted to a 0.5 McFarland standard with 0.85% NaCl solution or normal saline solution (NSS) to achieve a concentration of ~1.5 × 10⁶ c.f.u. ml⁻¹. One hundred microlitres of the culture, containing ~10⁶ c.f.u. micro-organism ml⁻¹, was inoculated in 80 μl MH broth supplemented with 20 μl rhodomyrtone. The microtitre plates were incubated at 37 °C for 16–18 h. A control culture with 1% DMSO was incubated under the same conditions. The experiment was carried out in triplicate. The MIC was recorded as the lowest concentration of the bioactive compound that did not result in any turbidity of the tested organism. Aliquots from the broth with no growth were spread onto fresh MH agar plates using a sterile loop and incubated at 37 °C overnight. The MBC was defined as the lowest concentration that completely killed the microorganism.

**Time–kill assay.** The bactericidal activity of rhodomyrtone against a strain of epidemic meticillin-resistant *S. aureus* (EMRSA), *E. faecalis* ATCC 29212 and a strain of vancomycin-resistant enterococci (VRE) was studied using a time–kill assay. The bacterial suspension and concentrations of rhodomyrtone at 8 ×, 4 ×, 2 ×, 1 × and 0.5 × MIC were prepared as described above. The culture was inoculated in MH broth supplemented with the active compound and incubated at 37 °C with shaking (200 r.p.m.). The samples were collected at various time intervals. Any surviving bacteria were cultured on MH agar and incubated under the same conditions. A tube containing 1% DMSO was used as a growth control. The experiment was performed in triplicate.

**Localization of rhodomyrtone in *S. aureus*.** *S. aureus* ATCC 29213 was inoculated in MH broth, incubated at 37 °C for 3–5 h with shaking and the turbidity adjusted to a number 4 McFarland standard (~1.2 × 10⁸ c.f.u. ml⁻¹). An inoculum of 18 ml culture was added to 2 ml rhodomyrtone dissolved in 10 % DMSO to give a final concentration of 8 μg ml⁻¹. The treated organisms were grown at 37 °C for 18 h. The cultures were then centrifuged at 5000 r.p.m. in a Hettich Mikro 120 centrifuge at 4 °C for 10 min and the pellets washed extensively with buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 5% (v/v) ethanol until no rhodomyrtone could be detected in the buffer after washing. The pellets were lysed by sonification on ice. The lysed cells were centrifuged at 5000 r.p.m. at 4 °C for 10 min. The pellets (containing the cell wall and cell membrane) and the supernatant (cytoplasm) were extracted with ethyl acetate. The combined organic layer was washed with distilled water, dried over anhydrous sodium sulphate and evaporated to dryness. The crude products were resuspended in ethyl acetate and spotted onto pre-coated TLC silica gel 60 F254 plates (Merck). The spots were separated using n-hexane : ethyl acetate (6:1, v/v), visualized under UV light at 254 nm, sprayed with anisaldehyde/H₃SO₄ reagent and heated. The experiment was carried out in triplicate.

**Time course of leakage of intracellular components.** Loss of 260 nm-absorbing material was carried out following a modified method by Oonmetta-aree et al. (2006). Colonies of *S. aureus* ATCC 29213 in the stationary phase of growth were prepared by culturing the bacteria on MH agar, which was incubated at 37 °C for 16–18 h. The colonies were suspended in 1 ml NSS, washed by centrifugation at 5000 r.p.m. in a Hettich Mikro 120 centrifuge for 5 min and resuspended in NSS. The suspension was adjusted to a number 0.5 McFarland standard. Rhodomyrtone was added at final concentrations equivalent to 4 ×, 2 × and 1 × MIC. A microbial suspension supplemented with 1% DMSO was used as a control. The bacterial suspensions were incubated at 37 °C and samples were collected at 0, 4, 8, 12, 16, 20 and 24 h after treatment. The cell pellets were removed by centrifugation at 10 000 r.p.m. for 5 min, after incubation as described above. Controls without bacterial suspension were carried out in triplicate.

### Table 1. Antibacterial activity of rhodomyrtone and vancomycin against 12 clinical isolates of major hospital-acquired drug-resistant pathogenic bacteria

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MIC/MBC (μg ml⁻¹)</th>
<th>Rhodomyrtone</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMRSA-15</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>EMRSA-16</td>
<td>0.5/0.5</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>EMRSA SOTON9</td>
<td>0.5/1</td>
<td>1/2</td>
<td></td>
</tr>
<tr>
<td>MRSB BB270</td>
<td>0.5/0.5</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>MRSB USA300</td>
<td>1/1</td>
<td>1/2</td>
<td></td>
</tr>
<tr>
<td>VISA Mu3</td>
<td>0.5/1</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>VISA Mu50</td>
<td>0.5/0.5</td>
<td>8/16</td>
<td></td>
</tr>
<tr>
<td>VRE-2</td>
<td>2/&gt;32</td>
<td>&gt;256/NA</td>
<td></td>
</tr>
<tr>
<td>VRE-3</td>
<td>1/16</td>
<td>&gt;256/NA</td>
<td></td>
</tr>
<tr>
<td>VRE-4</td>
<td>1/32</td>
<td>&gt;256/NA</td>
<td></td>
</tr>
<tr>
<td>VRE-7</td>
<td>2/&gt;32</td>
<td>&gt;256/NA</td>
<td></td>
</tr>
<tr>
<td>VRE-8</td>
<td>2/&gt;32</td>
<td>&gt;256/NA</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> ATCC 29212</td>
<td>2/32</td>
<td>2/32</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
<td>0.5/1</td>
<td>1/1</td>
<td></td>
</tr>
</tbody>
</table>

NA, Not applicable.
out under the same conditions. Low-molecular-mass metabolites known to leak from cells include nucleic acids, metabolites and ions. The levels of these metabolites in the supernatant were determined by measuring absorbance at 260 nm ($A_{260}$) using a UV/VIS spectrophotometer. The experiment was carried out in triplicate.

**Bacteriolysis.** Bacteriolysis was determined using a modified method as described by Carson et al. (2002). Suspensions of *S. aureus* ATCC 29213 and concentrations of rhodomyrtone at 4×, 2× and 1× MIC were prepared as described above. Positive and negative controls were treated with 5% Triton X-100 and 1% DMSO, respectively. The treatments were mixed with vortex mixer and incubated at 37°C. The optical density at 620 nm was measured at 0, 4, 8, 12 and 24 h to detect cell lysis, demonstrated as a decrease in OD$_{620}$. Dilutions of test agents only were used as blank controls. The results were indicated as a ratio of the OD$_{620}$ at each time point versus the OD$_{620}$ at 0 min. The assay was carried out in triplicate.

**Stepwise isolation of *S. aureus* with increasing resistance to rhodomyrtone.** Rhodomyrtone-resistant cells of *S. aureus* ATCC 29213 and EMRSA-16 were obtained by culturing wild-type cells directly onto defined-medium agar plates containing the active compound. The assay was performed as described elsewhere with some modifications (Tenney et al., 1983; Kruger et al., 1988). Briefly, 100 ml wild-type *S. aureus* ATCC 29213 and EMRSA-16 ($10^8$ c.f.u. ml$^{-1}$ in MH broth) were spread evenly onto LB agar plates supplemented with rhodomyrtone at 0.25× MIC and the plates were incubated at 37°C overnight. Resistant colonies were picked and subcultured on plates containing rhodomyrtone at the same concentration for five passages. After growth, the resistant colonies of the last culture were transferred to agar plates containing twofold incremental concentrations of the compound and subcultured for five passages before being transferred to agar plates supplemented with higher concentrations of rhodomyrtone. To investigate the stability of resistant bacterial cells, the colonies from the plates containing the highest concentration of rhodomyrtone that permitted growth were transferred sequentially three times into rhodomyrtone-free LB broth. The cultures were subjected to the MIC assay described above. Oxacillin, penicillin G and vancomycin were used as controls for *S. aureus* ATCC 29213, as described above.

**Erythrocyte haemolysis assay.** Haemolytic activity was determined following a modified method by Dathe et al. (1996). Briefly, freshly isolated human erythrocytes were washed three times with NSS. The erythrocytes were diluted to a concentration of $2.5 	imes 10^8$ cells ml$^{-1}$ in NSS and treated with rhodomyrtone in 10% DMSO at a final concentration of 128× MIC. The cells were incubated at 37°C for 30 min with gentle shaking. The samples were placed on ice for 5 min and the cell pellets removed by centrifugation at 3000 r.p.m. in a Hettich Mikro 120 centrifuge for 15 min. The levels of haemoglobin released from the treated erythrocytes were assayed by measuring the $A_{330}$ of the supernatants of tenfold dilutions in NSS using a spectrophotometer. Cell suspensions supplemented with distilled water were used to obtain a positive-control haemolysis value (100% haemolysis), and NSS was used as a negative control. Results for blank controls without erythrocyte suspensions were determined under the same conditions. The experiment was carried out in duplicate.

### RESULTS

**In vitro antibacterial activity**

In order to perform subsequent microbiological studies with rhodomyrtone and compare the results with the available antibiotic (vancomycin), comparative MICs were determined for the bacterial strains. The compound was tested in broth microdilution assays to determine MICs. The activities of rhodomyrtone against the tested human pathogens are indicated in Table 1. The results demonstrated that rhodomyrtone showed a pronounced antibacterial activity against meticillin-resistant *S. aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA) and VRE pathogens. The MIC and MBC values of rhodomyrtone against EMRSA and MRSA strains ranged from 0.5 to 1 μg ml$^{-1}$, which was very close to that of vancomycin (1–2 μg ml$^{-1}$). Moreover, the MIC and MBC of the active compound against VISA Mu50 were 0.5 μg ml$^{-1}$, and that of vancomycin was 8 and 16 μg ml$^{-1}$, respectively. The overall level of potency of rhodomyrtone against VISA Mu50 was approximately 16-fold greater than that of vancomycin, making it a highly potent agent against EMRSA and VISA. In addition, the active compound displayed good antibacterial activity against VRE strains, with MICs of 1–2 μg ml$^{-1}$. In contrast, vancomycin was inactive against the resistant strains.

**Effect of rhodomyrtone on the growth and survival of important hospital-acquired antibiotic-resistant pathogenic bacteria**

Rhodomyrtone displayed a pronounced activity against important drug-resistant Gram-positive bacteria. Therefore, the strains EMRSA-16, *E. faecalis* ATCC 29212 and VRE-3 were grown to mid-exponential phase and exposed to various concentrations of rhodomyrtone, and the effects of the active compound on their survival were examined in more detail.

The results for EMRSA-16 indicated that the growth rate of the organism was inhibited after incubation for 24 h at all concentrations of the compound. When the micro-organism was assessed for its survival ability after exposure to rhodomyrtone at 8× MIC (4 μg ml$^{-1}$) for 10 h and at 4× and 2× MIC for 24 h, the bacterial population decreased by ~2 logs, whilst exposure to rhodomyrtone at 0.5× MIC was predominantly bacteriostatic. The growth kinetics of the bacterial cells treated with 1% DMSO remained unchanged (Fig. 1).

The results of time–kill curves of strain VRE-3 after treatment with rhodomyrtone showed that the rhodomyrtone-treated bacteria failed to grow after treatment for 24 h at all concentrations tested. At 8× MIC (8 μg ml$^{-1}$), the compound reduced the survival ability of the pathogen by ~2 logs after treatment for 24 h. The addition of rhodomyrtone to the culture at 4×, 2× and 1× MIC also resulted in decreased bacterial growth. However, rhodomyrtone at 0.5× MIC had little effect on the viability of the bacterial strain (Fig. 2a).

Rhodomyrtone-treated *E. faecalis* ATCC 29212 exhibited poor growth under all concentrations tested. At 8× (16 μg ml$^{-1}$) and 4× MIC, a remarkable reduction in the viability of the bacterial culture cells was observed by at least 2 logs after 4 and 10 h after exposure, respectively. The viable cell
count after incubation with rhodomyrtone at $2 \times$ MIC for 2 and 24 h decreased by at least 1 and 2 logs, respectively. The active compound at $1 \times$ and $0.5 \times$ MIC also inhibited the bacterial growth rate, although to a lesser extent (Fig. 2b).

**Localization of rhodomyrtone, disruption of the cytoplasmic membrane and bacteriolysis in *S. aureus***

To determine the antibacterial mechanisms of rhodomyrtone in *S. aureus*, localization of rhodomyrtone in the bacteria was performed. Rhodomyrtone was extracted from the cell pellet and cytoplasmic fractions and detected using a thin layer chromatography technique. The results indicated that the compound was clearly visualized only in the cytoplasmic fraction of rhodomyrtone-treated *S. aureus* after detection by UV absorption and spraying methods. In contrast, only a very fine band was observed in the cell wall and cell-membrane fraction of the treated cells after spraying and heat treatment. Rhodomyrtone was not observed in either fraction of DMSO-treated *S. aureus* (Fig. 3).

We next sought to determine the effects of rhodomyrtone on the cell membrane and cell wall of *S. aureus* by assaying leakage of intracellular components of the treated cells. The results showed that no significant differences in $A_{260}$ were observed between the tested and control cells after treatment for 24 h (Fig. 4). In addition, damage to the bacterial cell wall was determined by measuring the decrease in turbidity after treatment with the active compound. The proportion of the initial OD$_{620}$ at each time interval is shown in Fig. 5. The OD$_{620}$ of suspensions was not significantly decreased at 24 h after treatment with rhodomyrtone at $4 \times$, $2 \times$ and $1 \times$ MIC. In contrast, a decrease in OD$_{620}$ occurred after 4 h of treatment with 5% Triton X-100, which was used as a positive control.

**Stepwise selection of *S. aureus* after treatment with rhodomyrtone**

Strains of *S. aureus* ATCC 29213 and EMRSA-16 were monitored for their ability to develop resistance to rhodomyrtone *in vitro*. A stepwise selection technique was used to select rhodomyrtone-resistant cells of the organisms. The initial MIC/MBC values of the original strains of *S. aureus* and EMRSA were 0.5/1 and 0.5/0.5 mg ml$^{-1}$, respectively. After culture on agar containing twofold incremental concentrations of rhodomyrtone for 45 passages, *S. aureus* ATCC 29213 and EMRSA-16 could grow on LB agar supplemented with rhodomyrtone at 32 and 16 mg ml$^{-1}$, respectively (data not shown). The resistant cells were subjected to MIC and MBC assays. The results showed that, after exposure to rhodomyrtone, both isolates exhibited MICs that were two- to fourfold higher than those of the original isolates. However, even after just three passages on rhodomyrtone-free medium, the MIC and MBC values of the active compound against both isolates decreased to 1–2 mg ml$^{-1}$ (Table 2). The MIC value of vancomycin against vancomycin-treated *S. aureus* was 0.5 mg ml$^{-1}$, the same as the MIC value of the untreated cells. For oxacillin, after serial passage on agar.
containing the drug, *S. aureus* showed a higher MIC than the original strain (eightfold increase from the original MIC of 0.125 µg ml\(^{-1}\)). In addition, penicillin G-treated *S. aureus* showed reduced susceptibility to this drug, with the MIC for the treated cells increasing from 0.03 µg ml\(^{-1}\) to 0.5 µg ml\(^{-1}\) (Table 2).

**Haemolytic property of rhodomyrtone against human erythrocytes**

Our results showed that rhodomyrtone exhibited good antibacterial activity against important pathogenic bacteria. Therefore, in order to assess the *in vitro* toxicity of the active compound, the percentage haemolysis of human erythrocytes was measured. The results demonstrated that, when human red blood cells were tested for haemolysis after exposure to rhodomyrtone at 128 × MIC for 30 min, the levels of released haemoglobin from treated erythrocytes decreased by at least 98%, the same as for NSS-treated cells, compared with the positive control (Fig. 6).

**DISCUSSION**

The widespread and increasing prevalence of drug-resistant pathogens such as *S. aureus* and enterococci is being identified increasingly in both hospital- and community-acquired infections (Gould, 2008; Bogomolova *et al.*, 2011; Rivera & Boucher, 2011). The best-known drug-resistant forms of these include MRSA, VISA and VRE. Most of these infections are associated with treatment failures (Cunha, 2005; Menichetti, 2005; Moore *et al.*, 2011). Moreover, increases in the incidence of such infections pose a problem by creating a significant economic burden (McKay *et al.*, 2009). Given the major problems being experienced with resistance to classical antibiotics, there seems to be considerable scope for the development of effective antibiotics from natural products (Chusri & Voravuthikunchai, 2011). We found that rhodomyrtone showed pronounced antibacterial activity against key antibiotic-resistant bacteria such as MRSA, VISA and VRE pathogens. VISA strains have been reported to possess a thickened cell wall, which greatly reduces the penetration of vancomycin through the cell wall (Smith *et al.*, 1999; Cui *et al.*, 2003). For example, the Mu50 VISA isolate has been found to contain 30–40 layers of peptidoglycan in its cell wall (Howden *et al.*, 2006), whereas a vancomycin-susceptible strain of *S. aureus* had only 20 layers (Cui *et al.*, 2006; Howden *et al.*, 2006). This thus acts as a physical barrier to block the access of vancomycin. Rhodomyrtone has been reported to be a small molecule of low molecular mass (442.6 g mol\(^{-1}\)) (Salni *et al.*, 2002; Mohamed & Ibrahim, 2007). Therefore, the active
compound could easily penetrate into bacterial cells and be effective against pathogens. Moreover, the results from localization of rhodomyrtone in *S. aureus* demonstrated that the active compound was found in the cytoplasmic fraction of the treated cells. Thus, the main target of action of rhodomyrtone on the pathogen is likely to be inside the cell.

Disruption of the cytoplasmic membrane and the bacteriolytic activity of rhodomyrtone against *S. aureus* were tested to investigate the mechanisms of action of the compound against the organisms. Exposure to rhodomyrtone at 4×, 2× and 1× MIC for 24 h produced no significant effect on the bacterial cell membrane and cell lysis was not observed. Similarly, Limsuwan *et al.* (2009) reported that rhodomyrtone exhibited no bacteriolytic activity against *Streptococcus mutans*. In addition, proteome studies showed that rhodomyrtone at a subinhibitory concentration (0.174 μg ml⁻¹) suppressed the expression of extracellular proteins of MRSA involved in cell wall hydrolysis such as staphylococcal secretory antigen (Visutthi *et al.*, 2011). In contrast, close observation by transmission electron microscope in a parallel study on cellular proteomic analysis illustrated that some cell lysis occurred in rhodomyrtone-treated MRSA cells (Sianglum *et al.*, 2011). However, the mechanisms of action of this compound are still unclear and need further investigation.

Treatment of EMRSA-16 and *S. aureus* ATCC 29213 with incremental concentrations of rhodomyrtone followed by growth on rhodomyrtone-free medium showed that the MIC and MBC values of the compound reverted to 1–2 μg ml⁻¹ against the tested strains after just three passages on rhodomyrtone-free medium, similar to the initial MIC and MBC values. In contrast, the resistant rate of *S. aureus* increased after culture on LB agar supplemented with penicillin G. Tenney *et al.* (1983) reported that the MIC of norfloxacin for *Pseudomonas aeruginosa* and *Escherichia coli* after serial passages on agar containing the antibiotic increased by up to 512-fold compared with the untreated strains. However, the development of resistance in vivo is unpredictable and can be determined only after clinical evaluation of an agent for a prolonged period (Liebowitz *et al.*, 1988).

The heat stability of rhodomyrtone under autoclave conditions (121 °C for 15 min) was investigated. The results showed that the antibacterial property of heat-treated rhodomyrtone displayed a similar activity against *S. aureus* ATCC 29213 as untreated rhodomyrtone (data not shown). In order to support medical applications of the compound, in vitro toxicity on human erythrocytes was tested. Selectivity, or the killing of bacteria at a concentration not harmful to human erythrocytes, is a highly desirable characteristic of an antibacterial agent. The results from this study demonstrated that released haemoglobin was not observed after treatment with rhodomyrtone at 128× MIC. Lv *et al.* (2007) reported that amphotericin B at a concentration of 30–100 μg ml⁻¹ lysed 50–100 % of erythrocytes, respectively. In contrast, chloramphenicol at the very high concentration of 4000 μg ml⁻¹ resulted in lysis of only 14–15 % of erythrocytes.

In conclusion, our results indicated that rhodomyrtone exhibited pronounced antibacterial activity against key antibiotic-resistant Gram-positive pathogens including strains of EMRSA, VISA and VRE. EMRSA-16, *E. faecalis* and VRE-3 demonstrated a significant decrease in their survival ability following treatment with rhodomyrtone at

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**Table 2.** Stepwise selection of *S. aureus* ATCC 29213 and EMRSA-16 cells after treatment with rhodomyrtone and antibiotics

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Antibiotic</th>
<th>MIC/MBC (μg ml⁻¹)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Initial</td>
<td>After culture on medium containing antibiotic</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
<td>Rhodomyrtone</td>
<td>1/1</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0.125/0.25</td>
<td>1/1</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.03/0.03</td>
<td>0.5/1</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.5/0.5</td>
<td>1/1</td>
</tr>
<tr>
<td>EMRSA-16</td>
<td>Rhodomyrtone</td>
<td>0.5/0.5</td>
</tr>
</tbody>
</table>

**Fig. 6.** Erythrocyte haemolysis activity of rhodomyrtone against human red blood cells determined as change in A₅₅₀. Distilled water was used to obtain the positive-control haemolysis value (100 % haemolysis), and NSS was used as a negative control. Results are shown as means±SEM of at least duplicate results.
$1 \times (0.5 \, \mu g \, ml^{-1})$, $2 \times$, $4 \times$ and $8 \times$ MIC for 24 h. Moreover, the compound was observed in the cytoplasmic fraction of rhodomyrtone-treated S. aureus and only a very fine band of the compound was detected in the cell-wall and cell-membrane fraction of the treated cells. In addition, exposure of S. aureus to rhodomyrtone at $4 \times$, $2 \times$ and $1 \times$ MIC for 24 h produced no significant effect on the bacterial cell membrane and cell lysis, suggesting that these are not the main targets of rhodomyrtone action in these organisms. Stepwise isolation of bacterial cells with increasing resistance to rhodomyrtone was not induced in either S. aureus or EMRSA-16 after 45 passages on LB agar supplemented with the compound. In addition, in vitro toxicity of rhodomyrtone towards human erythrocytes at $128 \times$ MIC was not observed. These results provide evidence to support therapeutic challenges of rhodomyrtone against Gram-positive pathogens.

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