Antibiofilm activity of α-mangostin extracted from *Garcinia mangostana* L. against *Staphylococcus aureus*

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ABSTRACT

Objective: To isolate α-mangostin (AMG) from the peels of mangosteen (*Garcinia mangostana* L.), grown in Vietnam, and to investigate antibiofilm activity of this compound against three *Staphylococcus aureus* (*S. aureus*) strains, one of which was methicillin-resistant *S. aureus* (MRSA) and the other two strains were methicillin-sensitive *S. aureus* (MSSA).

Methods: AMG in *n*-hexane fraction was isolated on a silica gel column and chemically analyzed by HPLC and NMR. The antibiofilm activity of this compound was investigated by using a 96-well plate model for the formation of biofilms. Biofilm biomass was quantified using crystal violet. The viability of cells was observed under confocal microscopy using LIVE/DEAD BacLight stains. Biofilm composition was determined using specific chemical and enzyme tests for polysaccharide, protein and DNA. Membrane-damaging activity was assayed by measuring the hemolysis of human red blood cells in presence of AMG.

Results: The results indicated that the isolated AMG, with a purity that exceeded 98%, had minimal inhibitory concentrations in the range of 4.6–9.2 µmol/L for the three strains tested. Interestingly, the MSSA strains were more sensitive to AMG than the MRSA strain. Minimal bactericidal concentrations were 2-fold higher than the minimal inhibitory concentration values for the three strains, indicating that AMG was a bactericidal compound. AMG also prevented biofilm formation effectively, albeit that again the MRSA strain was the most resistant. Interestingly, biofilms of the MRSA strain contained protein as a main component of the extracellular matrix, whereas this was polysaccharide in the MSSA strains. This might relate to the resistance of the MRSA 252 strain to AMG. Assays using human red blood cells indicated that AMG caused significant membrane damage with 50% of cell lysis occurred at concentration of about 36 µmol/L.

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Conclusions: Our results provide evidence that the isolated AMG has inhibitory activity against biofilm formation by *S. aureus*, including MRSA. Thus, isolated AMG proposes a high potential to develop a novel phytopharmaceutical for the treatment of MRSA.

1. Introduction

Biofilm-forming bacteria account for about 2/3 of human bacterial infections [1]. The bacteria in biofilms are highly tolerant to antimicrobials due to genetic and metabolic adaptations of the cells in the films. *Staphylococcus aureus* (*S. aureus*), including antibiotic resistant strains, are strongly biofilm-producing bacteria and are dangerous factors of human common infectious diseases [2,3]. In particular, methicillin-resistant *S. aureus* (MRSA) is a worldwide challenge and there is an urgent need to search for new and effective alternatives for the treatment of the bacteria. Therefore, control of diseases by inhibition of biofilm production is a novel approach to treat bacterial infections [4–8].

α-Mangostin (AMG), a yellow xanthone extracted from the peel of *Garcinia mangostana* L. (*G. mangostana*), is known for its potent anti-cancer, inflammatory, antimicrobial and antifungal activities [9–14]. Recently, it was found to be a potential antibiofilm agent against *Staphylococcus epidermidis* (*S. epidermidis*) [15] and the dental pathogen *Streptococcus mutans* [16,17]. However, antibiofilm activity against SA, including clinical isolates and MRSA, has not been investigated. Thus, it is worthwhile to examine the activity of AMG against MRSA biofilm formation and to explore the therapeutic applications of AMG. The present study reported antibiofilm activity of AMG isolated from the peels of *G. mangostana* grown in Vietnam against three SA strains.

2. Materials and methods

2.1. Extraction and isolation of AMG

The dried powder of *G. mangostana* peels were collected from the South of Vietnam and voucher specimen (NO. 15062014) was deposited at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi. The dried powder was extracted with ethanol at room temperature, followed by evaporation of the solvent to give a dark brown gummy ethanolic extract. This residue was partitioned between water and *n*-hexane. The *n*-hexane fraction was then evaporated and dried under reduced pressure. Chromatographic separation was performed using silica gel column chromatography (Merck Silica gel 60, 70–230 mesh). The procedure for isolation of AMG was described previously [16]. In brief, a 12.0 g residue of *n*-hexane fraction was separated on silica gel column chromatography (Merck Silica gel 60, 70–230 mesh) using an eluting system of *n*-hexane–ethyl acetate–methanol (6:3:0.1, by volume). Partially purified AMG (4.0 g) was separated from the active fractions and then further purified by silica gel column chromatography (Merck Silica gel 60, 70–230 mesh), eluting with *n*-hexane–chloroform–ethyl acetate–methanol (4:1:0.5:0.3, by volume), to give a

single compound AMG as yellow crystals (105 mg). The purified AMG was identified by ^{1}H and ^{13}C-nuclear magnetic resonance (NMR), mass spectrometry (MS) and high performance liquid chromatography (HPLC).

2.2. Bacteria and growth conditions

SA strains included the reference strain NCTC 6571, and the two clinical isolates MRSA 252 [18] and MSSA 15981 [19]. SA strains were aerobically cultured in tryptic soy broth (TSB) medium (Difco) at 37 °C. For biofilm growth, 0.5% glucose as a biofilm inducer was added (TSBg).

2.3. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determinations

A modified broth microdilution method according to the Clinical and Laboratory Standard Institute Guidelines [20] was used to determine the MIC and MBC of AMG. Two-fold serial dilutions of AMG were made in TSB using 96-well flat-bottom microtiter plates. A suspension of mid-logarithmic growth phase bacteria in TSB adjusted to 1×10^5 cfu/mL was added to each well. The final concentrations of AMG ranged from 1.145 to 36.600 µmol/L. The MIC was the lowest concentration of AMG showing no visible growth of microorganisms after incubation at 37 °C for 24 h. The MBC was determined by plating 20 µL from the broth with no visible growth in the MIC tests onto TSB agar. The MBC was the lowest concentration where no bacteria grew after 24 h incubation at 37 °C. All tests were repeated in triplicate.

2.4. Biofilm assay in 96-well microtiter plate

SA was cultured overnight in TSBg and diluted for biofilm growth in a 96-well polystyrene plate. The plates were incubated for 48 h at 37 °C on a 3-dimensional plate rocking machine. Media were freshly changed after 24 h growth. The cell suspension was then removed and biofilms were washed 3 times with sterile PBS. The plates were dried for 1 h at 60 °C, and biofilms were then stained with crystal violet solution (0.1% w/v) for 15 min. The crystal violet was then removed, and plates were washed gently with water. The absorbed crystal violet was dissolved in 30% v/v acetic acid and the absorbance was quantified at $\lambda = 595$ nm (A_{595}) [21].

2.5. Confocal microscopy

Polyvinyl plastic coverslips (22 mm × 22 mm) were sterilized in absolute isopropanol, dried and placed in wells of a 6-well cell culture plate. An aliquot (2 mL) of the diluted bacterial suspension of MRSA 252 in TSBg was added. To test inhibition of

the formation of biofilms, AMG was added to the wells at the start of biofilm growth. To test disruption and/or killing of biofilms, biofilms were grown for 24 h, followed by removal of planktonic cells and addition of AMG in fresh medium. The coverslips in the 6-well plate were incubated at 37 °C for a further 24 h, then the culture medium was removed and the coverslips were 3 times washed with sterile water. To assess the effectiveness of the agents, biofilms were stained with 0.3% v/v LIVE/DEAD BacLight mixture of dye solution in sterile water. The coverslips were left for 15 min in the dark prior to washing again with sterile water. Then the coverslips were mounted on glass slides and sealed with nail varnish. Stained biofilms were observed using laser scanning confocal fluorescence microscopy (Olympus, Tokyo, Japan). The image data were processed with the Imaris software (Bitplane AG, Zürich, Switzerland) [21,22].

2.6. Membrane activity assay

To determine the activity of AMG against mammalian membranes, hemolysis of human red blood cells (RBCs) was determined. RBCs were collected by centrifugation at $800{\times}g$ for 5 min, washed 4 times with PBS and diluted to 4%. The test compound was added at a concentration of 100 μg/mL and the RBCs were incubated for 1 h at 37 °C. Intact RBCs were then discarded by centrifugation and the release of hemoglobin in the supernatant was measured spectrophotometrically at 414 nm. The control for 100% lysis was RBCs lysed in water [22].

2.7. Chemical and enzyme tests for biofilm composition of SAs

Biofilms were grown in 96-well microtitre plates by inoculating the bacteria in TSBg and incubating the plates at 37 °C for 24 h. Following incubation, the media were removed and either replaced with 0.2 mL PBS (positive controls) or with 10 mmol/L sodium periodate in PBS (for removal of polysaccharide in biofilms), 0.1 mg/mL proteinase K in PBS (for removal of protein in biofilms) or 0.1 mg/mL bovine DNase I in 150 mmol/L NaCl and 1 mmol/L $CaCl_2$ (for removal of eDNA existing in biofilms). Plates were subsequently incubated at 37 °C for 4 h, then washed vigorously 3 times with PBS, dried at 60 °C for 30 min and stained with 0.1% leuco crystal violet dye. The absorbance was measured at A_{595} to detect biofilm biomass left after treatments [23].

2.8. Statistical analyses

Data are presented as the mean ± standard deviation (SD). Student's *t*-test was used to calculate the significance of the difference between the mean expression of experimental and control samples. The level of significance was set at 5%.

3. Results

3.1. Isolation of AMG from the peels of G. mangostana

Isolation of AMG was performed as described in the method section. The purity of compound exceeded 98% as determined by HPLC (Figure 1). ^{1}H and ^{13}C NMR spectra were measured and interpreted data are presented in Table 1. Based on the analyzed and reference data [24,25], the chemical structure of AMG (Figure 2) was confirmed.

3.2. MIC and MBC determinations

The results for determination of isolated AMG against all tested SA strains indicated that the MICs for isolated AMG were 4.58, 4.58, and 9.15 μmol/L for NCTC 6571, MSSA 15981 and MRSA 252, respectively. These concentrations are in the same range as has been reported for SA (including MRSA) and other bacterial strains [12,26–29], corroborating our results. The MBC values were only 2-fold higher, indicating that AMG was bactericidal rather than bacteriostatic. Our data on killing of MRSA 252 in suspension at 5 × MIC concentration for 10 min treatment also indicated a reduction in>−3log CFU (99.9% killing) compared to the control (data not shown).

3.3. Inhibition of biofilm formation by SA

AMG was used to test the effect on biofilm formation by SA and isolates, by adding AMG at the start of biofilm growth. The data in Table 2 show that only at a concentration of 24 μmol/L (= 5 × MIC) or higher, there was a significant effect on biofilm formation of SA NCTC 6571 and MSSA 15981. At a concentration of 48 μmol/L (= 10 × MIC), the biomass was inhibited up to > 81.0% for NCTC 6571 and > 93.5% for MSSA 15981 isolate. In contrast, MRSA 252 was more resistant to AMG. At a concentration of 96 μmol/L (= 10 × MIC), the biomass of the biofilm
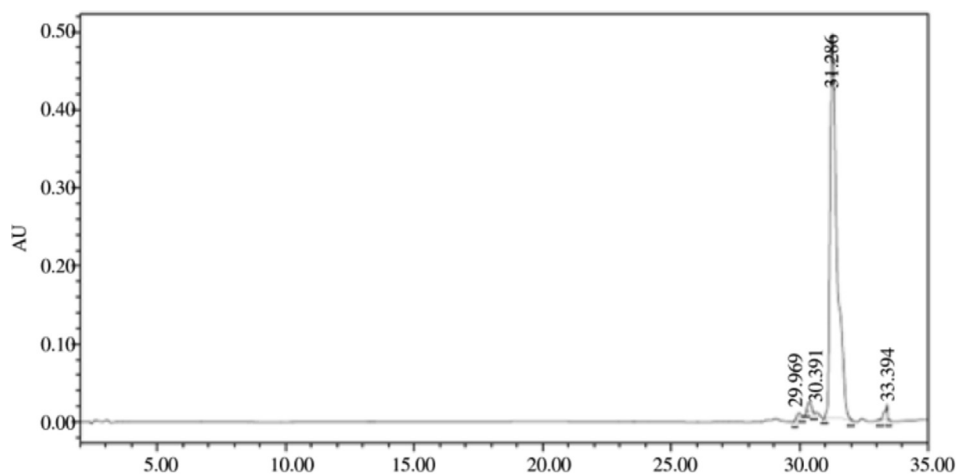

Figure 1. HPLC data on α-mangostin (AMG) isolated from *G. mangostana* grown in Vietnam.

Table 1

^{1}H and ^{13}C NMR data of AMG isolated from *G. mangostana* grown in Vietnam.

Pos.	$\delta_C{}^{a,b}$	$\delta_H{}^{a,c}$ (mult., J = Hz)
1	159.8	–
2	109.9	–
3	162.2	–
4	92.2	6.34 (s, 1H)
4a	154.5	–
5	101.8	6.80 (s, 1H)
6	156.8	–
7	143.3	–
8	136.3	–
8a	109.6	–
9	181.2	–
9a	101.7	–
10a	154.1	–
11	20.9	3.21 (d, 2H, J = 7.0 Hz)
12	123.7	5.17 (t, 1H, J = 7.0 Hz)
13	130.3	–
14	25.4	1.62 (s, 3H)
15	17.9	1.62 (s, 3H)
16	25.5	4.02 (d, 2H, J = 7.0 Hz)
17	122.5	5.16 (t, 1H, J = 7.0 Hz)
18	130.2	–
19	25.7	1.73 (s, 3H)
20	17.6	1.77 (s, 3H)
1′	60.1	3.70 (s, 3H)
OH-1	–	13.71 (s, 1H, chelated OH)

^{a} Measured in DMSO. ^{b} 125 MHz. ^{c} 500 MHz.

was only reduced by about 40% compared to the control. At the highest concentration tested, 386 μmol/L (= 40 × MIC), the biofilm was reduced by 77.0%. Our results clearly indicate that SA in biofilms was more resistant to AMG than planktonic SA.

We also tested the activity of AMG on pre-formed biofilms. In this experiment, biofilms were grown for 24 h before adding AMG in fresh growth media, followed by further 24-h incubation. However, no inhibitory activity was found for any of the strains (data not shown). This result shows that AMG exhibited inhibitory activity only on biofilm formation (early stage), and had no activity on mature (late stage) biofilms.

3.4. Confocal microscopy

In an attempt to visualize the activity of AMG, biofilms of MRSA 252 that were grown on polyvinyl coverslips were treated

Table 2

AMG inhibits biofilm formation by SA strains MRSA 252, NCTC 6571, and MSSA 15981.

AMG (μmol/L)	MRSA 252	NCTC 6571	MSSA 15981
0	0.941 ± 0.215	0.850 ± 0.176	4.160 ± 0.223
6	–	0.650 ± 0.172*	4.262 ± 0.170*
12	–	0.461 ± 0.153*	3.773 ± 0.331*
24	0.780 ± 0.129*	0.166 ± 0.036**	0.281 ± 0.006**
48	0.648 ± 0.070**	0.164 ± 0.009**	0.180 ± 0.053**
96	0.469 ± 0.112**	0.162 ± 0.010**	0.194 ± 0.082**
128	0.401 ± 0.092*	NA	NA
386	0.212 ± 0.050*	NA	NA

Biofilms were grown in TSBg media containing AMG at different concentrations for 24 h at 37 °C. Biofilm biomass was assessed by staining with 0.1% crystal violet solution and dissolving the stain in 30% acetic acid, followed by measuring the absorbance at λ = 595 nm (A_{595}). Data are expressed as the mean ± standard deviation; all experiments were performed in triplicate, in at least three independent experiments. Data marked with * are significantly different with $P < 0.05$ and ** with $P < 0.01$ from that for the vehicle control (pair-wise comparison using Student's t test).

with or without 48 μmol/L AMG (= 5 × MIC) from the start of biofilm for 48 h and stained with the Baclight Live/Dead reagent. Biofilms were then observed using laser scanning confocal fluorescence microscopy (Figure 3). It can be seen that in the presence of AMG, biofilms were still formed, but the biomass was markedly reduced. Also, in the presence of AMG, significantly more cells were dead (red) as compared to the control. Thus, AMG showed bactericidal activity with MRSA 252 in biofilms, similar to observations with other bacterial strains [15,17,30].

3.5. AMG membrane activity

In this study, the effect of different AMG concentrations on the membrane of human RBCs was also tested to check again the effects of AMG on mammalian cells. Results in Table 3 showed that at concentration of 36 μmol/L, about 50% of cells had lysed. Thus, the isolated AMG showed a clearly membrane-disrupting activity on human RBCs at concentrations higher than 4 × MIC. Our results, together with reported data, indicated that the killing activity of AMG at the concentration of 4 × MIC and higher on MRSA 252 was due to the strong surfactant-like action of the compound.
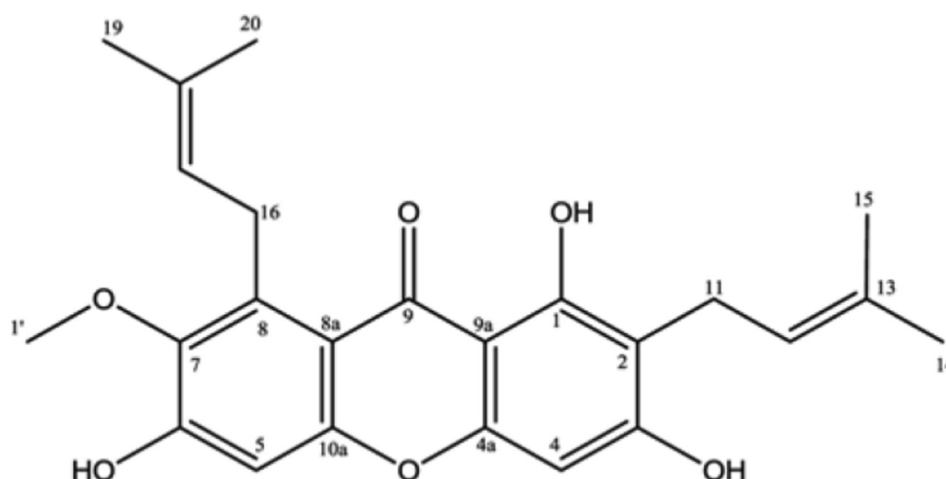

Figure 2. Chemical structure of α-mangostin (AMG) isolated from *G. mangostana* grown in Vietnam.
Molecular formula: $C_{24}H_{26}O_6$. Molecular weight: 410.459 6. ESI-MS at *m/z* 411.4 [M+H]$^+$.
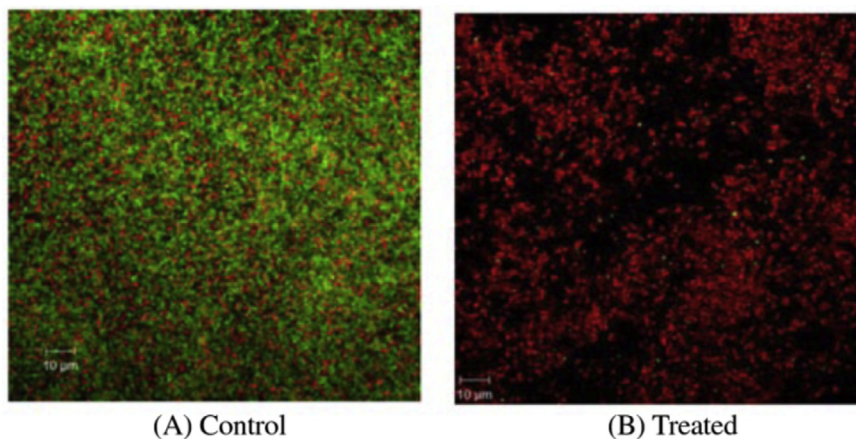
Figure 3. Confocal microscopy images of MRSA 252 biofilms grown on coverslips.
Biofilms of MRSA 252 were treated with for 48 h at 37 °C in fresh TSBg medium (Control) or in TSBg medium supplemented with 48 µmol/L α-mangostin (AMG) (Treated). The coverslips were then stained for 15 min with LIVE/DEAD BacLight mixture (50:50 v/v). Stained biofilms were observed using laser scanning confocal fluorescence microscopy (Olympus, Tokyo, Japan). The "dead" cells are in red, while "live" cells are in green.

Table 3
Membrane activity of AMG on human red blood cells.

Samples	% lysis RBCs
366.00 µmol/L AMG	100.0
183.00 µmol/L AMG	100.0
73.20 µmol/L AMG	79.5 ± 5.0
36.60 µmol/L AMG	56.8 ± 4.6
18.30 µmol/L AMG	18.3 ± 2.2
9.15 µmol/L AMG	9.2 ± 2.6
H_2O	100.0
100 µmol/L ampicillin	0.0
PBS	0.0
Vehicle	0.0

Data are expressed as the mean ± standard deviation for at least three separate experiments.

3.6. Biofilm composition of the test strains

In this experiment, we determined the composition of our test strains using specific enzymatic and chemical treatments. The data in Table 4 showed that more than 82.3% biofilm biomass of MRSA 252 was removed after treatment with proteinase K compared to the control, while the biomass was not changed when biofilms were treated with sodium periodate and DNase.

Table 4
Treatment of NCTC 6571, MSSA 15981, and MRSA 252 biofilms with sodium periodate, proteinase K and DNase I.

Treatments	MRSA 252	NCTC 6571	MSSA 15981
Control	1.99 ± 0.21	4.39 ± 0.11	4.39 ± 0.11
Proteinase K	0.53 ± 0.28[**]	4.48 ± 0.04[**]	4.25 ± 0.12[**]
Periodate	3.24 ± 0.18[**]	0.47 ± 0.07[**]	0.84 ± 0.03[**]
DNase I	3.69 ± 0.25[**]	4.29 ± 0.24[**]	4.27 ± 0.16[**]

Biofilms were grown in 96-well microtitre plates at 37 °C for 24 h and treated with PBS (control), 10 mmol/L sodium periodate, 0.1 mg/mL proteinase K or 0.1 mg/mL bovine DNase I. Plates were incubated at 37 °C for 4 h, followed by staining of the biofilms with crystal violet and measuring the absorbance at A_{595}. Data are expressed as the mean ± standard deviation for at least three separate experiments. Data marked with [**] are significantly different with $P < 0.01$ from that for the vehicle control (pair-wise comparison using Student's t test).

This confirmed that MRSA 252 used in our experiments produces a protein-based biofilm. In contrast, NCTC 6571 and MSSA 15981 had positive reactions with sodium periodate, demonstrating that NCTC 6571 and MSSA 15981 produced polysaccharide-based biofilms.

4. Discussion

In this study, the antibiofilm activity of AMG against SAs is investigated for the first time. Comparison to MIC and MBC tests with this compound indicated that SA in biofilms is more recalcitrant to AMG than planktonic cells. Our data contrast, to some extent, the findings by Sivaranjani et al [15], who showed that sub-MIC concentrations were sufficient to inhibit biofilm formation of S. epidermidis. Thus, biofilm formation by S. epidermidis seems to be more sensitive to AMG as compared to biofilm formation by S. aureus. Moreover, we realized that AMG exhibited inhibitory activity only on biofilm formation (early stage), and had no activity on mature (late stage) biofilms. This was also found in our previous tests with S. mutans biofilms (data not shown) [17]. Similarly, Sivaranjani et al indicated a less effective bactericidal activity of AMG on mature (24 h old) S. epidermis biofilms [15]. Thus, bactericidal activity of AMG on biofilms is strongly dependent on biofilm age, and AMG may be more efficient when used on combination with other antimicrobial compounds to maximize its activity.

Phenolic compounds may serve as surface active agents to disrupt lipid–protein interfaces [31]. Shapiro and Guggenheim studied the antibacterial mechanism of thymol, a phenolic plant-derived compound, against oral bacteria Porphyromonas gingivalis, Streptococcus artemidis, and Streptococcus sobrinus and found that the antibacterial activity was mainly due to rapid disruption of cell membranes by a surfactant-like action [32]. For AMG, its membrane damaging activity to bacteria was reported previously [14,16,29,30]. However, Koh et al tested membrane activity of AMG on rabbit RBCs and found the compound did not have significant lysis activity at concentrations around MIC value, and the authors concluded that AMG selectively targets bacterial membranes [29]. Phitaktim et al [30] and Koh et al [29] proposed that benzene ring and the isoprenyl group of AMG may play a significant role in inhibiting the growth of MRSA strains by direct interactions with the membrane.

However, the concentrations of AMG used for testing against rabbit erythrocytes were lower than that needed for biofilm eradication, and did not test the effect of AMG on human erythrocytes [29]. Interestingly, in our experiment, significant membrane-disrupting activity on human red blood was clearly found at a concentration of 4 × MIC and higher, corresponding to the concentration of AMG that is bactericidal for MRSA 252. This result confirmed the strong surfactant-like action of AMG.

SA strains produce either a PIA/PNAG (*ica*-dependent pathway to form polysaccharide-type biofilm) or protein-mediated biofilms (*ica*-independent pathway) depending on the environmental conditions [4,30,33–36]. Interestingly, protein-mediated biofilm seems to be formed frequently among the highly virulent MRSA isolates, demonstrating the special role of biofilm structure. A relation between methicillin resistance and protein-dependent multicellular behavior in SAs has been reported [35,37]. The four different surface proteins, Bap, SasG, protein A, and fibronectin-binding proteins (FnBPs) have been indicated in proteinaceous biofilms. In SAs, FnBPs are proven to play an important role in *ica*-independent biofilm formation by human MRSA isolates and during the *in vivo* colonization of cathartic. In case of MRSA 252 here, the organism can produce surface proteins FnBPA [38] and possibly other proteins. The finding that antibiofilm activity of AMG exhibits differently among test strains in this study might be explained in terms of biofilm structure. One hypothesis is that components of the extracellular matrix in biofilms (polysaccharides for NCTC 6175 and MSSA 15981, and surface proteins such as FnBPA for MRSA 252) interact differently with AMG. A number of studies that show that AMG can bind to various unrelated proteins such as human serum albumin, transferrin [39], and the ATP-binding cassette drug transporter ABCG2 in cancer cells [40]. The data on molecular docking of this research reveal that the main interaction between AMG and human serum albumin is through hydrophobic interactions, while the main interaction between AMG and transferrin is by hydrogen bonding and Van der Waals forces. We previously showed that AMG interacts with membrane bound enzymes F-ATPase and phosphotransferase system in *S. mutans*, as well as with catalytic domain of glucosyntransferase C, a key enzyme responsible for biofilm formation by this organism [16,17]. Thus, it is conceivable that AMG interacts with extracellular matrix proteins in the biofilm of MRSA 252, while it may not interact with the polysaccharides in the biofilm matrix of NCTC 6715 and MSSA 15981. If so, the proteinaceous biofilm matrix of MRSA 252 provides a protective effect by binding to AMG. However, further investigations to assess the global impact of AMG on key proteins involved in biofilm formation, as well as fully explore the interactions of AMG and surface proteins in MRSA are needed to understand the underlying mechanism and might be exploited in the development of new strategies to better prevent and treat SA infections.

In conclusion, our findings provide evidence that isolated AMG from the peels of *G. mangostana* L. has antibiofilm activity against SAs by killing the bacteria in biofilms, particularly during the early stages of biofilm formation. AMG provides potential to develop a useful of novel agent for the treatment of SA and MRSA. Future studies should disclose a full picture of actions of AMG against SAs and MRSAs, as well as its toxicity in animals and humans for therapeutic application.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NTMP designed the project, supervised and performed the experiments, analyzed data and wrote the manuscript. AB supervised the experiments, revised the research and wrote the manuscript. TTM, NVQ, NVA performed the experiments, and analyzed data. VR and CK helped in isolation of AMG, spectroscopic identifications of the isolates and editing the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

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