

# In vitro Antimicrobial Activity and the Mechanism of Berberine Against Methicillin-Resistant *Staphylococcus aureus* Isolated from Bloodstream Infection Patients

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**Purpose:** To investigate the antimicrobial activity of berberine and the mechanism by which it combats methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from patients with bloodstream infections.

**Methods:** Fifteen clinical MRSA isolates were collected, and their Multi-locus Sequence Types (MLST) were examined. The minimum inhibitory concentration (MIC) and combined antibacterial activity of berberine alone, and when combined with clindamycin and rifampicin separately, were determined. Additionally, two MRSA strains (ST239 and ST5) were selected to perform the time-killing assay and biofilm formation test. Cell wall alterations and cell membrane integrity were measured by confocal laser scanning microscopy (CLSM) and electron microscopy to assess the influence on cell morphology.

**Results:** Our data showed berberine was effective against MRSA at MIC values varying from 256 to 64 mg·L<sup>-1</sup> for different MLST types. Berberine alone, and when combined with clindamycin and rifampicin separately, displayed excellent antibacterial activity which reduced the bacterial counts by 2lgCFU·mL within 24h and significantly weakened biofilm formation compared with control strain. Additionally, bacterial cytological profiling indicates that berberine destroyed the structure of the cell walls, membrane integrity and further changed the cell morphology with concentration increased.

**Conclusion:** In our study, berberine has excellent anti-MRSA activities and has synergistic antibacterial property when combined with clindamycin and rifampicin separately, and the mechanism of activities involves the destruction of cell wall and membrane.

**Keywords:** methicillin-resistant *Staphylococcus aureus*, berberine, multi-locus sequence types, synergistic effects, biofilm, mechanism

## Introduction

*Staphylococcus aureus* (*S. aureus*), a major pathogen of healthcare-associated infections, causes many infectious diseases in hospital and community (eg, pneumonia, endocarditis, and bacteremia).<sup>1</sup> Due to its well-known exotoxins and ability to form biofilms,<sup>2</sup> the *S. aureus* infections are particularly difficult to treat. Furthermore, *S. aureus* rapidly develops resistance to the  $\beta$ -lactams antibiotics via the acquisition of the staphylococcal chromosome cassette carrying *mecA* gene.<sup>3–5</sup> The Methicillin-resistance *S. aureus* (MRSA) has become one of the most lethal pathogen in bacteremia. The case fatality rate of MRSA bacteremia has been reported to be up to 27.3%, with a mortality rate approximately 2–3

times higher than those infected with methicillin-susceptible *S. aureus*.<sup>6</sup> Although vancomycin remains the most effective antibiotic currently, the treatment of MRSA infections is still challenging due to the dramatic increase in MRSA isolation and the limited clinical treatment options.<sup>7,8</sup> Thus, there is a strong need to develop novel antibiotics for the treatment of MRSA.

Berberine (BER) is an isoquinoline quaternary alkaloid (5,6-dihydrodibenzo quinolizinium derivative) isolated from many medicinal plants, such as *Hydrastis canadensis*, *Berberis aristata*, *Coptis chinensis*, *Coptis rhizome*, *Coptis japonica*, *Phellodendron amurense*, and *Phellodendron chinense schneid*.<sup>9</sup> BER is used to treat diarrhea and bowel disorders in China, with the potential to be used for the treatment of many diseases.<sup>10</sup> Furthermore, BER has received extensive attention for its role as an antimicrobial,<sup>11–13</sup> antifungal,<sup>14</sup> anti-tumor,<sup>15,16</sup> and anti-diabetes drug.<sup>17</sup> However, the anti-MRSA mechanism of BER is still unclear.

Therefore, the current study evaluated the effects of BER on MRSA using the minimum inhibitory concentration (MIC) and time-killing test. Moreover, the potential mechanism of BER's activities on MRSA, including biofilm formation, cell membrane integrity, and cell morphology variation were investigated. We also explored the synergistic effect of BER combined with rifampicin and clindamycin against MRSA in vitro.

## Materials and Methods

### Bacterial Collection

Fifteen MRSA isolates were collected from blood cultures between March and September 2021 at Beijing Friendship Hospital, affiliated with the Capital Medical University of China. After collection, the bacteria were labeled A–O and stored in skim milk at  $-80^{\circ}\text{C}$  for further use. Subsequently, American Type Culture Collection (ATCC) 43300 was selected as the control strain. Bacteria were routinely grown in a Columbia blood plate (Thermo Fisher Scientific, Waltham, MA, USA) at  $37^{\circ}\text{C}$  for 18h unless otherwise specified.

### Minimum Inhibitory Concentration Assay

The minimum inhibitory concentration (MIC) of each antibacterial agents is defined as the lowest concentration of the drug that resulted in no visible growth.<sup>18</sup> Berberine (BER), clindamycin (CLI), and rifampicin (RIF) were acquired from the National Institutes for Food and Drug Control (Beijing, China). The MICs of BER were determined based on the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, PA, USA). Mueller-Hinton agar (MHA, OXOID) was mixed with BER in dishes, where the final concentration of BER was 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048  $\text{mg}\cdot\text{L}^{-1}$ . The MICs of antibiotics, including RIF and CLI, against MRSA strains were determined using a standard broth microdilution method recommended by the CLSI.<sup>19</sup> Cation adjusted Mueller-Hinton broth (CAMHB, OXOID) was mixed with RIF and CLI separately, where the final concentration of RIF and CLI was twofold series dilution concentration from 0.015625 to 2048  $\text{mg}\cdot\text{L}^{-1}$  in triplicate. Then, the tested bacterial were spotted on the medium and incubated at  $37^{\circ}\text{C}$  for 18h. All experiments were conducted at least three times on different days, and the most consistent results were presented.

### MLST Determination

MLST was determined by the PCR amplification of seven housekeeper genes (*aro*, *arc*, *glp*, *gmk*, *pta*, *tpi*, and *yqi*), according to the conditions published in <https://pubmlst.org/>. The PCR products were bidirectionally sequenced, and all sequences in the online database were analyzed to determine the allele number and respective ST types.

### Synergistic Effects on Checkerboard Assay

Checkerboard assays were used to determine the interactions between BER and antibiotics on the tested MRSA strains, as recommended by the CLSI guidelines. The concentration gradients of BER and antibiotics were selected based on the MICs. After MRSA cells were cultured in CAMHB medium with continuous shaking at  $37^{\circ}\text{C}$ , the cells were grown to 0.5McF ( $1.5\times 10^8$  colony forming unit $\cdot\text{mL}^{-1}$ , CFU $\cdot\text{mL}^{-1}$ ); then, the cultures were diluted 32-fold with sterile broth (approximately  $5\times 10^6$ CFU $\cdot\text{mL}^{-1}$ ). Delivering 10 $\mu\text{L}$  aliquots of cultures to 96-well plates containing 100 $\mu\text{L}$  of two-fold

diluted BER and antibiotics in cation-adjusted Mueller Hinton broth, where the final concentration of bacterial culture was approximately  $5 \times 10^5$  CFU\* $\text{mL}^{-1}$ .<sup>20</sup> The plates were incubated for 18 h at 37°C. Finally, each well was examined for signs of MRSA growth. For each interaction, the fractional inhibitory concentration (FIC) was calculated for each agent: synergism, antagonism, and indifference correlated with  $\text{FIC} \leq 0.5$ ,  $\text{FIC} > 4$ , and  $0.5 < \text{FIC} \leq 4$ , respectively.<sup>21</sup>

## Time-Killing Curves

Growth kinetics were used to assess the antimicrobial effect of BER against MRSA and sensitization to other antibiotics.<sup>22</sup> The tested MRSA strain was selected based on MLST types. The experiments were performed using CAMHB in 10mL with an initial inoculum of  $5 \times 10^5$  CFU/mL. The cultures were transferred to tubes with individual compounds and in combination ( $2 \times \text{MIC}$ ,  $1 \times \text{MIC}$ ,  $1/2 \times \text{MIC}$ ,  $1/4 \times \text{MIC}$  of BER,  $1/8 \times \text{MIC}$ ,  $1/32 \times \text{MIC}$  of RIF, or  $1/4 \times \text{MIC}$  of CLI) treated and incubated. The concentration of BER and antibiotics were chosen based on the MIC assay and synergistic effects assay. A kinetic growth or inactivation curve was constructed for each treatment using the viable cell counts performed at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24h of incubation at 37°C. The cultures containing 1% DMSO and bacterial inoculum served as the bacterial DMSO control (DC) and growth control (GC) separately.

## Biofilm Formation

The effect of BER on MRSA biofilm formation was tested. Static biofilm formation assay was performed in 96-well microtiter plates, as previously reported.<sup>23</sup> Delivering approximately  $5 \times 10^5$  CFU\* $\text{mL}^{-1}$  of MRSA cells to 96-well plates containing diluted BER and antibiotics in brain heart infusion broth (BHI, OXIOD) supplemented with 0.5% glucose.<sup>21</sup> Wells containing only the BHI were used as the negative control, whereas wells containing bacteria grown in BHI were used as the positive control. The suspension was transferred to 96-well plates with individual compounds and combinations ( $4 \times \text{MIC}$ ,  $2 \times \text{MIC}$ ,  $1 \times \text{MIC}$ ,  $1/2 \times \text{MIC}$ ,  $1/4 \times \text{MIC}$  of BER,  $1/16 \times \text{MIC}$ ,  $1/32 \times \text{MIC}$  of RIF, or  $1/4 \times \text{MIC}$  of CLI) treated and incubated for 18 h at 37°C. The concentrations of BER, RIF, and CLI were selected based on the previous MIC assays and synergistic effects assays. The tested MRSA strain was selected based on the MLST types, consistent with kinetics assay. After incubation, each well was washed three times with PBS to remove the planktonic cells, fixed for 15 min with methanol to stabilize biofilm, stained for 5 min with crystal violet (0.5% in methanol), and washed two times again with deionized water. The adhering dye was dissolved in 200 $\mu\text{L}$  of 33% acetic acid and the absorbance was measured at  $\text{OD}_{600}$  to quantify biofilm formation.

## Cell Membrane Integrity

Cell membrane permeability of MRSA cells was assessed by confocal laser scanning microscopy (CLSM) (TCS-SP5, Leica, GER), similar to the previous description.<sup>24</sup> To test membrane integrity, fluorescence images were acquired using fluorochrome, which were carboxyfluorescein diacetate (CFDA SE; Sigma-Aldrich, St. Louis, MO, USA) and propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) at final concentrations of 10  $\text{mmol} \cdot \text{L}^{-1}$  and 5  $\text{mg} \cdot \text{L}^{-1}$ , separately. CFDA SE is a nonfluorescent precursor that only passing through intact cell membranes, producing a fluorescent compound. When the cell membrane is damaged, PI can enter the cells and bind to the DNA to form a red fluorescent DNA-complex.<sup>25</sup> *S. aureus* are gram-positive cocci with a cytoderm structure. However, cytomembrane was essential to maintain cell function and prevent cytoplasmic leakage. Fluorescent staining was used to examine cell membrane integrity as previously reported.<sup>24</sup> After MRSA cells were grown to an approximately  $1.5 \times 10^8$  CFU\* $\text{mL}^{-1}$  in CAMHB at 37°C, delivering 10 mL of volume cultures to tubes. Then, the cells were treated with BER at  $0 \times \text{MIC}$ ,  $1 \times \text{MIC}$ , or  $2 \times \text{MIC}$  for 4 h, centrifuged at 5000 $\times g$  for 5 min, and resuspended with equivalent volumes of 0.9% NaCl. After incubation with cFDA SE for 15 min and PI for 1 min at room temperature away from light, the suspension was fixed on a glass slide using agar. Finally, the bacteria were examined by confocal laser scanning microscopy (CLSM, OLYMPUS, FV1000, Japan) at the excitation-emission wavelengths of 530nm for PI and 488nm for CFDA SE.

## Cell Morphology by Electron Microscopy

Field emission scanning electron microscopy (FESEM, Joel JSM-7900F, Japan) was used to detect morphological changes after BER treatment, according to the published protocols.<sup>26</sup> Subsequently, approximately  $1.5 \times 10^8$  CFU\* $\text{mL}^{-1}$

of MRSA cells were incubated in CAMHB of 10mL volume supplemented with BER at  $0 \times \text{MIC}$ ,  $1 \times \text{MIC}$ ,  $2 \times \text{MIC}$ , and  $4 \times \text{MIC}$  for 4 h at  $37^\circ\text{C}$ . The treated cells were washed with PBS three times and fixed with 2.5% glutaraldehyde for 4 h and 1% osmic acid for 2 h. Then, the cells were washed with PBS again. After dehydrating with different concentrations of ethanol, the cells were fixed on silicon slices, sputter-coated with gold under vacuum, and observed using FESEM.

## Statistical Analysis

Assays were conducted with at least two biological replications. The Kruskal–Wallis analysis was used to determine the differences between the multiple groups. Data are expressed as mean  $\pm$  SD, and p-values  $<0.05$  are considered statistically significant. No adjustments were made for the p-values reported in this study.

## Results

### MLST Determination, MICs, and FIC Index

The MLST determination showed 15 MRSA clinical isolates and distinguished them into five subtypes. The MIC values of BER varying from 256 to  $64 \text{ mg} \cdot \text{L}^{-1}$  (Table 1); however, among ST5 subtypes, only one strain had a MIC significantly different from the others (ie,  $>2048 \text{ mg} \cdot \text{L}^{-1}$ ). Excluding this strain, the highest MIC value was  $256 \text{ mg} \cdot \text{L}^{-1}$  for ATCC 43300, and the lowest MIC value was  $64 \text{ mg} \cdot \text{L}^{-1}$  for ST239 and ST5. Six clinical isolates were resistant to CLI, and eight strains were susceptible, whereas 11 isolates were resistant to RIF, and three isolates were susceptible. The MRSA control strain was resistant to CLI at MIC of  $1024 \text{ mg} \cdot \text{L}^{-1}$  and susceptible to RIF at MIC of  $0.06 \text{ mg} \cdot \text{L}^{-1}$  (Table 2). Using the checkerboard assay, BER in combination with RIF and CLI showed a significant effect against the MRSA strain, leading to a reduction in MIC values. Synergistic activity was observed for the combination of BER with CLI or RIF with FIC index values of 0.19–0.5 (Table 2). However, two isolates showed indifferent effects compared to others when BER was combined with CLI, and one isolate showed indifferent effects when BER was combined with RIF (Table 3).

### Time-Killing Curves

The time-killing curves for BER alone and in combination with RIF or CLI against ATCC 43300 (control strain) and clinical isolate ST239 are presented in Figure 1. Growth cultures without BER treatment increased to  $7 \text{lg} (\text{CFU} \cdot \text{mL}^{-1})$  for the control strain and  $9 \text{lg} (\text{CFU} \cdot \text{mL}^{-1})$  at ST239 after 24 h of incubation. The DMSO control group showed similar results to the growth cultures, suggesting that DMSO does not affect the growth of cells. Furthermore, BER at its MIC value showed dramatic inhibition of bacterial growth during 24 h of incubation, and showed stronger inhibition at  $2 \times \text{MIC}$  than at MIC after 12 h of incubation with similar bacterial growth compared to that at MIC after 24 h of incubation (reduction of  $2 \text{lg} (\text{CFU} \cdot \text{mL}^{-1})$  and  $1 \text{lg} (\text{CFU} \cdot \text{mL}^{-1})$  for ATCC 43300 and ST239, separately). BER at sub-MIC had a weak effect on the growth of the MRSA strain over 24 h; however, bacterial growth was positive (Figure 1A and D). Importantly, the combination of BER with CLI showed a synergistic effect against the MRSA control strain and clinical isolate ST239 with  $\sim 2 \text{lg} (\text{CFU} \cdot \text{mL}^{-1})$  killing compared to the single drug applied during 24 h (Figure 1B and E). In

**Table 1** The MIC Value of Berberine Among Different MLST Types of MRSA

MLST Type	Labels	MIC ( $\mu\text{g}/\text{mL}$ )
ST239	A, B, D, E, H, L, M, O	128.64
ST5	C, G, I	64, 128, $>2048^*$
ST2123	F	128
ST344	J, K	128
ST59	N	128
ST39	Control	256

**Notes:** \*Berberine can not be dissolved completely at this high concentration ( $>2048 \text{ mg} \cdot \text{L}^{-1}$ ).

**Table 2** MIC and FIC Index of CLI and RIF in the Presence of BER Against MRSA Strains

Labels	MIC( $\mu\text{g/mL}$ )		FIC	MIC( $\mu\text{g/mL}$ )		FIC
	CLI	CLI +BER		RIF	RIF+BER	
A	0.50	0.02	0.28	128.00	8.00	0.31
B	0.50	0.13	0.75	256.00	8.00	0.28
C	0.06	0.03	0.75	0.13	0.00	0.52
D	1024.00	256.00	0.50	256.00	8.00	0.28
E	1024.00	32.00	0.16	256.00	8.00	0.28
F	0.13	0.02	0.38	256.00	8.00	0.28
O	512.00	64.00	0.38	0.13	0.00	0.27
H	0.25	0.02	0.31	256.00	8.00	0.28
J	0.13	0.02	0.38	256.00	8.00	0.28
K	512.00	32.00	0.31	256.00	8.00	0.28
L	512.00	32.00	0.31	256.00	16.00	0.31
M	0.25	0.02	0.31	256.00	8.00	0.28
N	512.00	32.00	0.19	0.06	0.00	0.27
O	0.25	0.02	0.19	256.00	8.00	0.31
Control	1024.00	32.00	0.28	0.06	0.00	0.28

addition, the combination of BER with RIF showed more remarkable synergistic effects against MRSA than those of BER and CLI. As such, the combination of BER with RIF resulted in a  $> 2\text{L g (CFU}\cdot\text{mL}^{-1})$  inhibition compared to the combination of BER with CLI at 24 h (Figure 1C and F).

## Effects of BER Alone and in Combination with RIF or CLI on Biofilm Formation

According to the assay of biofilm formation, BER possessed an excellent inhibitory effect on the biofilm in a dose-dependent manner, especially at the MIC or higher concentrations, which was more prominent than that of the sub-MIC and control groups. Interestingly, biofilm formation sharply increased at sub-MIC in the ST 239 strain (Figure 2). In addition, the combination of BER with RIF or CLI at sub-MIC significantly inhibited biofilm formation compared to each drug separately in the control strain and clinical isolate (Figure 3). The percentages of biofilm reduction, which were compared with positive control in each group, are presented at Table 4.

## Cell Membrane Integrity

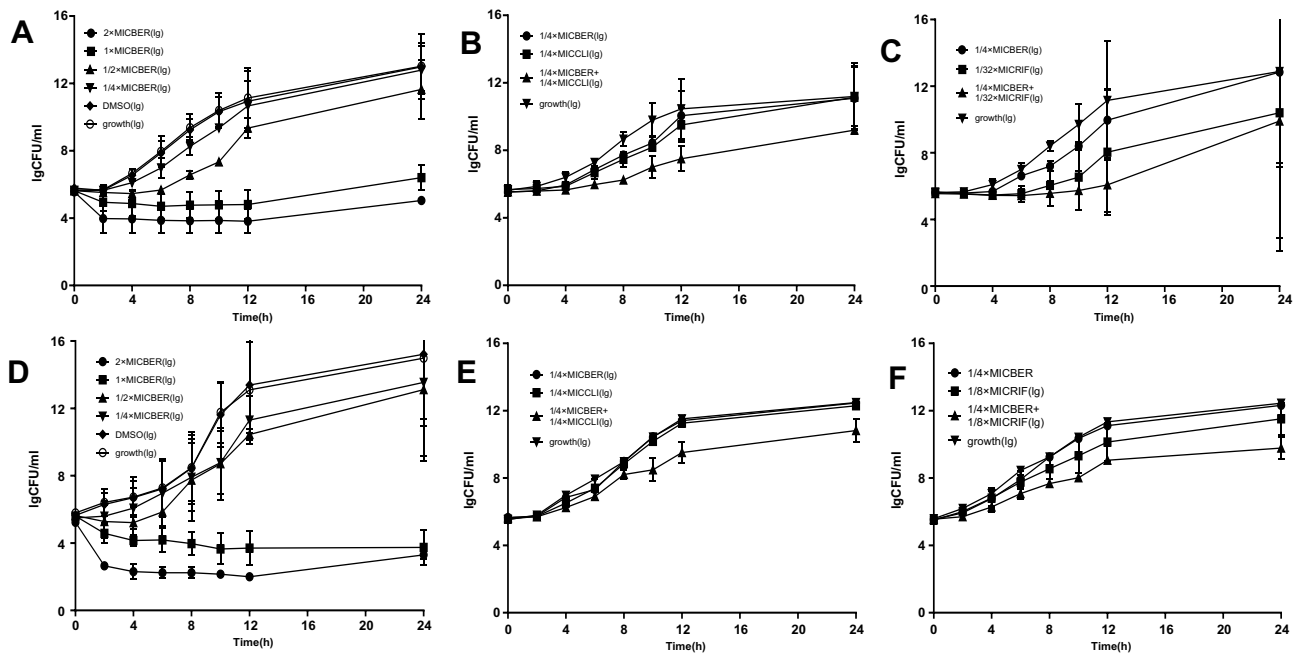
According to the images, untreated MRSA cells emitted green fluorescence, showing that the cell membrane was intact. Conversely, BER-treated MRSA appeared red and the proportion of red fluorescence increased with increasing BER concentrations, suggesting a damaged cell membrane (Figure 4). Meanwhile, the total number of cells declined after BER treatment, and the reduction was proportional to the BER concentration.

## Alteration in Morphology

The FESEM images illustrated significant changes in the morphology of MRSA cells with BER (Figure 5) at low magnification (10,000–15,000 $\times$ ) and high magnification (45,000–50,000 $\times$ ). Untreated control *S. aureus* exhibited arrangement in clusters and smooth surface and spherical contour at 10,000 $\times$  and 50,000 $\times$ , separately (Figure 5A and E). However, the

**Table 3** The Antibacterial Activity of BER in Combination with CLI and RIF Against MRSA Strains (% , n/N)

Antibiotics	Synergism	Indifferent	Antagonism	Unknown
CLI	80.0 (12/15)	13.3 (2/15)	0 (0/15)	6.7 (1/15)
RIF	86.7 (13/15)	6.7 (1/15)	0 (0/15)	6.7 (1/15)

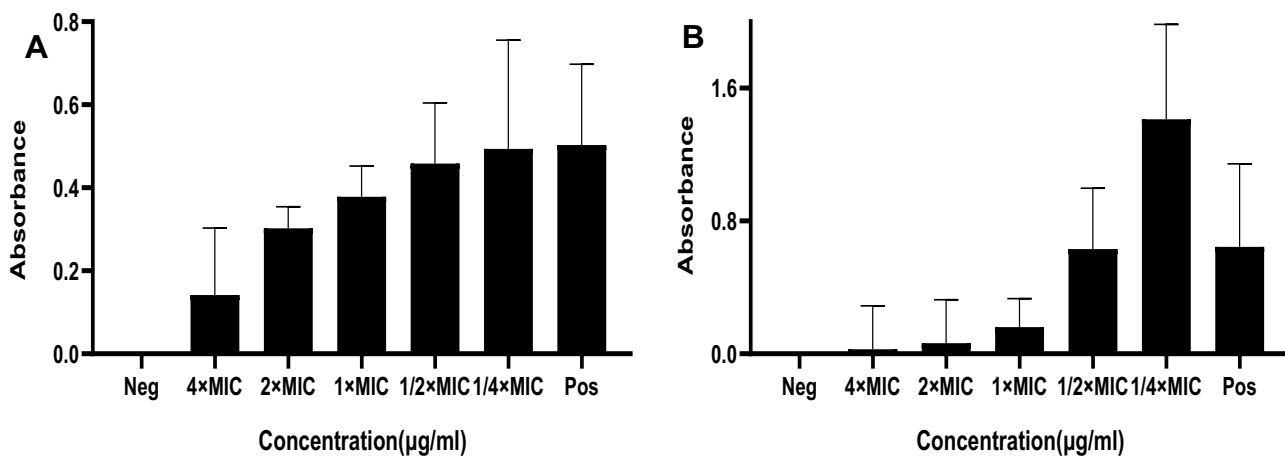


**Figure 1** The time-killing curves for BER alone and in combination with RIF or CLI against MRSA strains; (A–C): Control (D–F): ST239. The data are the mean  $\pm$ SD of colony counts at least two biological replicates in each group.

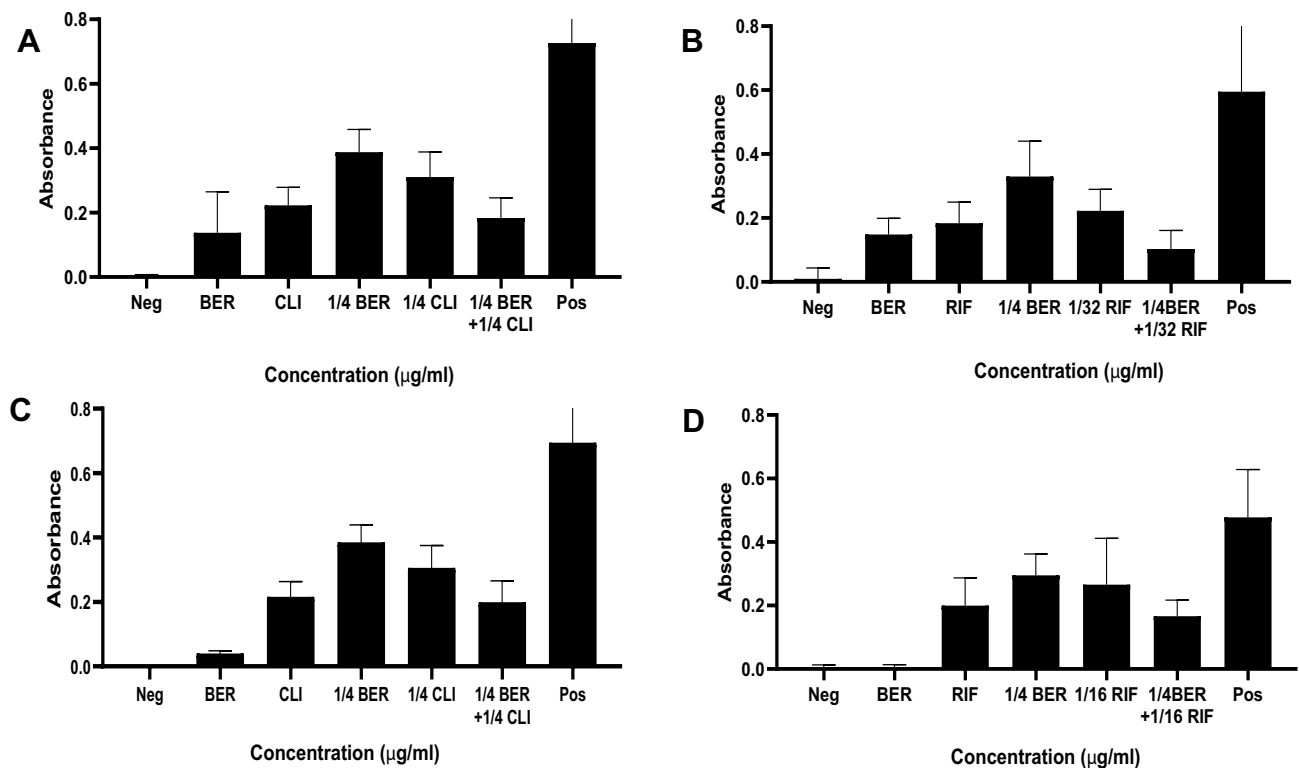
number of MRSA cells in the field of vision decreased and the morphology of the clusters was changed with increasing BER concentrations (Figure 5B–D). Remarkably, numerous flocculent substances were adhering to the cells that were not significantly cleaned by PBS. These flocculent substances increased with the increase in BER concentration compared to the untreated control. Furthermore, the cells were less uniform in size and had a rough surface (50,000 $\times$ ) compared to the untreated cells. When treated with MIC or higher concentrations of BER, the cell morphology exhibited shrinkage and deformation (Figure 5F); some cells even had a defective and punched surface, suggesting that the content was released (Figure 5G and H).

### Discussion

The dramatic increase and global spread of MRSA strains pose a serious threat to human health since it was first identified in 1960.<sup>27</sup> Vancomycin and daptomycin are recommended as the first-line antibiotics by the 2011 Infectious



**Figure 2** The effects of BER alone on biofilm formation of MRSA strains. (A) Control (B) ST 239; relative biofilm formation levels were represented as mean $\pm$ SD of at least two biological replicates; significant difference was determined at  $P < 0.0001$  with comparison between the groups.



**Figure 3** The effects of BER in combination with RIF or CLI on biofilm formation of MRSA strains. (**A** and **B**): Control (**C** and **D**): ST 239; relative biofilm formation levels were represented as mean±SD of at least two biological replicates; significant difference was determined at  $P < 0.0001$  with comparison between the groups.

Diseases Society of America guidelines for MRSA.<sup>28</sup> However, each antimicrobial agent has limitations<sup>29</sup> related to its effectiveness against bacteria<sup>30</sup> or its increasing resistance.<sup>31,32</sup> What is more, the emergence of vancomycin-resistant *S. aureus* leaves physicians with limited therapeutic options. Therefore, there is an urgent need in the development of new therapeutics to aid in the treatment of infectious diseases caused by MRSA. BER, an alkaloid extracted from a traditional Chinese herb, has emerged as a promising antibacterial agent.

In the current study, we observed that BER had anti-MRSA activities at MIC of 64–256 mg\*L<sup>-1</sup>, which related to the subtype of MRSA strains. As previously reported, subtype strains within the same bacterial species may have different clinical uses.<sup>2</sup> In this study, 15 strains were divided into five subtypes. We found that the MIC value of BER differed among various MLST types, and many strains showed diverse BER MIC values even in the same subtype. The MIC showed three values for ST5 and two values for ST239, unlike what has been reported in previous studies.<sup>2</sup> In this study, a subtype was matched with a MIC value. However, the MIC value of ST239 and ST39 in our study which was 64 or 128 mg\*L<sup>-1</sup> and 256mg\*L<sup>-1</sup>, was different with this study (64 mg\*L<sup>-1</sup> and 512 mg\*L<sup>-1</sup>).<sup>2</sup> Therefore, we recommend that the types of MLST should be expanded to verify the results.

In the current investigation, a synergistic effect of BER with CLI and RIF was observed against MRSA. As such, BER can markedly reduce the MIC value of antibacterial agents against MRSA. A synergistic effect of BER has also been reported in combination with oxacillin,<sup>33</sup> azithromycin,<sup>34</sup> and levofloxacin,<sup>34</sup> whereas an additive effect has been reported for BER in combination with ampicillin<sup>33,34</sup> and cefazolin.<sup>34</sup> This study is the first one to report the synergistic effects of BER in combination with CLI and RIF.

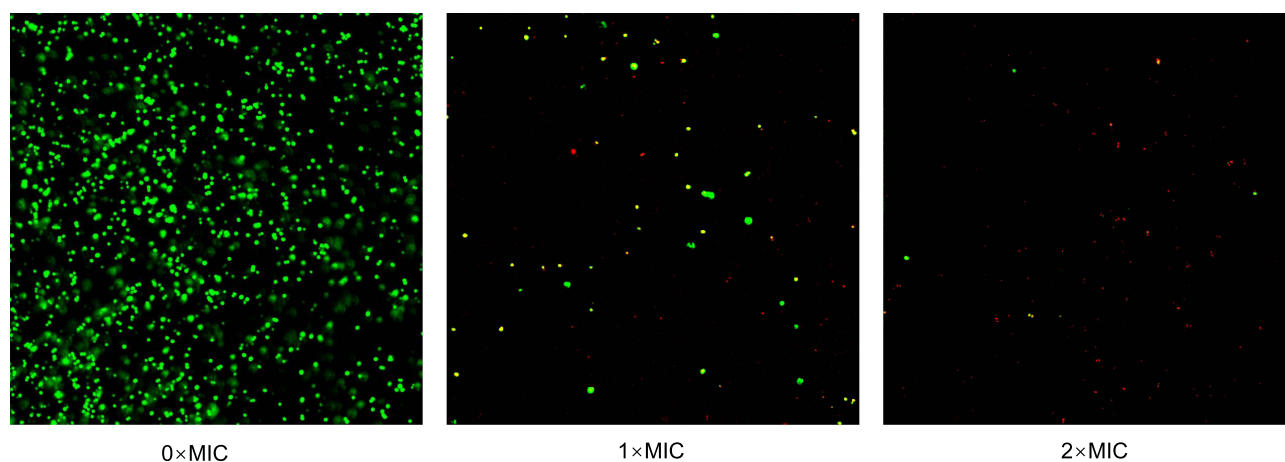
The time-killing assays showed similar results. The time-killing test of the control strains, the strain ST239 for BER alone, and the combination of BER with CLI or RIF separately showed significant growth inhibition. The inhibition of growth by BER alone strengthened with the increasing concentration. Notably, the test strain ST239 and the control strain showed attenuation of inhibition with the increase in curve after 12 h, suggesting that BER alone may not kill all the cells, but it maintains the bacteria at low levels when the BER gradually expands. When this occurs, the remaining cells

**Table 4** The Percentage of Biofilm Formation Inhibited

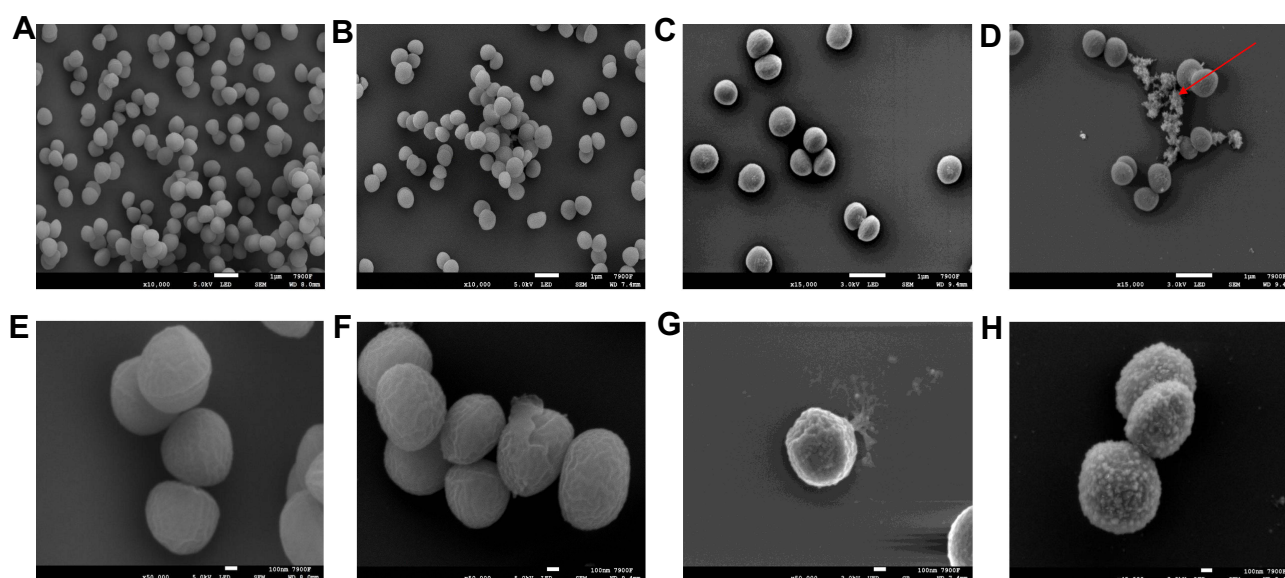
Strains	BER Alone					BER in Combination with CLI					BER in Combination with RIF				
	4×MIC BER	2×MIC BER	1×MIC BER	1/2×MIC BER	1/4×MIC BER	BER	CLI	1/4 BER	1/4 CLI	1/4 BER + 1/4 CLI	BER	RIF	1/4 BER	1/32 RIF	1/4BER +1/32 RIF (1/16 RIF)
Control	71.80%	39.84%	24.73%	8.81%	1.80%	81.09%	69.33%	46.58%	57.26%	74.75%	75.11%	69.17%	44.64%	62.64%	82.67%
ST239	95.65%	89.92%	75.03%	2.04%	-119.20%	94.48%	69.09%	44.69%	56.07%	71.46%	99.06%	58.17%	38.19%	44.36%	65.24%

**Notes:** The percentage of inhibited was that biofilm formation compared with positive control in each group and was calculated using the mean.





**Figure 4** The effect of BER on cell membrane integrity of MRSA strain by confocal laser scanning microscopy.



**Figure 5** The changes in the morphology of MRSA cells with BER by field emission scanning electron microscope. (A and E): 0xMIC; (B and F): 1xMIC; (C and G): 2xMIC; (D and H): 4xMIC. Shown by the arrow is flocculent substances.

are reduced, which is similar to the results of a previous study on the antimicrobial activity of a Chinese herb.<sup>35</sup> Therefore, there is a significant effect of BER concentration on its antimicrobial activity, suggesting that BER must maintain a persistently high blood concentration. In addition, the combined treatment resulted in obvious reduction in the cell quantity within 8–24 h of incubation in the control strain and 10–24 h of incubation in ST239, compared to CLI or RIF separately. These results suggest that BER has excellent anti-MRSA activity, which is enhanced when combined with other antibiotics, such as CLI and RIF.<sup>33,34,36–39</sup>

Biofilm formation plays an essential role in the persistence of pathogens.<sup>34,40</sup> Biofilms harbor bacteria that are irreversibly attached to cell surfaces and prevent antibiotic exposure.<sup>41</sup> In light of this, BER showed excellent inhibition of biofilm formation with increasing concentrations, similar to previous studies.<sup>2,42</sup> However, biofilm formation increased at sub-MIC in the ST 239 strain in our study, similar to other studies.<sup>2</sup> The concentration of BER cannot increase slowly when treating MRSA infections, because low concentration of BER may promote biofilm formation. Importantly, BER, in combination with CLI or RIF separately, also inhibited biofilm formation better than either drug alone. These data are consistent with previous studies that suggest the existence of synergistic effects of BER with those of CLI and RIF

against MRSA. Therefore, antibacterial activity and anti-biofilm formation character of BER can be used as the basis of clinical practice and the synergistic effect of BER with CLI and RIF can be used as the alternative treatment when the bacterial infection was difficult to treat. However, the specific mechanism underlying the effects of BER in combination with CLI or RIF separately remains unclear and should be explored in future studies.

Morphological analysis was used to explore the mechanism of the BER effect against MRSA. In the current study, we observed cell wall alteration and its punched appearance due to BER exposure at high magnification. Flocculent substances were observed to adhere to the cells. Zhang et al found that BER could damage the cell surface of MRSA.<sup>43</sup> Therefore, it is reasonable to speculate that the substances may include the contents of punched bacterial cells due to BER treatment. This observation is consistent with the above findings, illustrating that BER can destroy MRSA cell walls in a concentration-dependent manner.

The cytoplasmic membrane is an important dynamic structure that allows cells to interact with other cells and environmental molecules.<sup>44</sup> While the cytoplasmic membrane integrity of MRSA has not been studied previously, the function of the gram-positive bacterial membrane is essential for subsisting membrane proteins and transportation.<sup>45</sup> As assessed by CLSM, our results showed that the integrity of the MRSA cytoplasmic membrane was compromised at increased BER concentrations. This finding showed that BER possessed anti-MRSA activities by damaging the cell wall and cytoplasmic membrane to perturb cell functions and release intracellular contents. Although BER could exert an influence on the cell wall and cytoplasmic membrane, the mechanism here is unclear and needs to be investigated further. Meanwhile, combination antibacterial assays would verify the hypothesis that BER could damage the cytoplasmic membrane to kill MRSA. Because CLI and RIF play antibacterial roles inside the cells,<sup>46,47</sup> they may easily enter the cells in the presence of BER.

## Conclusions

The current study demonstrated that BER has remarkable antimicrobial effects against MRSA with favorable MIC. It can also be used as an adjuvant to RIF or CLI to treat MRSA infections. BER alone and in combination with RIF or CLI reduces biofilm formation. In addition, BER significantly damages the cell wall and membrane of MRSA to kill the bacteria. This study provides novel mechanistic insight into supporting the development of BER as an antibacterial drug.

## Data Sharing Statement

The datasets generated for this study are available from the corresponding author Miaorong Xie.

## Ethics Statement

This study was approved by the Ethics Committee of Beijing Friendship hospital, Capital Medical University (20210208). Adult patients wrote the informed consent and a parent or legal guidance of patients under 18 years of age provided informed consent prior to the experiment described below. This study was conducted in accordance with the Declaration of Helsinki.

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## Disclosure

The authors declare no conflicts of interest for this study.

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