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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Potent anti-MRSA activity and synergism with aminoglycosides by flavonoid derivatives from the root barks of *Morus alba*, a traditional Chinese medicine

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Abstract

Infections of clinical methicillin-resistant *Staphylococcus aureus* (MRSA) is a very tough public health problem and a challenge of new drug development. Nearly 90 Diels-Alder adducts (DAAs) have so far been isolated from *Morus* plants, but only a few of them have been evaluated for their anti-MRSA activities. To study the antibacterial compounds of DAAs from the root barks' section of *Morus alba* L. and their synergism with antibacterial agents against clinical MRSA strains, bioassay-guided phytochemical methods were used to screen the active components. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were assayed through broth serial microdilution. The synergism were evaluated by checker board microdilution and dynamic time-kill experiments. Three DAAs (multicaulisin (**1**), sanggenon G (**2**) and albanin G (**3**)) were isolated and identified from *M. alba* root barks. They were determined with potent effect against MRSA isolates with MICs/MBCs at 2–8/16–128 mg/L. They also showed synergy with conventional antibacterial agents, especially the aminoglycosides, with fractional inhibitory concentration indices (FICIs) ranged from 0.19 to 0.50 and the dose reduction indices (DRIs) ranged from 16 to 2. The MRSA resistance to the antibiotics could be reversed by compounds **1**–**3**. The dose-dependent bactericidal synergism against MRSA was observed as well. The study released for the first time the anti-MRSA synergism of DAAs from *M. alba* root barks with antibacterial agents and the reversal of MRSA resistance to aminoglycosides. The results may be valuable for further development of new antibacterial drugs and synergists against MRSA infections.

Keywords MRSA · *Morus alba* · Diels-Alder adduct · Aminoglycoside · Synergy · Resistance reversal effect

List of abbreviations

AG	albanin G
Ak	amikacin
AMR	antimicrobial resistance
CFU	colony forming unit
CBMD	checker board microdilution
CLSI	Clinical and Laboratory Standards Institute
DAA	Diels-Alder adduct
DRI	dose reduction index
Em	etimicin
Gm	gentamicin
FICI	fractional inhibitory concentration index
I	intermediate
Le	levofloxacin
MBC	minimal bactericidal concentration
M-H	Mueller-Hinton medium
MIC	minimal inhibitory concentration
MSSA	methicillin-susceptible *Staphylococcus aureus*
MRSA	methicillin-resistant *Staphylococcus aureus*
Mu	multicaulisin
NMR	nuclear magnetic resonance
R	resistant
S	susceptible

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SBP	Sang-Bai-Pi (root barks of *Morus alba* L. (Moraceae))
SG	sanggenon G
T-KC	time-killing curve
Va	vancomycin

Introduction

Staphylococcus aureus is a clinical prevalent pathogen causing diseases from mild skin and soft tissue to life-threatening infections, especially the infections with methicillin-resistant *S. aureus* (MRSA) by higher rates of in-hospital complication and mortality, and greater health care costs (Guillamet et al. 2018). Currently, MRSA can be resistant to most conventional antibacterial agents (Mercer et al. 2017), worse yet the occurrence of vancomycin-resistant *S. aureus* (VRSA) (Okada et al. 2018). A serious lack of new anti-MRSA drugs has promoted the search for novel chemical entities from natural sources. For a number of years, we are working on finding the novel phytochemicals from medicinal plants based on their uses in traditional Chinese medicine (TCM), especially those which can potentiate the anti-MRSA effect and even reverse the MRSA resistance to conventional antibacterial agents (Zuo et al. 2018).

Morus alba L. (Moraceae) is a typical plant of genus *Morus*, the perennial deciduous shrub or tree with a height of 3–15 m. It is distributed throughout Asia, Africa, Europe, and South and North America. The root bark of *M. alba* is a TCM known as Sang-Bai-Pi (SBP) and has been documented in the Chinese Pharmacopoeia. SBP has been widely used for asthma, edema, and other diseases since 500 BC. Over 110 compounds have been found from SBP, including flavonoid derivatives of Diels-Alder adducts (DAAs), 2-arylbenzofurans and stilbenes. The pharmacological effects of these compounds, such as anti-microbic, anti-inflammatory, anti-tumor and anti-oxidative activities, were also reported (Zuo et al. 2018; Wei et al. 2016).

It was claimed that nearly 90 DAAs have been found from SBP and other *Morus* plants, however, only a few of them have been subjected to the evaluation of anti-MRSA properties (Ross et al. 2008; Ku et al. 2010; Farooq et al. 2015). As part of our continuing studies on the new anti-MRSA phytochemicals from SBP (Zuo et al. 2018), we herein report the anti-MRSA activity and potentiation effect of three known DAAs from SBP, i.e., multicaulisin (Mu, **1**; Ferrari et al. 2000), sanggenon G (SG, **2**; Fukai et al. 1983) and albanin G (kuwanon H, moracenin A) (AG, **3**; Cui 2008; Nomura et al. 2009), mainly dealing with their synergism on aminoglycosides against clinical MRSA isolates by classic checker board microdilution (CBMD) and time-killing curve (T-KC) methods.

Materials and methods

General phytochemical procedures

^{1}H- and ^{13}C-NMR spectra (TMS as internal standard) were determined on a Bruker AM-400 NMR spectrometer. MS spectra were recorded on a VG Auto Spec-3000 mass spectrometer. Column chromatography was accomplished on si-gel (200–300 mesh and 300–400 mesh, Qingdao Marine Chemical Co., Ltd., Qingdao, China) and Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Fractions and isolated compounds were monitored by TLC (GF254, Qingdao Marine Chemical Co., Ltd., Qingdao, China) through visualizing by 5% $FeCl_3$ (in 80% ethanol) and 10% H_2SO_4 (in ethanol) reagents, respectively.

Plant materials

The root barks of *M. alba* (SBP; voucher specimen KUN 0515476, Herbarium of KIB) were purchased from Yunnan Lv Sheng Pharmaceutical Co., Ltd, Kunming, China in Aug. 2010. The samples were washed thoroughly, air-dried at room temperature, coarsely powdered by a grinder (Suining General Machinery Factory, Sichuan, China) and stored in sterile airtight containers.

Antibacterial agents and disks

The four antibacterial agents including three aminoglycosides and a floroquinolone were purchased from the manufacturers in China, i.e., amikacin (Ak) (Jiangsu Wuzhong Pharmaceutical Group Co., Ltd.); etimicin (Em) (Wuxi Jiming Kexin Shanhe Pharmaceutical Co., Ltd., Wuxi China); gentamicin (Gm) and levofloxacin (Le) (Yangtze River Pharmaceutical Group Co., Ltd., Taizhou China). Vancomycin (Va) was bought from Eli Lilly Japan K. K., Seishin Laboratories. Antibiotic impregnated disks of cefoxitin (0.03 mg) and others were supplied by Tiantan biological products Co., Ltd., Beijing, China. The three DAAs **1–3** were prepared from SBP (purity ≥95% by ^{13}C NMR spectral analysis; Supplementary material, S1).

Bacterial strains and media

Ten clinical MRSA strains of SCCmec III type were obtained and characterized using cefoxitin disk and multiplex PCR tests as previously reported (Zuo et al. 2018). MSSA (ATCC 25923) was used as the control strain. Mueller-Hinton (M-H) agar and broth (Tianhe Microbial Agents Co., Hangzhou, China) were used as bacterial culture media. All susceptibility assays including time-kill curves were carried out in M-H broth. The bacteria were

grown at 35 °C for 24 h and examined in daylight. Colony counts in M-H agar plates were manually made under microscope.

Preparation of DAAs 1–3 from SBP

Preparation of extracts

The pulverized plant sample (5000 g) was immersed and extracted with 80% ethanol at room temperature for three times (7, 3 and 3 days, respectively). The filtered liquid sections were combined and evaporated the solvent at 40 °C under reduced pressure to get the crude extracts (635 g, 12.7%). The crude extracts were suspended in deionized water (1000 mL) and further sequentially extracted using ethyl acetate and n-butanol (Zuo et al. 2018). The resulted sub-sections 240 and 40 g, together with 355 g from water phase were stored at −20 °C until tested for the inhibition against MRSA.

Bioassay-guided isolation of the DAAs

The ethyl acetate extracts (240 g) which showed the most effective in the three sub-sections on MRSA were chromatographed through si-gel column (200–300 mesh), gradient elution with petroleum ether-ethyl acetate (10:1–1:1) to give 20 fractions (Sfr-1–20); further screening of the active fractions and repeated column chromatography of Sfr-12 with si-gel (300–400 mesh, chloroform-methanol (30:1 and 15:1, respectively)) to furnish compound **3**. Further repeated chromatography of Sfr-13 with si-gel (300–400 mesh, chloroform-methanol (30:1 and 10:1, respectively) and Sephadex LH-20 (methanol) afforded compounds **1** and **2**, respectively. Compounds **1**–**3** were subjected to spectral analyses and compared the data with those of previously reported (Ferrari et al. 2000; Fukai et al. 1983; Cui 2008).

Multicaulisin

Mu (**1**) was isolated as reddish brown powder (methanol); ESI-MS: *m/z* (%) 715 (100) $[M + Na]^+$; ^{1}H-NMR (400 MHz in CD_3OD) δ: 7.34 (1H, d, J = 8.8, H-6′), 7.15 (1H, b d, H-27), 6.81 (1H, br s, H-3), 6.15 (1H, br d, H-24), 6.49 (1H, d, J = 2.3 Hz, H-3′), 6.46 (1H, dd, J = 8.8, 2.3 Hz, H-5′), 6.42 (1H, br s, H-8), 6.13 (1H, d, J = 2.2 Hz, H-30), 6.08 (1H, dd, J = 2.2, 8.2 Hz, H-32), 6.06 (1H, dd, J = 2.4, 8.8 Hz, H-24), 5.93 (1H, d, J = 2.4 Hz, H-24), 5.29 (1H, br s, H-10), 5.22 (1H, brt, H-14), 4.92 (1H, br m, H-20), 4.34 (1H, br d, H-9), 3.70 (1H, br m, H-19), 1.45–1.64 (2H, br m, H-12, 13, 18), 1.35 (3H, s, H-16), 1.28 (3H,s,H-17). ^{13}C-NMR (100 MHz in CD_3OD) δ: 210.3 (C-21), 184.0 (C-4), 166.0 (C-23), 165.8 (C-25), 162.6 (C-7,4′), 161.9 (C-2,5), 161.2 (C-2′), 157.9 (C-31), 156.9 (C-29), 134.5 (C-11), 132.9 (C-33), 132.7 (C-6′), 132.3 (C-27), 125.0 (C-10), 124.8 (C-14), 123.1 (C-28), 116.8 (C-22), 113.8 (C-6), 108.7 (C-1′), 108.3 (C-5′), 108.1 (C-3), 107.9 (C-32), 105.7 (C-26), 103.8 (C-3′), 103.6 (C-24), 103.0 (C-30), 98.6 (C-8), 48.7 (C-20), 39.2 (C-9), 26.1 (C-12, 18), 24.9 (C-19), 23.6 (C-13), 23.3 (C-16), 17.8 (C-17). These data were in agreement with that of multicaulisin as the previous report (Ferrari et al. 2000).

Sanggenon G

SG (**2**) was isolated as brown powder (methanol)), ESI-MS: *m/z* (%) 717 (100) $[M + Na]^+$; ^{1}H-NMR (400 MHz in CD_3OD) δ: 7.61 (1H, br d, J = 8, H-27), 7.17 (1H, d, J = 8, H-6′), 6.82 (1H, d, J = 8, H-33), 6.31 (1H, d, J = 2, H-3′), 6.29 (1H, dd, J = 2, 8 Hz, H-5′), 6.12 (1H, d, J = 2.5, H-30), 6.07 (1H, dd, J = 2.5, 8 Hz, H-26), 6.06 (lH, dd, J = 2.5, 8 Hz, H-32), 5.97 (1H, d, J = 2.5, H-24), 5.70 (1H, s, H-8), 5.54 (1H, br d, J = 13, H-2), 5.20 (1H, m, H-14), 3.02 (1H, dd, J = 13, 16 Hz, trans-H-3), 2.50–2.68 (1H, m, cis-H-3), 2.05–2.16 (4H, m, Hx2-12, 13), 1.63, 1.68 (each 3H, s, H-15). ^{13}C-NMR (100 MHz in CD_3OD) δ: 210.8 (C-21), 198.4 (C-4), 166.2 (C-7), 166.0 (C-5), 163.4 (C-23, 25), 162.6 (C-8a), 159.7 (C-4′), 157.3 (C-29, 31), 156.8 (C-2′), 132.2 (C-33), 129.1 (C-15), 128.9 (C-27), 125.7 (C-14), 118.0 (C-28), 116.2 (C-22), 110.5 (C-1′), 108.5 (C-5′), 107.9 (C-6), 107.7 (C-32), 107.4 (C-26), 103.8 (C-24), l03.4 (C-4a), 103.0 (C-30), 102.7 (C-3′), 96.2 (C-8), 75.6 (C-2), 43.2 (C-3), 38.7 (C-12), 27.5(C-13), 26.1 (C-16), 18.0 (C-17). These data were in agreement with that of sanggenon G as the previous report (Fukai et al. 1983).

Albanin G

AG (**3**) was isolated as reddish brown powder (methanol)), ESI-MS: *m/z* (%) 783 (100) $[M + Na]^+$; ^{1}H-NMR (400 MHz in CD_3OD) δ (ppm): 1.47 (3H, s, H-13), 1.50 (3H, s, H-7″), 1.54 (3H, s, H-25″), 1.60 (3H, s, H-12), 1.65 (3H, s, H-24″), 1.94 (1H, m, H-6″), 2.00 (1H, overlapped, H-6″), 3.11 (4H, d, J = 7.2 Hz, H-9, 21″), 3.22 (1H, m, H-5″), 4.42 (1H, br d, J-9.6 Hz, H-3″), 4.65 (1H, br s, H-4″), 5.05 (1H, t, J = 7.2 Hz, H-22″), 5.16 (1H, t, J = 9.6 Hz, H-10), 5.20 (1H, br s, H-2″), 5.98 (1H, d, J = 8.4 Hz, H-13″), 5.99 (1H, s, H-6), 6.05 (1H, br d, J-8.4 Hz, H-19″), 6.20 (1H, br s, H-17″), 6.55 (1H, br d, J = 8.4 Hz, H-5′), 6.65 (1H, br s, H-3′), 6.85 (1H, d, J = 8.4 Hz, H-20″), 7.26 (1H, d, J = 8.4 Hz, H-6′), 7.82 (1H, d, J = 8.4 Hz, H-14″); ^{13}C-NMR (100 MHz in CD_3OD) δ (ppm): 209.5 (C-8″), 183.9 (C-4), 163.6 (C-10″), 163.0 (C-8[a],12″), 161.7 (C-4′), 161.3 (C-2), 161.0 (C-7), 157.7 (C-5, 18″), 156.8 (C-2″, 8″), 134.4 (C-1″), 132.7 (C-23″), 132.1 (C-11, 20″), 131.6 (C-

6′), 130.7 (C-14″), 124.6 (C-2″), 123.6 (C-15″, 22″), 122.9 (C-10), 121.5 (C-3), 115.7 (C-11″), 115.3 (C-9″), 113.7 (C-1′), 108.7 (C-5′, 19″), 107.8 (C-8), 107.3 (C-13″), 105.6 (C-4a), 103.6 (C-3′, 17″), 98.5 (C-6), 47.5 (C-4″), 38.1 (C-3″, 5″, 6″), 25.9 (C-12, 24″), 24.7 (C-9), 23.1 (C-7″), 22.3 (C-21″), 17.8 (C-25″), 17.7 (C-13). These data were in agreement with that of albanin G as the previous reports (Cui 2008).

Susceptibility testing

The minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of DAAs **1–3** were measured by standard method of CLSI as previous report (Zuo et al. 2018). Briefly, test samples of DAAs **1–3** were dissolved in 5% aqueous DMSO to obtain a stock solution of 1024 mg/L. Serial broth microdilution of the samples with M-H broth from 1024–2 mg/L were performed in the 96-well flat-bottomed microplates. The well containing no sample served as a negative control. Each well contained final bacterial inoculums of 5×10^5 CFU/ml. The plates were incubated at 35 °C for 24 h. MIC was defined as the lowest concentration at which no turbidity was observed after incubation. Aliquots (0.02 mL) removed from wells with no bacterial growth were streaked onto M-H agar plates and incubated under the same conditions. MBC was defined as the lowest concentration at which the growth of colonies counted less than five after incubation. They were determined in duplicate. Vancomycin was used as the positive control drug.

Synergy testing

The bacteriostatic synergism of a combination was assessed as previously reported (Zuo et al. 2018), using CBMD method by criteria of the fractional inhibitory concentration indices (FICIs) as synergy, FICI ≤ 0.5; indifferent, 0.5 < FICI ≤ 4; antagonism, FICI > 4. The degree of potentiation was determined according to a dose reduction index (DRI) which was calculated as $DRI = MIC_{alone}/MIC_{combined}$. The corresponding bactericidal synergism was evaluated by the T-KC experiments through the increase of killing in log_{10}CFU/mL at the 24 h incubation (ΔLC_{24}), i.e.: $\Delta LC_{24} \geq 2$ log_{10}CFU/mL, synergy; $\Delta LC_{24} = 1$–2 log_{10}CFU/mL, additive; $\Delta LC_{24} = \pm 1$ log_{10}CFU/mL, indifferent; $\Delta LC_{24} > -1$ log_{10}CFU/mL, antagonism.

Cell cytotoxicity

The cytotoxicity of compounds (**2** and **3**) were tested against human cell lines of liver HL-7702 and lung cancer A549 (Procell, Wuhan, China) using the MTS assay as the previous report (Zuo et al. 2018).

Results

The identified active DAAs 1–3 from SBP extracts

Bioassay-guided fractionation of the SBP extracts resulted in the identification of three anti-MRSA compounds DAAs **1–3** (Fig. 1). Their spectral data were in agreement with those reported in the literature (Ferrari et al. 2000; Fukai et al. 1983; Cui 2008; S1).

Potent activity and synergism of DAAs 1–3 on MRSA

Tables 1 and 2 show the activity of DAAs **1–3** with MIC_{90} ≤8 and 2 mg/L ($n = 10$) used alone and in combination with the antibacterial agents, respectively. Generally, DAAs **1–3** each used alone was determined with activity more potent than any of the combined aminoglycosides (Ak, Em, and Gm) and a floroquinolone (Le), with the exception of Va. There are four to nine MRSA strains ($n = 10$) showing synergy in the six combinations of DAAs **1–3** with the three aminoglycosides Ak, Em and Gm, with $FICI_{50}$ ranged from 0.375 to 0.563 and DRI_{50} from 8 to 4 (Table 2). None of the combinations showed antagonism.

Reversal of MRSA resistance to aminoglycosides by DAAs 1–3

Table 3 shows the reversal effect of DAAs **1–3** on MRSA resistance to the tested aminoglycosides. According to the CLSI interpretive criteria (i.e., MICs ≤ 16 mg/L) (CLSI 2012), all combinations of Ak with DAAs **1–3** against the ten MRSA isolates showed susceptible (S) because the combined MICs were 2–16 mg/L, which revealed that the resistance of all MRSA strains ($n = 10$) to Ak was reversed. Similarly, Mu caused the resistance of five MRSA isolates against Gm to be reversed, but AG showed no resistance reversal on Le against MRSA (Table 3). Em also showed MICs ≤1 mg/L in the combinations with Mu and SG against nine MRSA isolates, the same potency against the MSSA (Tables 1–3).

Dose dependent killing effects on MRSA by aminoglycosides in combination with DAAs 1 and 2

The potential bactericidal synergism by DAAs **1–3** were further evaluated through the classical dynamic T-KC experiment using MR09, a MRSA strain listed in Table 1. As the results displayed in Table 4 and Fig. 2, there are three combinations (i.e., SG + Ak, AG + Ak and AG + Le) exhibiting synergy of bactericidal effect at 1 × MIC of the interacted concentrations revealed by the criterion of increase in killing at 24 h ($\Delta LC_{24} \geq 2$ log_{10} CFU/mL) (CLSI 2012), as the ΔLC_{24} were calculated as 2.59, 4.11, and 2.11

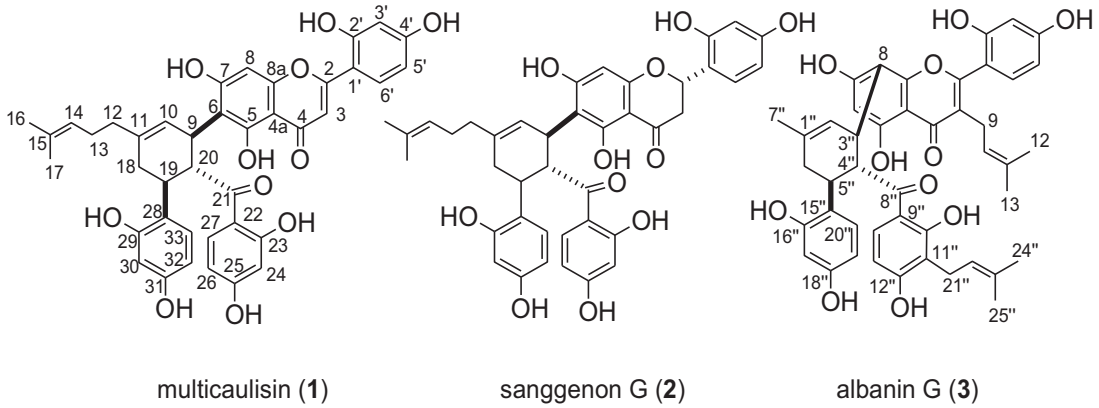

Fig. 1 The structures of DAAs **1**–**3**

Table 1 MICs and MBCs of DAAs (**1–3**) and some antibacterial agents alone against MSSA and MRSA strains (mg/L)

Agent		SA	MA01	MA02	MA03	MA04	MA05	MA06	MA07	MA08	MA09	MA10
Mu (**1**)	MIC	4	8	4	4	4	4	4	2	8	4	8
	MBC	16	32	32	32	16	16	16	16	32	32	32
SG (**2**)	MIC	4	4	4	4	4	4	4	4	4	4	4
	MBC	8	8	8	8	8	8	8	16	16	8	8
AG (**3**)	MIC	4	8	4	4	4	8	8	4	4	2	16
	MBC	32	64	64	64	32	64	64	64	128	32	128
Ak	MIC	16	16	64	32	32	16	32	32	32	32	32
	MBC	32	64	256	128	128	64	128	256	256	128	128
Em	MIC	4	4	8	4	8	4	8	8	8	4	4
	MBC	16	8	32	32	32	16	32	32	64	16	16
Gm	MIC	32	16	32	16	32	32	32	32	32	32	32
	MBC	128	64	64	64	256	128	256	128	256	128	128
Le	MIC	4	64	32	32	32	32	32	16	8	16	32
	MBC	16	128	64	64	64	128	128	64	32	128	64
Va	MIC	0.5	1	0.25	1	0.5	2	1	1	1	1	1
	MBC	2	1	1	1	2	2	2	2	2	2	2

SA MSSA (ATCC25923), *MA* MRSA, *Mu* multicaulisin (**1**), *SG* sanggenon G (**2**), *AG* albanin G (**3**), *Gm* gentamicin, *Ak* amikacin, *Em* etimicin, *Le* levofloxacin, *Va* vancomycin

$\log_{10}$ CFU/mL, respectively ($\Delta LC_{24} = LC_{24Md} - LC_{24co}$; Table 4).

The dose-dependent synergistic effects were demonstrated by (1–1/4) × MIC of the interacted aminoglycosides with 1 × MIC of DAAs **1** and **2**, respectively. The results are shown in Table 4. In the combinations of Ak with SG, decreasing concentrations of Ak to 1/2 MIC also displayed equally significant synergy with 1 × MIC of SG with $\Delta LC_{24} = 2.59$ $\log_{10}$ CFU/mL, but only the additive effect ($\Delta LC_{24} = 1.89$ $\log_{10}$ CFU/mL) at 1/4 MIC of Ak was observed (Table 4). Beside 1 × MIC, Em at 1/2 and 1/4 MIC all showed additive effects with 1 × MIC of SG as well. Meanwhile, Em with the concentrations of (1–1/4) × MIC showed interaction with Mu as additive, additive and indifferent effects, respectively (Table 4). Nevertheless, the differences of ΔLC_{24} were yet statistically significant with the changes of concentration for the antibacterial agents. For the concentrations of DAAs other than 1 × MIC used alone, the increases of killing effects dose-dependently against MRSA at the concentrations from 1 to 4 × MICs were significantly observed, with even the LC_{24} of SG under 2 and 4 ×MICs both reduced to zero (Fig. 2h).

In vitro cell cytotoxicity of sanggenon G and albanin G

The cytotoxicity of Mu and SG were assayed with IC_{50} values as 3.6, 4.4 mg/L and 3.4, 3.0 mg/L on HL-7702 and A549 cell lines, respectively.

Table 2 MICs (mg/L), DRIs and FICIs of DAAs (**1**–**3**) and the antibacterial agents used alone or combinatorially against clinical MRSA isolates ($n = 10$)

Combn	Activity[a]	DAAs			Antibacterial agent			FICI[b]	Syn (%)[c]
		MIC_{al}	MIC_{co}	DRI	MIC_{al}	MIC_{co}	DRI		
Mu + Ak	Range	2–8	0.5–2	8–2	16–64	2–16	16–2	0.375–1	40
	50%	4	2	2	32	2	8	0.563	
	90%	8	2	2	32	16	4	0.75	
	MSSA	4	1	4	16	4	4	0.5	
Mu + Em	Range	2–8	1–2	8–2	4–8	0.5–4	8–2	0.25–1	60
	50%	4	1	4	4	1	8	0.5	
	90%	8	2	2	8	1	4	0.75	
	MSSA	4	1	4	4	1	4	0.5	
Mu + Gm	Range	2–8	1–2	4–2	16–32	2–8	16–4	0.375–0.75	70
	50%	4	1	4	32	4	4	0.5	
	90%	8	2	2	32	8	4	0.563	
	MSSA	4	1	4	32	8	4	0.5	
SG + Ak	Range	4–4	0.5–1	8–4	16–64	4–16	4–2	0.375–0.75	60
	50%	4	1	4	32	8	4	0.5	
	90%	4	1	4	32	16	2	0.75	
	MSSA	4	1	4	16	8	2	0.75	
SG + Em	Range	4–4	1–2	4–2	4–8	0.5–2	8–4	0.375–0.75	90
	50%	4	1	4	4	1	8	0.375	
	90%	4	1	4	8	1	4	0.5	
	MSSA	4	1	4	4	1	4	0.5	
AG + Ak	Range	2–16	1–2	8–2	16–64	2–8	16–4	0.188–0.625	80
	50%	4	1	4	32	4	8	0.375	
	90%	8	2	2	32	4	4	0.563	
	MSSA	4	2	2	16	2	8	0.625	
AG + Le	Range	2–16	1–4	8–1	16–32	2–32	16–1	0.375–1.5	20
	50%	4	2	2	32	8	4	0.625	
	90%	8	4	2	32	16	2	1.5	
	MSSA	4	2	2	16	2	8	0.625	

Combn Combination, *Mu* multicaulisin (**1**), *SG* sanggenon G (**2**), *AG* albanin G (**3**), *Gm* gentamicin, *Ak* amikacin, *Em* etimicin, *Le* levofloxacin, *al* alone, *co* combined, *DRI* dose reduction index = MIC_{al} /MIC_{co}, arranged from the maximal to the minimal

[a]50% ($n = 10$) and 90% ($n = 10$): inhibitory effects against 50 and 90% of the 10 MRSA strains, e.g., MIC_{50}, (MIC_{90}) etc

[b]FICI (of A combined with B) = $((MIC_A)_{co}/(MIC_A)_{al}) + ((MIC_B)_{co}/(MIC_B)_{al})$; FICI ≤ 0.5, synergy (Syn); 0.5 < FICI ≤ 4, indifferent; FICI > 4, antagonism

[c]Syn%: percent of strains that showed synergy ($n = 10$), all the rest been indifferent.

Discussion

MRSA was first reported by Jevons in 1961 (Jevons 1961), and has since been evolved as a seriously global healthcare problem. There is the emergence of MRSA with reduced susceptibility to vancomycin and even VRSA occurred (Okada et al. 2018). It was estimated that antimicrobial resistance (AMR) would cause 10 million deaths a year by 2050 (De Kraker et al. 2016). For this reason, the World Health Organization (WHO) has already listed anti-MRSA new drug development as high priority (WHO 2017). Unfortunately, however, the lack of innovation in antibiotics development and the "paradox" in the practical superiority validation for the proposed drug over the existing drugs may make AMR problem (including MRSA) more tricky, and thus there remains concerning the crisis that we may return to the pre-antibiotic era (Fears and Ter Meulen 2014; Rex et al. 2017).

Therefore, the rational treatment regimens of current antibacterial agents, and strategies of making the existent antibacterial agents to be sustainably effective and reversing the resistant agents to return to be susceptible to AMR pathogens become vital important. Alternatively, synergism between plant natural products and antibiotics has been

Table 3 **a** Collective MIC Breakpoints of aminoglycosides against *Staphylococcus* spp. from CLSI Performance Standards in three different years' edition

Interpretive Categories	Years	MIC Breakpoints (mg/L)				
		Gentamicin	Amikacin	Kanamycin	Netilmicin	Tobramycin
S (susceptible)	2018	≤4	–	–	–	–
	2012	≤4	≤16	≤16	≤8	≤4
	2007	≤4	≤16	≤6	≤12	≤4
I (intermediate)	2018	8	–	–	–	–
	2012	8	32	32	16	8
	2007	–	–	–	–	–
R (resistant)	2018	≥16	–	–	–	–
	2012	≥16	≥64	≥64	≥32	≥16
	2007	≥8	≥32	≥25	≥32	≥8

b The susceptibility spectrum of MRSA strains to the antibacterial agents in combination with DAAs

Agent	MIC interpretive criteria (mg/L)		
	S	I	R
Ak	≤16	32	≥64
(Mu;SG;AG)[b]	(10;10;10)[b]	(0;0;0)	(0;0;0)
Gm	≤4	8	≥16
(Mu)	(5)	(5)	(0)
Em[c]	(≤1)	–	(≥1)
(Mu;SG)	(9;9)	(0;0)	(1;1)
Le	≤1	2	≥4
(AG)	(0)	(3)	(7)

The data shown in Table 3a are from CLSI 2007, 2012 and 2018.

Mu multicaulisin (**1**), *SG* sanggenon G (**2**), *AG* albanin G (**3**), *Gm* gentamicin, *Ak* amikacin, *Em* etimicin, *Le* levofloxacin, *S* susceptible, *I* intermediate, *R* resistant

[a]Numbers in brackets indicate the MRSA strains (up to 10) which showed S, I or R to antibacterial agents in the combinations with indicated DAAs corresponding to the leftmost column

[b]Em has no MIC criteria in CLSI standards and we took the MIC values against MSSA (Table 2) for the judgment

proposed as approaching a new generation of phyto-pharmaceuticals (Hemaiswarya et al. 2008; Wagner and Ulrich-Merzenich 2009). Zacchino et al. (2017) also reviewed the plant phenolics and terpenoids as adjuvants of antibacterial and antifungal drugs. Hence, results of the present report added new phytochemical potentials of the approach.

In this report, we demonstrated that the DAAs **1**–**3** from SBP showed potent anti-MRSA activities used alone. More significantly, we also firstly found the synergism of them in combination with current antibacterial agents, especially aminoglycosides, against MRSA with potent effects in fighting against the problematic AMR pathogen. Other antibiotics, such as penicillins and cephalosporins, showed no synergism with these compounds in the preliminary experiment. Apart from anti-MRSA effects of the DAAs herein, AG also showed other pharmacological effects, including anti-inflammatory, anti-hypertensive, HIV, and tyrosinase inhibitory effects (Nomura et al. 2009; Wei et al. 2016), and used as a weight loss ingredient (Yimam et al. 2017). SG was reported inhibition against the neuraminidases (Grienke et al. 2016), GABA (A) receptor (Çiçek 2018) and an apoptosis protein (XIAP) which was viewed as an attractive target for anti-cancer therapy (Seiter et al. 2014). AG and SG were also previously reported with potent anti-MRSA activity (i.e., MICs of 0.3–3 mg/L) (Ku et al. 2010). The MIC differences from our results might be attributed to the assay with varied MRSA strains.

Although previous reports have also involved the anti-microbial and cytotoxic activity of other SBP ingredients (Wei et al. 2016), including other *Morus* DAAs against MRSA, i.e., artonin I (Farooq et al. 2015), and Sorocenols G and H (Ross et al. 2008). However, the anti-MRSA and cytotoxic activity of Mu and SG, especially the synergism of DAAs **1**–**3** has so far been reported herein for the first time. It is noted that a natural DAA from *Morus* spp. is

Table 4 Dynamic bactericidal effects of the antibiotics at concentrations of (1–1/4) × MIC combined with DAAs (**1**–**3**) at concentrations of 1 or 4 × MIC against a MRSA clinical strain (MA07)

Agent	Conc	Mu (4Mu)			SG			AG		
		Md	ΔLC_{24} (Int)		Md	ΔLC_{24} (Int)		Md	ΔLC_{24} (Int)	
			12 h	24 h		12 h	24 h		12 h	24 h
Ak	1MIC	Mu	−0.12	0.26(i)	SG	1.48	2.59(s)	AG	0.96	4.11(s)
	1/2MIC	Mu	−0.12	0.43(i)	SG	1.13	2.59(s)	nt	nt	nt
	1/4MIC	Mu	−0.24	0.26(i)	SG	0.91	1.89(a)	nt	nt	nt
Em	MIC	Mu	0.36	1.26(a)	SG	0.46	1.24(a)	nt	nt	nt
	1/2MIC	Mu	0.00	1.13(a)	SG	0.29	1.22(a)	nt	nt	nt
	1/4MIC	Mu	0.52	0.83(i)	SG	0.48	1.16(a)	nt	nt	nt
Gm	1MIC	Mu (4Mu)	0.88(0.42)	1.73(a) (2.38(s))	nt	nt	nt	nt	nt	nt
	1/2MIC	Mu (4Mu)	0.32(0.54)	1.73(a) (2.38(s))	nt	nt	nt	nt	nt	nt
	1/4MIC	Mu (4Mu)	0.21(−0.49)	0.44(i)) (2.38(s))	nt	nt	nt	nt	nt	nt
	1MIC	nt	nt	nt	nt	nt	nt	AG	0.15	2.11(s)
Le	1/2MIC	nt	nt	nt	nt	nt	nt	nt	nt	nt
	1/4MIC	nt	nt	nt	nt	nt	nt	nt	nt	nt

Ak amikacin, *Em* etimicin, *Gm* gentamicin, *Le* levofloxacin, *Conc* concentration, *Mu* multicaulisin (**1**), *SG* sanggenon G (**2**), *AG* albanin G (**3**), *4Mu* 4 × MIC concentration of Mu, *Md* most effective single drug, ΔLC_{24} ΔLog_{10} CFU/mL at 24 h ($\Delta LC_{24} = LC_{24Md} - LC_{24co}$), *Int* interaction (*s* synergy ($\Delta LC_{24} \geq 2$), *i* indifferent ($-1 < \Delta LC_{24} < 1$), *a* additive ($1 < \Delta LC_{24} < 2$), *nt* not tested

formed by a Diels-Alder reaction between α, β-olefinic moiety of a chalcone and an isoprene moiety. Generally, they are phenolic phytochemicals. Therefore, they are likely to have similar effects on MRSA which warrant further investigations in the future.

DAAs **1**–**3** contain the same moieties of tetrahydroxyflavone (**1** and **3**) or tetrahydroxyflavanone (**2**), including the same double-resorcinol moieties in the structures (Fig. 1). It is thus not surprising that the (MICs) $_{MSSA}$ and the 50 and 90% of the combined $(MICs)_{MRSA}$ and DRIs in the combinations were not different significantly (Table 2). Moreover, DAAs in the combinations might have played a leading role in reducing the MIC values of combinatory usage as well (Tables 2–4, Fig. 2). It could be seen that the DRI value of an antibacterial agent in a combination is usually greater than that of a DAA, which well illustrates DAAs as the potentiators of the tested antibacterial agents (Table 2).

Bacterial pathogens have evolved AMR to current drugs via varied mechanisms including the receptor (or active site) modification and enzymatic modification /degradation of the drugs, and reducing accumulation of the drugs within the bacterial cells (decreased membrane permeability and active efflux). Therefore, the anti-AMR action of a phytochemical used alone or in combination with an antibacterial drug would be also to counteract one or more of these resistant mechanisms through (1) synergistic multi-target effects; (2) enzymatic inhibition or increasing bioavailability; (3) combined interactions on bacterial resistance mechanisms and (4) elimination or neutralization of adverse effects by the added agents, so that altogether a better effectiveness than without these additions or manipulations can be achieved (Wagner and Ulrich-Merzenich 2009; Hemaiswarya et al. 2008). It was revealed that artonin I, one of the DAA-type phytochemical with similar activity as DAAs **1**–**3**, could depolarize bacterial cell membrane and inhibit efflux mechanism and induce cellular destruction through cell membrane damage (Farooq et al. 2015), which implies that DAAs **1**–**3** may also exhibit the similar mechanisms and remains to be clarified.

Although DAAs **1**–**3** are extraordinarily active alone against MRSA, their potent cytotoxicity does not seem to be beneficial for anti-MRSA treatment. However, the combined use of DAAs **1**–**3** with the antibacterial agents could largely reduce their respective MIC_{50} to below the respective IC_{50} (Table 2), result in anti-MRSA potency approaching to vancomycin (Tables 1 and 2). Therefore, both the toxicity of DAAs and the agents could be reduced. There could also be possibly beneficial for the DAAs as potential antitumor agents, i.e., achieving multi-target chemotherapy of tumor patients with bacterial infection at the same time (Seiter et al. 2014), considering systematic studies have shown that the Gram-positive organisms are the leading cause of invasive bacterial disease in patients with cancer (Holland et al. 2014).

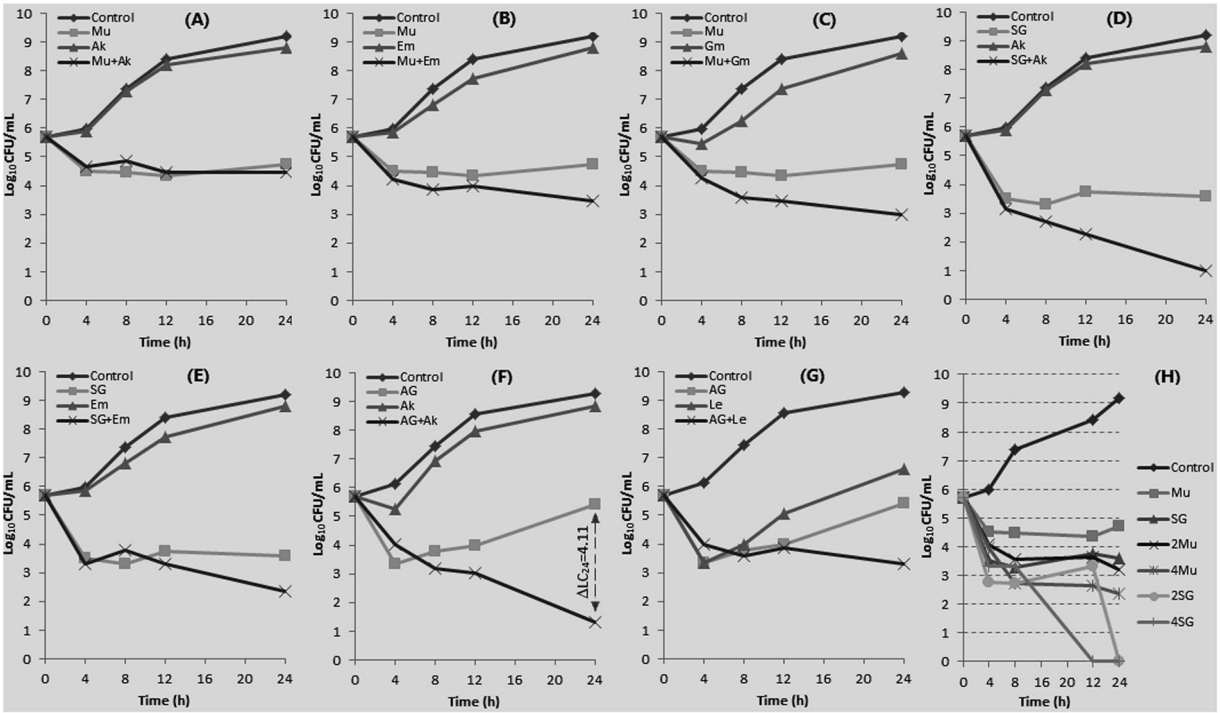

Fig. 2 Time-kill curves of the synergistic effect of the combination of multicaulisin (Mu), sanggenon G (SG) and albanin G (AG) at 1 × MIC (alone concentration) **a**–**g**[a], and (1–4) × MIC (alone concentrations) of Mu and SG **H**[b], respectively, against MA07, a clinical methicillin-resistant *Staphylococcus aureus* strains of SCCmec III type. Results are mean of triplicate samples. [a]$\Delta LC_{24} = LC_{24Md} - LC_{24co}$ (e.g., in **f** AG + Ak showed synergy as $\Delta LC_{24} = 4.11$, corresponding to the value in Table 4). [b]The trend of the fold lines in **H** indicates that as the concentration of Mu and SG increases, the CFU/mL value of LC_{24} gradually approaches zero, i.e., the time-kill effects following the order of Mu < SG < 2Mu < 4Mu < 2SG < 4SG

The broad-spectrum antibiotics of aminoglycoside suffer from issues of nephrotoxicity and ototoxicity. We also noted that Table 2C entitled "Zone Diameter and MIC Breakpoints for *Staphylococcus* spp." in the 28th CLSI Performance Standards only retained the gentamicin breakpoint (Table 3a) (CLSI 2018). Even so, in the last decade, the field of aminoglycoside antibiotics has experienced a renewed interest among the scientific community and efforts were made to counter the resistance and toxicity associated with these drugs. The reversal of MRSA resistances herein might add this interest in the renaissance to aminoglycoside (Fair and Tor 2014; Chandrika and Garneautsodikova 2016; Krause et al. 2016). In cases where aminoglycosides are required to be used, it is vital for controlling infection with a smaller dosage, highlighting the importance of which DAAs are used in combination with such antibacterial agents. In the TCM treatment, a crude drug is usually not used individually. Multiple crude drugs employed in a TCM decoction may produce synergistic effects of increased potency and decreased toxicity, similar to the synergism in the present research, i.e., the adverse effects of either the DAAs or the antibiotics would be reduced when they are used in combination attributable to the reduced dosage.

Therefore, the future work is necessary to perform in-depth studies on the in vivo efficacy. It is warrant to enrich more amounts of DAAs **1**–**3** and to isolate additional DAAs to explore the potential mechanisms and other pharmacological aspects, including evaluation of more antibacterial agents showing synergy on the compounds or their synthetic derivatives both with effectiveness but less toxicity.

Conclusion

Our study details DAAs, i.e., multicaulisin (**1**), sanggenon G (**2**) and albanin G (**3**) as the potent in vitro anti-MRSA constitution in the root barks of *M. alba* (SBP) extract, justifying the effects of DAAs **1**–**3** on synergism with conventional antibacterial agents. The data were the first time presented so far for the synergistic anti-MRSA effects of the novel chemical entities of DAAs **1**–**3** on three conventional antibacterial agents of aminoglycosides amikacin, etimicin, gentamicin, and a floroquinolone (levofloxacin), and the reversal of MRSA resistance to the aminoglycosides, especially amikacin. The results could be beneficial for developing new anti-infectious drugs or synergists

against MRSA and warrant further studies on mechanisms of action and in vivo pharmacological efficacy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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