



Anti-biofilm Potential of *Boesenbergia rotunda* L. Extract and Synergy with Cloxacillin on Biofilms of β -lactam-resistant *Staphylococcus aureus*

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ABSTRACT

Introduction: A biofilm is extracellular substances produced by microorganisms to protect themselves from harmful agents, including antibiotics. Bacteria living in biofilms are known to have higher resistance to antimicrobial therapy and is one of the most important mechanisms of resistance to antibiotics. Hence, development of a novel anti-biofilm agent is of far-reaching importance.

Objective: To investigate the anti-biofilm effect of *B. rotunda* extract (BRE) and its synergism with cloxacillin (CLX) on biofilms of β -lactam-resistant *Staphylococcus aureus*.

Methods: *B. rotunda* extract was carried out by macerating with 99.9% ethanol. The extracts were subsequently dried using a rotary evaporator and lyophilized. The anti-biofilm activity and synergistic effect of BRE were carried out by a crystal violet assay. Quantitation of biofilms were done by a spectrophotometer, while qualitative analysis of biofilm formation was observed under a light microscope.

Results: Following 24 h of treatment, the result from quantitative analysis of biofilm formation using a crystal violet assay revealed that BRE significantly eradicated biofilms of β -lactam-resistant *S. aureus*. Interestingly, the combination of BRE and CLX synergistically inhibited biofilm formation. In line with qualitative analysis of biofilm formation, bacteria treated with BRE and CLX, either alone or in combination, had explicitly lower biofilm than untreated control.

Conclusion: BRE potentially destroyed biofilms and had synergistic effect with CLX in eradication of biofilms of β -lactam-resistant *S. aureus*. These findings from the evidence that the combination of BRE and CLX could be a good candidate for further development as a novel agent for treatment of recalcitrant infection caused by β -lactam-resistant *S. aureus*.

Keywords: *Boesenbergia rotunda* extract; biofilms; synergism; *Staphylococcus aureus*

Introduction

A biofilm is a structured community of bacteria embedded in a self-produced extracellular polymeric substance (EPS) composing of polysaccharides, proteins, nucleic acids, lipids, and humorous-like substances [1]. This matrix makes it difficult to eradicate and causes chronicity of infection [2]. Biofilms are formed on many implanted medical devices, such as central venous catheters, heart valves, urinary catheters, orthopedic devices and even contact lenses [3,4]. The bacteria living in a biofilm can become

as much as 10-1000 times more resistant to antimicrobial agents compared with normally seen in planktonic cells [3]. Failed penetration of the agents, delayed diffusion of antibiotic, and slow-growing or non-growing state are the contributing factors for resistance to antibiotics [5]. Accumulation of β -lactamase with an increased upregulation of efflux pumps allows bacteria to survive in the biofilm [6]. Treatment of bacteria in the biofilm with antibiotics often fails to kill. Currently available antibiotics are

most effective against planktonic bacteria, but they are typically ineffective to eradicate bacterial biofilm and persistent infections. So, this indicates a necessity to research and develop novel agents targeting biofilms [7]. Plant-derived antibiofilm agents are of interest. Flavonoids isolated from *Moringa oleifera* seed extract had inhibitory effects on biofilms of *S. aureus*, *P. aeruginosa* and *C. albicans* [8]. Salviposone extracted from hairy roots of *Salvia sclarea* L. also showed anti-biofilm activity against *S. aureus* and *S. epidermidis* [9]. In addition, phytochemical/antibiotic or antibiotic/antibiotic combinations are a promising approach to eradicate bacterial biofilms. Coelho and Pereira (2013) also found the anti-biofilm activity of three essential oils, including cinnamon (*Cinnamomum zeylanicum*), tea tree (*Melaleuca alternifolia*) and palmarosa (*Cymbopogon martini*), either used singly or in combination with ciprofloxacin. They found that *P. aeruginosa* biofilms were inhibited by each of these essential oils [10]. N-acetylcysteine, EDTA, ethanol and talactoferrin (TLF) also revealed synergy with some antibiotics against *S. epidermidis* and *C. albicans* biofilms, which are common catheters-indwelling pathogens. These combinations were proposed to be new regimens for treatment of infected catheters [11]. *Staphylococcus aureus* is a Gram-positive coccus belonging to the family *Staphylococcaceae*. It is considered as the normal microbiota of human mucous, nasopharynx and skin. This bacterium is among the greatest threats to human health in both community onset and healthcare-acquired infections. It causes a variety of human illnesses, including pneumonia, meningitis, toxic shock syndrome, bacteremia, wound sepsis, osteomyelitis, and endocarditis [12]. Methicillin-resistant *S. aureus* (MRSA) was recorded in the year of the drug's launch. Since then, MRSA has become one of the most serious public health problems worldwide. Using a combination approach of a phytochemical and antibiotic is a promising avenue for combating multidrug-resistant bacteria and their biofilms. *B. rotunda*, or locally known in Thai as Kra-chai khaw, has been used as traditional ethnomedicine for treating several human ailments including rheumatism, muscle ache, fever, gouty arthritis, bowel disorder, abdominal distension, carminative, appetite promotion, gastric disturbances, anti-ulcer, anti-inflammation, antioxidant, anti-viral, anti-parasite and anticancer. Interestingly, this herbal plant exhibits strong antibacterial activity with a wide spectrum against pathogenic bacteria [13]. Due to scarce data, the present study therefore investigated the anti-biofilm activity and synergy effect of *B. rotunda* extract (BRE) with cloxacillin (CLX) against biofilms of β -lactam-resistant *S. aureus*.

Methodology

Bacterial strains and antibiotic

β -lactam-resistant *S. aureus* DMST 20651 was obtained from the Department of Medical Science,

Ministry of Public Health, Thailand. Cloxacillin antibiotic ($\geq 95\%$ purity) was purchased from Sigma-Aldrich, Singapore.

Extraction of *B. rotunda*

Rhizomes of *B. rotunda* were domestically obtained from Nakhon Ratchasima province, Thailand during August 2014. The plant sample was authenticated by comparing with the Forest Herbarium specimen (a voucher specimen BKF No. 192160). Finely ground of dried rhizome were macerated with 99.9% ethanol for a week at room temperature. The extracts were then filtered through Whatman no. 1 filter paper before removing the solvent using a rotary evaporator under reduced pressure. Subsequently, the extracts were dried by a lyophilized [14].

Quantitation of bacterial biofilm

The inhibitory effect of BRE either alone or in combination on biofilm development were evaluated as previously described by Gopal et al. (2014) with some modifications [15]. Briefly, an 18-h culture of test bacteria was adjusted in saline to give 5×10^6 CFU/mL. Twenty microlites of adjusted bacterial suspension were added to 190 μ L of broth medium supplemented with 0.2% glucose plus BRE, or antibiotic alone, or in combination. The concentration at half-MIC or FIC of BRE or antibiotic from a previous study was employed [14]. Following incubation at 37 °C for 48 h, the culture medium was discarded, and free-floating planktonic cells were washed with distilled water. The adhered biofilms were stained with 0.4% (w/v) crystal violet solution for 30 min. The excess stain was washed off thoroughly with distilled water. After the wells are air-dried, the stained biofilms were then solubilized with 100% ethanol before measuring an absorbance using a microplate reader at 595 nm. The optical density values represented adhering bacteria and forming a biofilm

Microscopic analysis of bacterial biofilm

The light microscope analysis of bacterial biofilm was examined following the method of Nithya and Pandian (2010) and Packiavathy et al (2014) with few modifications [16,17]. Two hundred microliters of adjusted bacteria (5×10^6 CFU/mL) were added to 1800 μ L of fresh medium (supplemented with 0.2% glucose) containing BRE either alone or in combination with antibiotics in a 24-well polystyrene plate. Wells with and without test bacteria were used as controls. The plate was incubated at 37 °C for 48 h. Afterwards, the planktonic cells were removed prior to staining the adhered with 0.4% crystal violet solution. The excess crystal violet was washed off by repeatedly submerging the plate into reservoirs of distilled water. The plate was then placed in an incubator at 37 °C until dry. The stained biofilm was viewed under a light microscope.

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM) from 6 repetitions. The results were analyzed and compared by One-way ANOVA followed by Tukey's HSD Post-hoc test. The different alphabetical characters represent a statistically significant difference between each group ($p < 0.01$).

Results

Ethanollic extraction of *B. rotunda* yielded $10.3 \pm 0.94\%$ (w/w). The antibiofilm activity of CLX and BRE either used singly or in combination against MRSA DMST 20651 using a standard quantitative biofilm assay is demonstrated in figure 1. After 48 of treatment, MRSA DMST 20651 grown in the absence of any antimicrobial agent showed the highest biofilm biomass. All treated groups significantly dislodged the biofilm of MRSA DMST 20651 compared with a control group, $p \leq 0.01$. CLX (256 $\mu\text{g/mL}$)-treated group exhibited lowest biofilm formation, but no significant difference compared with BRE (8 $\mu\text{g/mL}$) alone and BRE (8 $\mu\text{g/mL}$) plus CLX (1 $\mu\text{g/mL}$), $p > 0.01$.

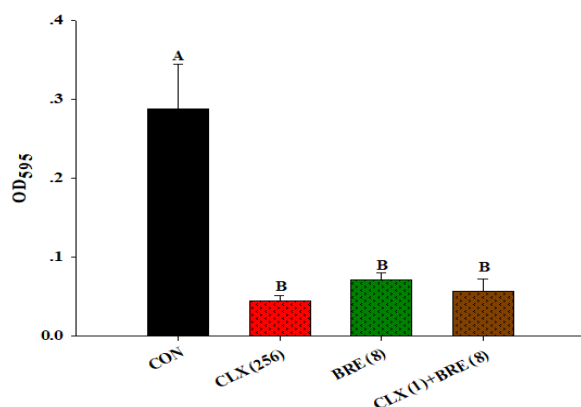


Figure 1 Quantitative analysis of biofilm formation of MRSA 20651 after exposure to no antibacterial agents, *B. rotunda* extract and cloxacillin either used alone or in combination. CON = control, CLX (256) = cloxacillin at a concentration of 256 $\mu\text{g/mL}$, BRE (8) = *B. rotunda* [18] (8) = cloxacillin at a concentration of 1 $\mu\text{g/mL}$ and *B. rotunda* extract at a concentration of 8 $\mu\text{g/mL}$.

The effect of 256 $\mu\text{g/mL}$ CLX, 8 $\mu\text{g/mL}$ BRE alone and the combination of 1 $\mu\text{g/mL}$ CLX plus 8 $\mu\text{g/mL}$ BRE on biofilm formation of MRSA 20651 were also qualitatively examined under a light microscope. The results of this assay are shown in figure 2. The crystal violet staining in a control group exhibited a thick coating of biofilm (figure 2A), whereas the explicit reduction in biofilm formation was observed in cells treated with BRE and CLX either alone or in combination. The CLX-treated group showed the greatest activity in the eradication of biofilm formation compared with BRE alone and BRE plus CLX. MRSA DMST 20651 cells were observed under light microscopy.

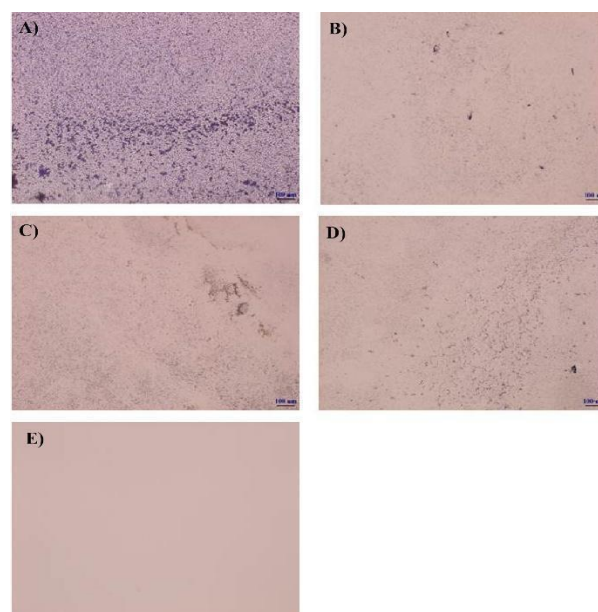


Figure 2 Qualitative microscopic analysis of biofilm formation using a light microscope. A= positive control, B = CLX (256 $\mu\text{g/mL}$)-treated group, C = BRE (8 $\mu\text{g/mL}$), D = CLX (1 $\mu\text{g/mL}$) plus BRE (8 $\mu\text{g/mL}$)-treated group, E = a negative control. Bar = 100 μm and magnification = 400x

Discussion

A biofilm is known to be problematic for several human ailments. Bacteria that live in a biofilm present high resistance to antibiotics leading to treatment failure. This is due to biofilms being highly resistant to immune killing and clearance, and to treatment with antimicrobial agents [19]. Bacterial biofilm can form on either environmental abiotic surfaces or biotic surfaces. the biofilm-formed cultures of *Actinomyces pyogenes*, *Corynebacterium renale*, *Corynebacterium pseudotuberculosis*, *S. aureus*, *Staphylococcus hyicus* and *Streptococcus agalactiae* in biofilm were resistant to ampicillin, ceftiofur, cloxacillin, oxytetracycline, penicillin G, streptomycin, tetracycline, enrofloxacin, and erythromycin. Whereas, all of these antibiotics were effective against those strains in planktonic cells [20]. Based on our previous study, β -lactam-resistant *S. aureus* used in the present study was highly resistant to a number of antibiotics including cloxacillin and ampicillin and cefazolin. Regarding anti-biofilm activity of BRE [14], this study is the first to show that BRE either alone or in combination with CLX prevents the development of biofilms of β -lactam-resistant *S. aureus*. The MRSA DMST 20651 biofilm was significantly minimized following exposure to BRE either used alone or in combination. This finding agrees with Yanti and colleagues who reported that Panduratin A isolated from *Kaempferia pandurata* Roxb (synonym *B. rotunda* extract) has the ability to disperse biofilm formed by multispecies oral bacteria [18]. Also, flavonoids from *Moringa oleifera* seed extract have

inhibitory effects on biofilms of *S. aureus*, *P. aeruginosa* and *C. albicans* [21]. In addition, other flavonoids, such as naringenin, kaempferol, quercetin, and apigenin have also been reported to have the ability to eradicate *Vibrio harveyi* and *E. coli* biofilms [22].

Conclusion

BRE possesses anti-biofilm activity and has a synergistic effect by potentiating CLX to eradicate bacterial biofilms of β -lactam-resistant *S. aureus*. The combination of BRE and CLX could be an attractive candidate of a novel pharmaceutical adjuvant for treatment of life-threatening β -lactam-resistant *S. aureus*.

Competing Interests

The authors have declared no conflict of interests.

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Author contributions

YT and GE conceived and conceptualized this project. PK and YT carried out experiments as well as analyzed data.

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