***Boesenbergia rotunda* (L.) Mansf*.* extract potentiate the antibacterial activity of some β-lactams against β-lactam-resistant Staphylococci**

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**ABSTRACT**

**Objective:** The purpose of this study was to investigate the effect of *Boesenbergia rotunda* (L.) Mansf. Extract (BRE) and peptidoglycan inhibitor antibiotics, either used alone or in combination against β-lactam resistant Staphylococci.

**Methods:** Antibacterial and synergistic activities of BRE alone and in combination with ampicillin (AMP), cloxacillin (CLX), cefazolin (CFZ) or vancomycin (VAN) were evaluated against two β-lactam-resistant *Staphylococcus aureus* (BRSA) and one β-lactam-resistant *Staphylococcus epidermidis* (BRSE). The activities were confirmed by killing curve assays. The preliminary antimicrobial action was elucidated by transmission electron microscopy (TEM) and cytoplasmic membrane (CM) permeability.

**Results:** All tested Staphylococci were inhibited by BRE at a MIC of 16 µg/mL. Two BRSA strains showed high resistance to CLX, AMP, and CFZ, while BRSE was resistant to CLX and AMP. All tested isolates remained susceptible to VAN. The chequerboard assay demonstrated the fractional inhibitory concentration index (FICI) at 0.502 of the BRE and CLX combination against the BRSA strains. Killing curve determinations confirmed the antibacterial and synergistic activities. The TEM study revealed collapse of the CM in BRE-treated cells and damage of both CM and peptidoglycan in BRE plus CLX-treated cells. The CM permeability assay showed that either BRE or nisin alone, and BRE plus CLX significantly induced leakage of OD260-absorbing materials.

**Conclusions:** The BRE potentiated β-lactams, particularly CLX against β-lactam-resistant Staphylococci by damaging the CM and peptidoglycan layer, leading to leakage of intracellular materials. The combination of BRE and β-lactams provides a potential way forward in developing a novel antistaphylococcal agent.

*Keywords: Boesenbergia rotunda* (L.) Mansf*.* extract; β-lactams; Staphylococci; cytoplasmic membrane

**1. Introduction**

 Resistance to penicillin antibiotics in *S. aureus* isolates has been reported to be approximately 90-95% worldwide [1]. Methicillin-resistant *S. aureus* (MRSA) has emerged to be one of the most life-threatening pathogens to human health [2]. In Asia, about 67.4% of MRSA infections are hospital-associated infections, and 25.5% are community-onset infections [3]. Resistance is mediated by expression of an additional transpeptidase enzyme (PBP2a) encoded by the *mecA* gene[4] and production of β-lactamase mediated by the *blaZ* gene [5]. Infections caused by MRSA have higher morbidity and mortality in both community and hospital settings compared with methicillin-sensitive *S. aureus* [6].

 Plant-derived antimicrobials have shown great potential for novel therapeutics [7, 8]. They have also been considered for use in either phytochemical/ phytochemical or phytochemical/antibiotic combinations in the treatment of drug-resistant bacteria [9]. The combination regimen has been explored and studied for combating multidrug-resistant bacteria [10, 11]. *Boesenbergia rotunda* (L.) Mansf. has been widely cultivated and used as a food ingredient and also in folk medicine in several Asian countries. Interestingly, previous findings found that BRE showed no haemolytic activity and displayed IC50 of higher than 4 mg/mL [12]. Furthermore, Saraithong et al. reported that the effect of BRE at 240 mg/KgBW/day on male rats showed no toxic effect on kidney, liver and all haematological parameters [13].With regard to antimicrobial resistance issues, they present an urgent necessity for research and development of novel antibacterial compounds and new strategies to overcome antibiotic-resistant bacteria. To achieve these aims, this study, therefore, investigated the antibacterial activity of BRE and its synergism with cell wall inhibitor antibiotics (ampicillin, cloxacillin, cefazolin, and vancomycin) against β-lactam-resistant Staphylococci. The primary mechanisms of actions, such as cytoplasmic membrane (CM) permeabilisation and cell wall damage were also elucidated in the present study.

**2. Materials and Methods**

*2.1 Bacterial isolates, antibiotics and chemicals*

 Clinical isolates employed included β-lactam-resistant *Staphylococcus aureus* (BRSA) DMST 20651, DMST 20652, and β-lactam-resistant *Staphylococcus epidermidis* (BRSE) DMST 14932. These strains were obtained from the Department of Medical Science, Ministry of Public Health, Thailand. *S. aureus* ATCC 29213 was obtained from the American Type Culture Collection (ATCC) and was used as a reference strain. All antibiotics employed in the present study, including cloxacillin (CLX), vancomycin (VAN), nisin (NIS), ampicillin (AMP), and cefazolin (CFZ) were obtained from Sigma-Aldrich.

*2.2 Plant specimen and extraction procedure*.

 *B. rotunda* was obtained locally from Pakthongchai district, Nakhon Ratchasima province, Thailand. The plant specimen was identified and authenticated by Dr. Paul J. Grote, Suranaree University of Technology, Thailand. A voucher specimen (BKF No. 192160) was deposited at the Forest Herbarium of Thailand. To extract the *B. rotunda*, the rhizomes of *B. rotunda* were washed thoroughly with tap water, dried in a hot air oven (40 °C), and were pulverised. One hundred grammes of the finely ground dried *B. rotunda* were macerated with 500 ml of 99.9% ethanol for a week at room temperature. The extracts were filtered, concentrated using a rotatory evaporator and lyophilised to obtain the dry extract.

*2.3 Preliminary phytochemical screening tests*

 Preliminary qualitative phytochemical screening analysis was executed in accordance with previously described literature with slight modifications [14, 15]. The extract was tested for its constituent: alkaloids, tannins, flavonoids, saponins, glycosides, steroids and coumarins.

Total flavonoid content was quantified by the aluminium chloride colourimetric assay following previous reports [16]. Briefly, 50 µL of the extract or quercetin at varying concentrations were added to 10 µL of 10% aluminium chloride accompanied by 150 µL of 95% ethanol. An aliquot of 10 µL of 1 M sodium acetate was then added to the mixture in a 96 well plate. The sample was measured at a wavelength of 415 nm. The total flavonoid content of the extract was quantified by comparing with standard quercetin. The data were expressed in mg quercetin equivalents (QE) per 100 mg BRE.

*2.4 Bacterial suspension standard curves*

 This assay was carried out to select bacterial suspensions with a known viable count following the previous reports with some modifications [17, 18].

*2.5 Minimum inhibitory concentration (MIC) determinations*

 The MIC was determined by a 96-well microtitre plate with resazurin according to the modified method as previously described [19, 20]. Antibiotics were prepared in sterile distilled water, and BRE was prepared with DMSO (final concentration 5% (v/v)). Resazurin was prepared in sterile distilled water to obtain 0.02% (w/v) and sterilised by filtration through a 0.2 μm-pore syringe filter. Each organism was adjusted according to bacterial suspension standard curve. Twenty microlitres of the adjusted organism (5 x 106 CFU/mL) were added to wells containing 20 µl of varying concentrations of BRE or antibiotics plus 140 µL of Mueller-Hinton broth (MHB) and 20 µL of 0.02% resazurin. Wells with 10% and 5% DMSO, but without antibacterial agents and bacteria were also used as the controls. The microplate was then incubated at 37 °C for 20 h. The lowest concentration showing no colourimetric change from blue to pink was considered as the MIC value.

*2.6. Chequerboard determinations*

 The interaction between BRE and the antibiotic combination was evaluated by a chequerboard assay following Bonapace and colleagues [21]. The bacterial culture and antibacterial agents were prepared and performed similarly to the MIC determination. The BRE and antibiotic were combined and incubated at 37 °C for 20 h. The lowest concentration of antibacterial agents in combination showing no visible growth was selected to calculate the fractional inhibitory concentration (FIC). The FIC index (FICI) was calculated to determine drug interaction and interpreted as shown below [22].

FIC index = FICA+FICB =

Conc. of A in MICs of A+B + Conc. of B in MICs of A+B

 MIC of A alone MIC of B alone

Where, FICI ≤ 0.5 denoted synergism; FICI > 0.5 − < 1.0 denoting partial synergism; FICI = 1 denoting addition; FICI > 1 − ≤ 4.0 denoting indifference; FICI > 4.0 denoting antagonism.

*2.7 Killing curve determinations*

 The combination of BRE and CLX that showed the lowest FIC index against BRSA 20651 was selected to confirm antibacterial and synergistic activities. The assay was performed according to a previous report with some modifications [18]. In brief, inocula (5 x 106 CFU/mL) were challenged with BRE alone, CLX alone and in combination at nine distinct exposure times (0, 0.5, 1, 2, 4, 6, 8 and 24 h). Aliquots (0.1 mL) of each exposed time were diluted in 0.9 mL normal saline as appropriate to enumerate 3-50 colonies by the Miles Misra technique. Ten microlitres from each dilution were dropped on MHA medium. Following incubation at 37 °C for 20 h, the growing colonies were counted and then plotted as killing curves. Decreases of ≥ 2 log10 in CFU/mL between the combination and those obtained by the most active agent alone at 24 h were termed synergistic interactions. Additive or indifferent interactions were defined as < 2 log10 CFU/mL reduction. Increases of > 2 log10 in CFU/mL at 24 h were denoted as antagonistic. Also, the bactericidal activity of the antibacterial agents was determined by a reduction in **≥** 3log10 CFU/mL in comparison to the initial inoculum, whereas a reduction in < 3log10 CFU/mL in comparison with the initial inoculum at 24 h was defined as bacteriostatic [23, 24].

*2.8 Transmission electron microscopy (TEM)*

 The samples were prepared following a previous report with slight modifications [25]. Briefly, BRSA (5 x 106 CFU/mL) was grown in antibacterial free (control), BRE alone, antibiotics alone at half-MICs and BRE plus antibiotic at sub-FIC concentrations, to get a final concentration of 5 x 105 CFU/mL, for 4 h. The culture was subsequently pelleted, fixed in 2.5% glutaraldehyde for 12 h, and then washed twice with 0.1 M phosphate buffer. The post-fixation was carried out with 1% osmium tetroxide for 2 h at room temperature. After washing in the buffer, the samples were gently dehydrated with graded acetone solutions (20%, 40%, 60%, 80% and 100%, respectively) for 15 min each. Afterwards, infiltration and embedding were performed using Spurs resin (EMS), the block resin was thin-sectioned and mounted on copper grids. Ultimately, the ultrathin sections were counterstained with 2% (w/v) uranyl acetate for 15 min and then 0.25% (w/v) lead citrate for 15 min. Following staining, the specimens were visualised and images captured with a Tecnai G2 electron microscope (FEI, USA), operating at 100 kV. In addition, the cell area from micrographs was calculated by measuring cell width multiplied by cell length (nm2) to confirm the effects of BRE either used singly or in combination with antibiotic on cell size.

*2.9 Cytoplasmic membrane (CM) permeability*

 The cytoplasmic membrane permeabilisation experiment was executed according to previous reports with some modifications [26]. BRE alone or in combination with antibiotic-induced CM damage resulted in the release of OD260-absorbing material measured using a UV-VIS spectrophotometer. An 18-h culture BRSA was harvested and adjusted in normal saline to give 5 x 106 CFU/mL. Five millilitres of adjusted cultures were added to 45 mL of 2.5 mM sodium HEPES buffer (pH 7.2) supplemented with 100 mM glucose plus BRE or antibiotic alone at half-MIC concentrations or the combination of antibiotic and BRE at sub-FIC concentrations. The flask containing no antibacterial was used as a negative control and with nisin was used as a positive control. The bacterial suspensions were incubated at 37 °C in a shaking water bath. The CM permeability was determined after the contact time of 0, 0.5, 1.0, 2.0, 3.0 and 4.0 h. An OD260 value of UV-absorbing materials released by the cells in the supernatant was measured. All the measurements were carried out in three replicate experiments in a Varian Cary 1E UV/ VIS spectrophotometer.

*2.10 Statistical analysis*

 The experiments were carried out in three replications at each concentration with an exception for suspension standard curve, killing curve assays, cell area, and the experiments were repeated three times. Data were expressed as a mean ± standard error of the mean (SEM). Significant differences of cell area in each treated group from TEM and CM permeability were analysed by one-way ANOVA. A *p*-value at < 0.05 and < 0.01 with Tukey’s HSD post-hoc test was considered as the statistically significant difference.

**3. Results and discussion**

 The resistance of microorganisms to commonly-prescribed antibiotics is becoming one of the most significant issues threatening human health. This problem motivates scientists to develop novel antimicrobial agents [27]. To prevent and delay the development of antibiotic resistance, the use of a drug combination therapy between naturally occurring antimicrobials and well-tried agents which have lost their original effectiveness is an area of far-reaching importance.

*3.1 Plant extraction and Preliminary phytochemical analysis*

 The percentage yield of the extract obtained was 10.3% (w/w). The preliminary qualitative phytochemical screening tests showed that there were alkaloids, flavonoids, tannins, glycoside and steroids in BRE, while saponin and coumarins were absent in this extract. The total flavonoids content was 4.73 ± 1.02 mg quercetin equivalent per 100 mg BRE (R2= 0.994).

*3.2 MIC determination*

 The MICs of BRE were 16 μg/mL against all of the tested Staphylococcal isolates. BRSA DMST 20651 exhibited high resistance to CLX, AMP, and CFZ with MICs 512, 256 and 256 μg/mL, respectively. Similarly, BRSA DMST 20652 had the MIC for CLX, AMP and CFZ of 512, 64, and 256 μg/mL, respectively. Whereas, BRSE DMST 14932 was resistant to CLX and AMP. All tested isolates were susceptible to VAN at a MIC value of 0.5 μg/mL (Table 1). The CLSI susceptibility breakpoints for CLX, AMP, CFZ and VAN against *Staphylococcus* spp. are ≤ 0.25, ≤ 0.25, ≤ 8 and ≤ 4 μg/mL, respectively [28]. CLX has been reported to be stable to staphylococcal penicillinases [29]. Therefore, it is possible that the resistance mechanism of the two BRSA strains used in the present study is mainly mediated by *mecA*-encoded PBP2a. This could also be the leading cause of β-lactam resistance in *S. epidermidis*.

*3.3 Chequerboard determinations*

 Table 2 illustrates the results of the interaction between BRE and CLX, AMP, CFZ or VAN against *Staphylococcus* spp.The lowest FIC index (0.502) was observed with the combination of BRE and CLX against BRSA DMST 20651 and DMST 20652, while the combination of BRE plus AMP or CFZ demonstrated similar results against these isolates (Table 2). The MICs of CLX, AMP and CFZ were dramatically reduced when used in conjugation with the BRE. A half reduction in the MIC was observed in BRE when employed in the combination against all tested bacteria. The combination of BRE and VAN exhibited no synergistic activity against all staphylococci tested including *S. aureus* ATCC 29213. In *S. epidermidis*, a synergistic activity was only observed with the combination of BRE plus AMP and partial synergistic activity was seen in the combinations of BRE plus CLX or CFZ. BRE is enriched with flavonoids and their derivatives [30, 31]. These results are consistent with a previous study suggesting that some flavonoids such as galangin, quercetin, and baicalein demonstrate synergistic interaction with β-lactam antibiotics against clinical isolates of *S. aureus* [32].

*3.4 Killing curve determinations*

 The combination of BRE and CLX showing the lowest FIC index against BRSA 20651 was chosen to confirm its antibacterial and synergistic activity. Figure 1 demonstrates the effect of BRE and CLX either alone or in combination, on cell viability of BRSA 20561. The results showed that untreated cells (control) grew exponentially after 2 h and increased in cell mass continuously until 24 h. The viable count of CLX or BRE treated alone grew steadily until 6 h compared to the starting inoculum and began a marked increase after 6 h to 24 h. The viable count of the combined BRE and CLX at FIC concentration displayed a gradual decline and slight increase in viable cells after 8 h. The viable cell reduction at 24 h of this combination was > 2 log10 CFU/mL compared to BRE or CLX treated alone. Furthermore, at higher concentrations of BRE and CLX (16 μg/mL and 2 μg/mL, respectively), bactericidal activity of the combination was demonstrated by a reduction of ≥ 3log10 CFU/mL in comparison to the initial inoculum after 24 h [24]. These findings provide evidence that BRE potentiates synergistically with CLX in inhibiting the growth of BRSA 20651. These results are similar to previous findings that *Stephania suberosa* extracts (SSE) plus ampicillin and ceftazidime plus flavonoids (galangin or baicalein or quercetin) exhibited synergistic activity against this strain [25, 32]. A previous study by Limsuwan and Voravuthikunchai (2013) found that *Streptococcus pyogenes* grew steadily after treatment with *Boesenbergia pandurata* extract at a concentration of 1/2 MIC, while was inhibited bactericidally at MIC, 2x MIC and 4x MIC in a dose-dependent manner [33].

*3.5 TEM study*

 The morphology of control cells grown in the absence of any antimicrobial agents is depicted in Figure 2. Untreated cells were round. The cytoplasmic membrane and peptidoglycan of these cells were unambiguously identified and distinguished (Figure 2a). TEM micrographs of cells treated with BRE alone are illustrated in Figure 2b. The cytoplasmic membrane of a number of these cells exhibited collapse and appeared detached from the peptidoglycan. These findings suggest that BRE could interact and damage the cytoplasmic membrane of the test strain. BRSA 20651 treated with CLX alone displayed little peptidoglycan damage to the cells (Figure 2c). BRE plus CLX treatment, many cells showed both cytoplasmic membrane and peptidoglycan damage (Figure 2d). In addition, the average cell areas of the BRE plus CLX treated cells were significantly smaller than those of controls (*p* < 0.05) (Figure 3). These results are consistent with those of Teethaisong et al. where SSE plus ampicillin caused peptidoglycan, cytoplasmic membrane damage to this strain and average cell areas significantly smaller than control [25]. Furthermore, the galangin plus cetazidime combination also produced cytoplasmic membrane and peptidoglycan collapse in this strain [32]. These findings provide evidence that the synergistic activity of this combination could occur and this mechanism of action may be caused by flavonoids and alkaloids contained in BRE.

*3.6 CM permeability*

 The results of this assay are illustrated in Figure 4. The CLX-treated group exhibited no leakage of OD260-absorbing materials in comparison with the control group during 4 h of treatment (*p* > 0.01). The cells treated with BRE or Nisin alone and the combination of BRE plus CLX showed a significant increase in OD260 compared with those of CLX treated and control (*p* < 0.01). However, no significant differences among those of cells treated with BRE, nisin, and BRE plus CLX were observed (*p* > 0.01). The increase of 260 nm absorbing material implies that mostly DNA, RNA, metabolites and ions are released from the cells [34]. Nisin primarily kills Gram-positive bacteria by forming pores in the cytoplasmic membrane [35]. Thus, in the current study, it was used as a positive control to induce CM permeability. Our findings found that BRE treated alone and the combination of BRE and CLX could increase the cytoplasmic permeabilisation of testing strain, while cells treated with CLX alone did not. These findings provide evidence that the interaction of BRE and the cytoplasmic membrane play a major role in inducing the disruption and dysfunction of the membrane. How BRE precisely interacts with the cytoplasmic membrane remains to be investigated.

**4. Conclusion**

 BRE possess an excellent inhibitory activity against β-lactam-resistant staphylococci. Its mechanism of action is probably by interacting with the bacterial cytoplasmic membrane, destroying the cytoplasmic membrane accompanied by leakage of the intracellular materials. Synergistic activity of BRE in combination with CLX against BRSA strains may involve co-inhibition of peptidoglycan synthesis and cytoplasmic membrane damage. *B. rotunda* has a high potential for the development of a novel adjunct phytopharmaceutical to β-lactam antibiotics, especially CLX in the treatment of infections caused by β-lactam-resistant Staphylococci. The active ingredients, efficacy and toxicity of this combination by *in-vivo* mammalian tests should be further investigated.

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**Competing interests**

 None declared.

**Ethical approval**

 None required.

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**Table 1**

Minimum inhibitory concentration (MIC) of selected peptidoglycan inhibitor antibiotics and BRE against β-lactam-resistant *S. aureus* strains.

|  |  |
| --- | --- |
| **Bacterial****Isolates** | **Minimum inhibitory concentration (μg/mL)** |
| BRE | CLX | AMP | CFZ | VAN |
| *S. aureus* DMST 20651 | 16*ND* | 512*R* | 256*R* | 256*R* | 0.5*S* |
| *S. aureus* DMST 20652 | 16*ND* | 512*R* | 64*R* | 256*R* | 0.5*S* |
| *S. epidermidis* DMST 14932 | 16*ND* | 2*R* | 32*R* | 4*S* | 0.5*S* |
| *S. aureus* ATCC 29213\* | 16*ND* | ≤0.25*S* | ≤0.25*S* | ≤0.25*S* | 0.5*S* |

*S* = Susceptible; *R* = resistant; *ND* = not determine in CLSI breakpoint; BRE = *B. rotunda* extract; CLX = cloxacillin; AMP = ampicillin; CFZ = cefazolin; VAN = vancomycin; \*A reference strain. The data expressed were obtained from three replications.

**Table 2**

Drug interaction betweenBRE and selected antibiotics against β-lactam-resistant staphylococci.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Bacterial Isolates** | **Combination of agents** | **MIC****(in combination)** | **FIC****index** | **Type of Interaction** |
| *S. aureus* DMST 20651 | BRE | 8 | 0.502 | Synergism |
|  | CLX | 1 |  |  |
|  | BRE | 8 | 0.504 | Synergism |
|  | AMP | 1 |  |  |
|  | BRE | 8 | 0.504 | Synergism |
|  | CFZ | 1 |  |  |
|  | BRE | 8 | 1.02 | Addition |
|  | VAN | ≤0.25 |  |  |
| *S. aureus* DMST 20652 | BRE | 8 | 0.502 | Synergism |
|  | CLX | 1 |  |  |
|  | BRE | 8 | 0.507 | Synergism |
|  | AMP | 0.5 |  |  |
|  | BRE | 8 | 0.504 | Synergism |
|  | CFZ | 1 |  |  |
|  | BRE | 8 | ≤1 | Addition |
|  | VAN | ≤0.25 |  |  |
| *S. epidermidis* DMST 14932 | BRE | 8 | ≤0.625 | Partial |
|  | CLX | ≤0.25 |  | Synergism |
|  | BRE | 8 | 0.516 | Synergism |
|  | AMP | 0.5 |  |  |
|  | BRE | 8 | 0.625 | Partial |
|  | CFZ | 0.5 |  | Synergism |
|  | BRE | 8 |  ≤1 | Addition |
|  | VAN | ≤0.25 |  |  |
| *S. aureus* ATCC 29213\* | BRE | ≤0.25 | 1.5 | Addition |
|  | CLX | ≤0.25 |  |  |
|  | BRE | 8 | 1.5 | Addition |
|  | AMP | ≤0.25 |  |  |
|  | BRE | 8 | 1.5 | Addition |
|  | CFZ | ≤0.25 |  |  |
|  | BRE | 8 | ≤1 | Addition |
|  | VAN | ≤0.25 |  |  |

BRE = *B. rotunda* extract; CLX = cloxacillin; AMP = ampicillin; CFZ = cefazolin; VAN = vancomycin; \*A reference strain. Fraction inhibitory concentration index (FICI) ≤0.5 denoting synergism; FICI >0.5 − <1.0 denoting partial synergism; FICI = 1 denoting addition; FICI >1− ≤4.0 denoting indifference; FICI >4.0 denoting antagonism.