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Antibiofilm Activity of a *Curcuma zedoaria* Rosc Rhizome Extract against Methicillin-Resistant and Susceptible *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) are major causes of hospital- and community-acquired infections. The treatment of biofilm-related infections caused by these bacteria is a global healthcare challenge. Therefore, the development of alternative therapeutics is required. An essential oil extracted from *Curcuma zedoaria* (CZ) Rosc, also known as white turmeric, has been reported to possess various antimicrobial activities. In the present study, we evaluated the antibiofilm activities of an ethanolic extract of the CZ rhizome against MRSA and MSSA. The results showed that the CZ extract with the highest sub-minimum inhibitory concentration (sub-MIC), 1/2 MIC (0.312 mg/ml), significantly inhibited biofilm production by up to 80-90% in both tested strains. Subsequently, we evaluated the ability of the CZ extract to prevent cell-surface attachment to a 96-well plate and extracellular DNA (eDNA) release from the biofilm. The CZ extract demonstrated an inhibitory effect on bacterial attachment and eDNA release from the biofilm biomass. The CZ extract may inhibit biofilm formation by preventing eDNA release and cell-surface attachment. Therefore, this CZ extract is a potential candidate for the development of alternative treatments for biofilm-associated MRSA and MSSA infections.

Keywords: ***Curcuma zedoaria*, antibiofilm, methicillin-resistant *Staphylococcus aureus,* methicillin-susceptible *S. aureus*, extracellular DNA, attachment**

Introduction

Antibiotics resistance in bacteria has increased dramatically worldwide and is a major cause of death and healthcare-associated infections (HAIs) [1]. One of the major pivotal characteristics that enhance bacterial resistance to antimicrobial agents is biofilm formation. Biofilm is a microbial community structure enclosed by a self-produced extracellular polysaccharide matrix that attaches to biotic or abiotic surfaces [2]. Biofilm-associated infections can complicate treatments involving catheter-related infections and lead to treatment failure [3]. Several studies have reported that biofilm-forming bacteria are more resistant to antibiotics than the planktonic form and also have an increased ability to persist within infected hosts, resulting in chronic infection [4, 5].

Currently, infectious diseases caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) are a major problem for HAIs and community-associated infections (CAIs). The diseases caused by these bacteria range from infection of the skin and surgical sites to catheter and prosthetic implant-related infections, as well as pneumonia, endocarditis, and bacteremia [6, 7]. HAIs caused by MRSA have been reported to be associated with inferior clinical

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outcomes and higher mortality rates than MSSA [8] and other gram-negative bacterial infections [9]. Nevertheless, infections with MSSA are still much more frequent than MRSA and remain an important problem in both HAIs and CAIs. Therefore, these pathogens, especially MRSA, have been categorized as a high priority requiring the research and development of new antibiotics to combat this infection [10].

The biofilm-forming ability of MRSA and MSSA strains is considered a key characteristic influencing its persistent infection in the host and results in complications of treatment, especially those associated with medical device-related infections such as catheters and medical implants [11]. The treatment of biofilm-associated infections of MRSA and MSSA is more difficult, leading eventually to chronic device-related infections [12]. The single use of standard antibiotics is ineffective, especially for infections in biofilm form. Several studies have demonstrated the use of alternative treatment strategies for combating biofilm-associated infections, especially plant extract or a combination of plant extract and antibiotics.

Curcuma zedoaria (CZ) Rosc, known as white turmeric, belonging to the Zingiberaceae family, is a perennial herb commonly found in India and Southeast Asia, especially Thailand. This herb is traditionally used to treat digestive disorders, including indigestion, nausea, flatulence, and bloating. Moreover, it has been applied externally to treat ulcers, wounds, and other skin disorders-related infections. Therefore, this study aims to investigate the antibiofilm activities of CZ extract against MRSA and MSSA strains.

Materials and Methods

Plant material and extraction

The ethanol extract of the rhizome of CZ was prepared as follows. Briefly, 20 g of mashed CZ rhizome was extracted with 150 ml of 70% ethanol (HPLC grade) in 250 ml Erlenmeyer flask and sonicated for 30 min; sonication was repeated 4 times until extraction was exhaustive. After sonication, the combined extract of each time was filtrated through Whatman filter paper no. 1. The filtrate was evaporated using a rotary evaporator for ethanol removal and drying in a water bath at 40℃ until the excess solvent was completely removed, and the percent yield of dry weight was recorded. The extract was stored at 20℃ until use.

High-performance chromatography (HPLC) analysis of phenolic compounds

Curcuminoids (curcumin, bisdemethoxycurcumin, and demethoxycurcumin), a major phenolic compound found in rhizome of *Curcuma* spp. [13], were qualified and quantified by comparing with pure standard curcuminoids compound (Chemfaces) and then analyzed by the HPLC system (Shimadzu, Japan). The extract was dissolved in methanol and filtered with a 0.22 μm nylon syringe filter. The separation was carried out by using an Inertsil ODS-3 (4.6 × 250 mm, 5 μm) column and an Inertsil ODS-3 guard column (4.0 × 10 mm, 5 μm). The mobile phase was 0.25% aqueous acetic acid solution (A) and acetonitrile (B) with the gradient concentrations as follows: 0–8 min, 40% A; 8–10 min, 60% A; 10–15 min, 60% A; 15–16 min, 40% A and 16–20 min, 40% A. Column temperature was 30℃ and flow rate was 0.8 ml/min. The injection volume of the sample was 5 μl and detection at 425 nm.

Bacterial strains and growth conditions

Methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA) and methicillin-susceptible *S. aureus* ATCC 25923 (MSSA) were used in this study. The bacteria were maintained in tryptic soy agar (TSA) (Hi-Media, India) and incubated at 37℃ for 24 h. All the experiments were initiated using an overnight culture in tryptic soy broth (TSB) (Hi-Media, India) containing 1.5% glucose, except for the disc diffusion and broth microdilution assays, Muller Hinton agar (MHA), and Muller Hinton broth (MHB) (Hi-Media) were used according to standard methods of CLSI [14].

Determination of minimal inhibitory concentration (MIC) of CZ extract

The broth microdilution method using a 96-well microplate (Nunclon, Thermo Scientific, China) was used to determine the inhibitory efficacy of CZ ethanolic extract against MRSA and MSSA. Two to three single colonies of the bacteria were inoculated into 3 ml MHB and incubated overnight at 37℃ with shaking at 250 ×*g* for 18 h. Two hundred microliters of the bacterial suspension were then transferred into fresh 10 ml MHB

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and continued incubation until attaining the exponential phase. The bacterial cells were adjusted to 0.5 at OD600 by a spectrophotometer (bacteria no.1-1.5 × 10^8 CFU). Bacterial broth cultures were diluted to correspond to the final inoculums of 5 × 10^5 CFU/ml upon inoculation into each well containing two-fold serial dilution of test samples. The extract was then tested with concentrations varying from 0.019 mg/ml to 5 mg/ml parallel with control antibiotics, vancomycin, and ampicillin.

Determination of the effect of CZ extract on biofilm production by MRSA and MSSA

The extract was prepared by two-fold serial dilution with TSB-glucose in a 96-well microplate at concentrations of 5 to 0.019 mg/ml. MRSA and MSSA without the extract were used as the positive control. A single colony of MRSA and MSSA from the overnight culture grown on TSA was inoculated into 3 ml TSB and incubated at 37℃ for 24 h with shaking at 250 rpm. The bacterial suspension was diluted to 1:50 in fresh TSB-glucose to give a final concentration of inoculation size of approximately 1 × 10^5 CFU/ml in a total volume of 200 µl in each well of a 96-well microplate (Nunc). The microplate was incubated at 37℃ for 24 h. After static incubation, the planktonic cells in each well were removed and washed twice with 200 µl phosphate buffer saline (PBS) and air-dried. Wells were fixed with 200 µl 99% methanol for 15 min and removed, and then the plates were allowed to dry. The plates were stained with 200 µl/well of 0.1% crystal violet for 5 min. Excess crystal violet stain was gently rinsed off with tap water, after which the plates were allowed to air dry. The stain was solubilized with 200 µl of 33% glacial acetic acid and shaken in a microplate orbital shaker for 30 min, and then measured at OD570 nm. The experiment was done in triplicate on different occasions.

Microplate attachment assay

The experiment was carried out based on methods previously described by Carolina Santiago *et al.* [15]. The extract was prepared in a two-fold dilution in TSB-glucose in a 96-well microplate, (Nunclon) at concentrations of 5 to 0.019 mg/ml. MRSA and MSSA suspensions at a final inoculum size of 1 × 10^7 CFU/ml were added into each well. Wells containing only MRSA and MSSA were used as a positive control. The microplate was incubated at 37℃ for 1 h. After incubation, the unattached bacteria were removed and washed with 200 µl PBS. The percentage of cell attachment to the microplate was determined by the crystal violet staining method and measured by spectrophotometry at OD595 nm. The experiment was done in triplicate on different occasions.

Quantification of eDNA in biofilm

The quantity of eDNA in biofilm biomass of MRSA and MSSA was determined using QuantiFluor dsDNA System (Promega, USA) in a 96-well black plate (Nunclon, Thermo Scientific, Denmark), as described previously by Pakkulnan *et al.* [16]. The 24 h biofilm treated with CZ extract in a 96-well black plate was rinsed three times with sterile distilled water. To quantify eDNA, 200 µl of freshly prepared QuantiFluor dsDNA dye in 1×TE buffer was added into each well and mix thoroughly using a plate shaker. Subsequently, the plate was incubated for 5 min at room temperature and protected from light. The fluorescence intensity (excitation 504 nm/emission 531 nm) was measured by using a fluorometer (Thermo Scientific™ Varioskan™ Flash Multimode Reader) with SkanIt Software 2.4.5 RE for Varioskan Flash. Lambda DNA was used to generate a standard control for each experiment.

Statistical analysis

The experiments were performed in triplicate. Significance was assessed by one-way analysis of variance (one-way ANOVA), Scheffe test, and Dunnett's T3 post hoc analysis using IBM SPSS Statistics version 20.0 software. The significance level was considered as a p-value < 0.05.

Results

HPLC analysis of curcuminoid content in CZ extract

The extraction yield of ethanolic extracts of CZ rhizome was 17.40 ± 0.58% w/w. Fig. 1 shows the HPLC chromatogram of curcuminoid content in CZ extract consisting of bisdemethoxycurcumin, demethoxycurcumin, and curcumin. Quantitative analysis of the extract based on a simple linear regression equation obtained from standard curcuminoids was determined in µg/ml.

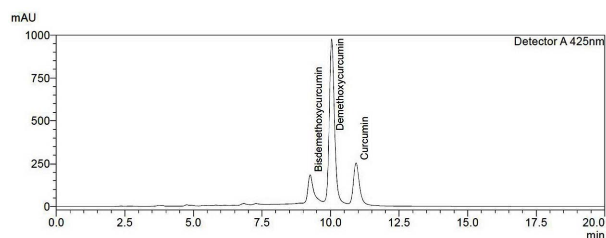

Fig. 1. HPLC chromatogram of CZ rhizome extracted with 70% ethanol showing three different main peaks of curcuminoids including bisdemethoxycurcumin, demethoxycurcumin, and curcumin at retention times of 9.255 min, 10.040 min, and 10.929 min, respectively (Milli absorbance unit; mAU).

The results showed that curcuminoid contents in the sample extract were bisdemethoxycurcumin (49.8 µg/ml), demethoxycurcumin (255.96 µg/ml), and curcumin (114.72 µg/ml). The highest curcuminoid content found in the CZ extract was demethoxycurcumin.

Antimicrobial activity of CZ extract against *S. aureus*

From the results of the disc diffusion method, an antibiotic disc containing 2.5 mg of CZ rhizome extract showed antimicrobial activity against both MRSA and MSSA with an inhibition zone of 8 mm. A standard disc containing 30 µg of vancomycin was used as a positive control and showed an inhibition zone of 16 mm. The negative control DMSO did not show an inhibition zone. To evaluate the MIC of CZ extract, a two-fold dilution of the concentration ranging from 5 to 0.019 mg/ml was performed in a 96-well microplate. The results showed that CZ extract exhibited antimicrobial activity against both MRSA and MSSA with the same MIC value of 0.625 mg/ml. The MIC of standard antibiotics control, including ampicillin, vancomycin, and oxacillin are also shown in Table 1.

Table 1. Minimum inhibitory concentration (MIC) of ethanolic extract of CZ rhizome and antibiotic control against MRSA ATCC 43300 and MSSA ATCC 25923.

Antibiotics and extract	MIC (mg/ml)	
	MRSA	MSSA
Ampicillin	0.128	0.008
Vancomycin	0.001	0.002
Oxacillin	0.032	0.0005
CZ rhizome extract	0.625	0.625

Anti-MRSA and MSSA biofilm formation activity of CZ extract

The results of crystal violet staining showed the inhibitory activity of CZ rhizome extract on both MRSA and MSSA biofilm formation. The total biofilm production of MRSA and MSSA after being treated with CZ was strongly decreased in a dose-dependent manner when compared with untreated control. To assess the growth of the planktonic cells under biofilm conditions, the turbidity of bacterial growth in a 96-well microplate was measured at OD600. The results showed that planktonic cell growth was comparable to the positive control (data not shown). A sub-minimal inhibitory concentration (sub-MIC) of CZ extract was used to evaluate the biofilm formation of tested strains. At the sub-MIC of CZ ranging from 1/32 MIC to 1 MIC, the percentage of biofilm production of MRSA was significantly reduced ($p < 0.05$)

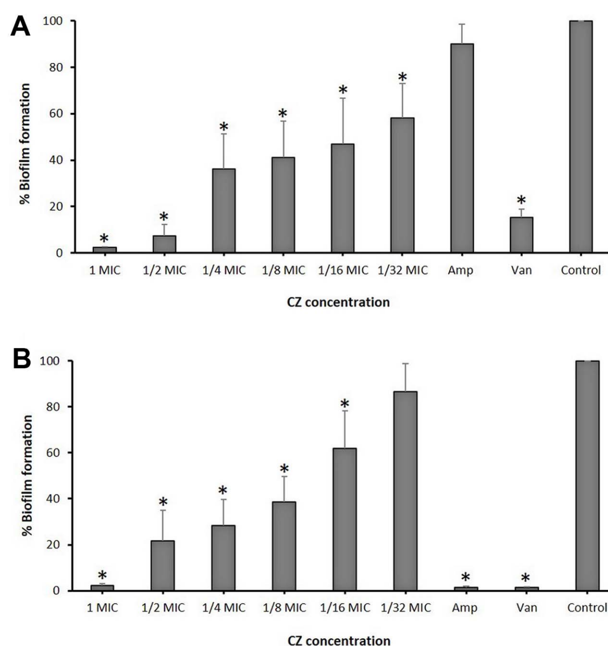

Fig. 2. Quantity of biofilm formation after crystal violet stained and measured at OD570 in a 96-well microplate containing 1/32 MIC-1MIC of CZ rhizome ethanolic extract. A percentage of biofilm formation of (A) MRSA ATCC 43300 and (B) MSSA ATCC 25923. Control; MRSA and MSSA grown on TSB medium without CZ treatment, Van; vancomycin (0.05 mg/ml) and Amp; ampicillin (0.05 mg/ml) were used as antibiotic control. An asterisk indicates a significant difference between the control and CZ treated group at a p-value < 0.05.

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by 41.97–97.70% (Fig. 2A). The biofilm production of MSSA was significantly decreased ($p < 0.05$) at sub-MIC ranging from 1/16 MIC-1 MIC, and the percentage of biofilm production was 38.01–97.69% (Fig. 2B). In addition, the antibiotic control showed that vancomycin reduced biofilm formation in both tested strains. The results indicated the antibiofilm activity of CZ extract against tested strains by showing better inhibitory effects against MRSA than MSSA.

Inhibitory activity of CZ extract against bacterial attachment to abiotic surfaces

To evaluate the properties of CZ extract on bacteria adherence ability to an abiotic surface, a bacterial attachment assay was performed in a 96-well microplate. The result of adherence cells was determined by the crystal violet staining method after 1 h of incubation. The results indicated the ability of bacterial adhesion to microplate surfaces was strongly inhibited by CZ extract in a dose-dependent manner when compared with untreated control. At sub-MIC concentrations of 1/32 MIC - 1 MIC, CZ extract showed significant ($p < 0.05$) inhibition of MRSA attachment ranging from 44.30 to 97.85% (Fig. 3A). Meanwhile, CZ extract showed significantly reduced ($p < 0.05$) MSSA adhesion to microplate surface ranging from 66.54 to 98.29% at sub-MIC of 1/8 MIC-1 MIC (Fig. 3B). The adherence ability of MRSA after treatment with CZ extracts was slightly lower than MSSA at sub-MIC of 1/32 MIC-1/8 MIC. In both MRSA and MSSA, the increased concentration of CZ extracts strongly inhibited bacterial adhesion (Figs. 3A and 3B).

CZ extract reduced eDNA release in biofilm

Based on the hypothesis that eDNA production is associated with bacterial attachment and biofilm formation of *S. aureus*, we further investigated the inhibitory effect of CZ extract on the production of eDNA in MRSA

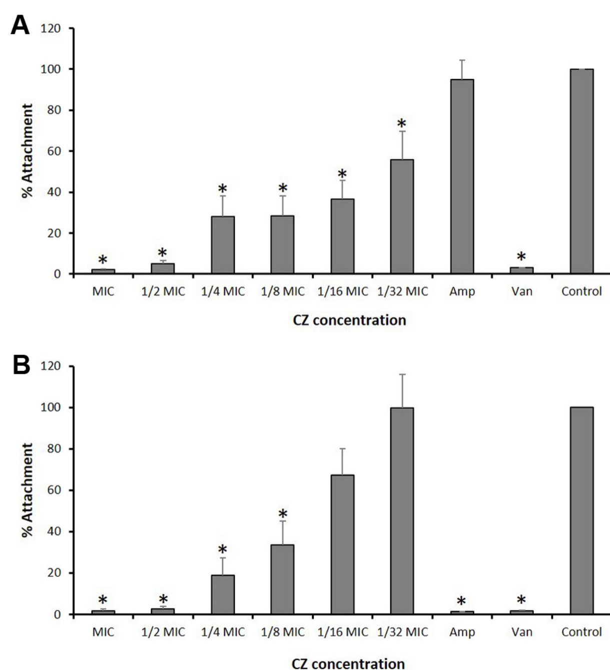

Fig. 3. Quantity of bacterial attachment to the surfaces of 96-well microplates containing CZ extracted at various concentrations ranging from 1/32 MIC to 1 MIC. A percentage of bacterial attachment of (A) MRSA ATCC 43300 and (B) MSSA ATCC 25923. Control; MRSA and MSSA grown on TSB medium without CZ treatment, Van; vancomycin (0.05 mg/ml) and Amp; ampicillin (0.05 mg/ml). An asterisk indicates a significant difference between the control and CZ treated group at a *p*-value < 0.05.

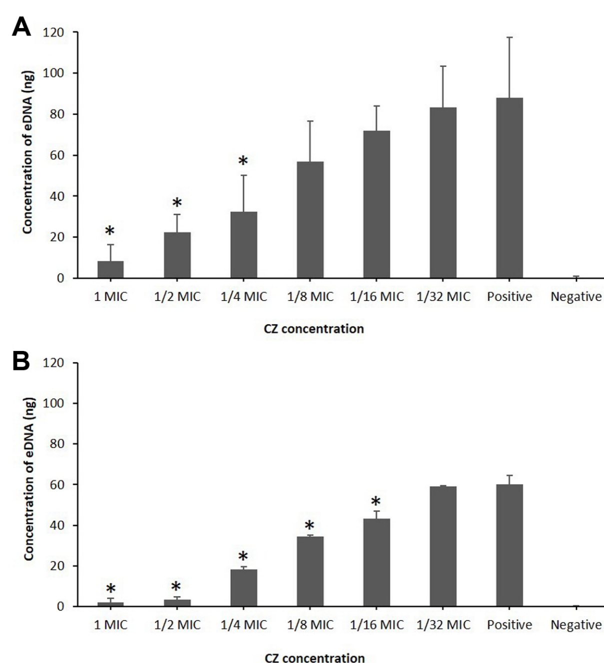

Fig. 4. eDNA quantity (ng) in 24-hours biofilm biomass of (A) MRSA ATCC 43300 and (B) MSSA ATCC 25923 after treatment with various concentrations of CZ extract, ranging from 1/32 MIC to 1 MIC and measured by QuantiFluor dsDNA System. The control included bacteria lacking CZ extract (positive) and TSB medium lacking bacteria (negative). An asterisk indicates a significant difference between the positive control and CZ treated group at a *p*-value < 0.05. The mean value ± SD of data was derived from triplicate independent experiments.

Table 2. Correlation analysis between eDNA quantity and biofilm biomass of *S. aureus* after treatment with the CZ extract.

eDNA quantity	Biofilm biomass of *S. aureus* strains	
	MRSA	MSSA
Pearson correlation's coefficient	0.871	0.965
p-value	0.011	< 0.001

and MSSA biofilm biomass. Twenty-four hours of biofilm form was assessed by using a QuantiFluor dsDNA system in a 96-well black plate. The results showed that eDNA release in biofilm biomass was decreased significantly ($p < 0.05$) as the concentration of CZ extract increased at 1/4 MIC to 1 MIC and 1/16 MIC to 1 MIC for MRSA and MSSA, respectively, when compared to a positive control bacterium without the extract (Figs. 4A and 4B). In addition, Pearson correlation analysis also demonstrated a significant positive correlation between eDNA quantity and biofilm biomass after treatment with CZ extract in each strain ($p < 0.01$) (Table 2).

Discussion

In this study, we evaluated the antimicrobial and antibiofilm properties of *C. zedoaria* (CZ) rhizome extract against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA). MRSA infection is a major threat to healthcare facilities worldwide, and the treatment of highly biofilm-forming strains remains challenging due to multiple forms of antibiotics resistance [17]. The global increase in antibiotic-resistant bacteria has led to a search for new strategies to combat their associated infections. Nowadays, the treatment of antibiotic-resistant bacteria with natural extracts has been proposed as an alternative and antimicrobial adjuvant [18]. Several studies have reported the antimicrobial activity of CZ extract against both pathogenic Gram-positive and Gram-negative bacteria [19–21]. In this study, the agar disc diffusion method showed an inhibition zone of 8 mm at a CZ extract concentration of 2.5 mg against both strains, MRSA and MSSA. This observation was similar to the study of Chachad *et al.* (2015), who reported the inhibitory effect of ethanol extract of CZ rhizome against *S. aureus* with an inhibition zone of 16 mm at a concentration of 40 mg/ml [22]. Another study also revealed that the water extract of rhizomes of CZ showed an inhibition zone of 9.8 mm at a concentration of 30 mg [23].

In previous studies of the minimum inhibitory concentration (MIC), CZ tubes extract showed inhibitory effects against six bacterial pathogens, both Gram-positive and Gram-negative, with MIC values ranging from 0.01 to 0.15 mg/ml, but did not inhibit the growth of *S. aureus* [20]. In contrast to our study, the ethanolic extract of CZ rhizome showed inhibitory effects against *S. aureus*. In another study by Huang *et al*. (2019), the steam extract of rhizomes of CZ Rosc showed inhibitory effects against MSSA (ATCC 29213) and four clinical isolates of MRSA with MIC values ranging from 1 to 2 mg/ml [24]. A similar pattern of antimicrobial results was obtained in this study. Interestingly, the ethanolic extracts of CZ rhizome were found to have more of a potent inhibitory effect against tested strains with lower MIC values of 0.625 mg/ml (Table 1). The difference in antimicrobial activity may be due to various conditions such as differences in extraction agent, age and time of harvesting, and geographical area. These results indicated that CZ rhizome extract demonstrated promising antibacterial activity against MRSA and MSSA.

Biofilm formation plays an important role in the pathogenesis and antimicrobial resistance of several pathogenic bacteria [25]. The biofilm-forming ability of MRSA has been reported to increase pathogenicity and antimicrobial resistance [26, 27]. Therefore, the treatment of highly biofilm-forming strains is more difficult than that for non-biofilm-forming strains [28, 29]. This is the first study to evaluate the effect of CZ extract on the biofilm formation of MRSA and MSSA. The results of our study showed that CZ extract at sub-MIC ranges from 1/16 MIC to 1 MIC significantly reduced the production of MSSA biofilm, while the MRSA biofilm was significantly decreased at lower concentrations of 1/32 MIC to 1 MIC (Figs. 2A and 2B). This result indicated that MRSA biofilm was more easily inhibited by CZ extract than that of MSSA biofilm. The antibiofilm activity of CZ extract has not been reported yet, while other species such as *C. longa* (turmeric), belonging to the Zingiberaceae family, have been widely evaluated in terms of their antimicrobial and antibiofilm activity against various microorganisms. Newton Suwal *et al.* (2021) reported that *C. longa* Linn. rhizome ethanolic extract

showed antibiofilm activity against clinical isolates of *S. aureus* and *Pseudomonas aeruginosa* at a concentration of 0.5–2 mg/ml [30]. This finding was consistent with our study; CZ extract reduced biofilm formation at concentrations ranging from 0.019 mg/ml (1/32 MIC) to 0.625 mg/ml (1 MIC) against MRSA and MSSA. In addition, Sumreen Hayat *et al.* (2018) also demonstrated that the chloroform extract of turmeric inhibits biofilm and exopolysaccharide (EPS) production at a sub-MIC concentration of 64 μg/ml in *S. aureus* [31]. Although the CZ extracts presented antibacterial and antibiofilm activities in the current study, little is known about the effects on biofilms produced by MRSA and MSSA. Since curcumin, bisdemethoxycurcumin, and demethoxycurcumin were identified and quantified in this extract, several studies have demonstrated the antibiofilm activity of curcumin through various mechanisms such as the inhibition of bacterial quorum sensing (QS) in *P. aeruginosa, Vibrio* spp., and *S. aureus* [32–34], decreasing of exopolysaccharide (EPS) production [31, 35], and decreasing of bacterial cell adhesion and attachment [36]. Batista de Andrade Neto *et al.* (2021) reported that curcumin was able to reduce the cell viability of MRSA biofilm formation [37]. In addition, Maya Moshe *et al.* (2011) also showed that curcumin reduces *S. aureus* biofilm formation by imposing membrane damage [38]. Moreover, demethoxycurcumin, a major constituent of curcuminoids found in this study, has also been reported for antibiofilm activity in *Candida albicans* [39], while bisdemethoxycurcumin reduced biofilm formation in MRSA [40].

Factually, the adhesion of the bacterial cell to abiotic or biotic surfaces is the initial step in biofilm formation. Therefore, the reduction of MRSA and MSSA biofilm after being treated with CZ extract was confirmed in this study by cell attachment assay on a 96-well microplate. The results indicated that bacterial cell-surface attachment to the microplate was significantly inhibited by CZ extract at sub-MIC concentrations ranging from 1/32 MIC to 1 MIC for MRSA and 1/8 MIC to 1 MIC for MSSA. These results demonstrated the inhibition activity of CZ extract against MRSA and MSSA biofilm formation by preventing bacterial cell-surface attachment. It is worth noting that the percentage of initial attachment is proportional to the percent of biofilm formation in both tested strains. The corresponding initial attachment and biofilm formation has been shown by several studies [41–43]. On the other hand, ampicillin failed to inhibit cell-surface attachment and biofilm production in MRSA. While, ampicillin can inhibit both cell-surface attachment and biofilm formation of MSSA. Previous studies have reported that low doses of β-lectam antibiotics such as ampicillin, penicillin, and amoxicillin enhanced MRSA biofilm formation [44, 45]. The mechanism of ampicillin-induced biofilm production and cell-surface attachment is not well understood. It was proposed that gene-encoding antibiotic resistance such as penicillin-binding protein PBP1, PBP1a/2, and PBP3, and many antimicrobial resistance proteins were up-regulated in an ampicillin-induced biofilm strain of *S. aureus*. In addition, gene encoding cell-surface proteins that promote bacterial adhesion and enhance biofilm viability were also up-regulated [46].

As a result of CZ extract reducing bacterial attachment and the development of biofilm, we further hypothesized that this extract may interfere with the release of eDNA, a component of extracellular matrix (ECM) released in biofilm biomass by several bacteria, including *S. aureus* [47, 48], and is essential for initial adhesion and biofilm maturation [49]. Interestingly, QuantiFluor dsDNA System analysis demonstrated that CZ extract reduced the amount of eDNA released in a dose-dependent manner after treatment with CZ extract in both tested strains (Fig. 4). Nonetheless, the underlying mechanism of action for CZ extract against eDNA released from bacterial cells is unknown and needs to be investigated further. In addition, the quantity of eDNA and the amount of biofilm biomass was positively correlated in both tested strains (Table 2), which is similar to the findings of Cortes *et al*. (2015), who reported a positive correlation between the amount of biofilm and eDNA quantity in biofilm biomass in different MRSA isolates [50]. This finding suggests that CZ extract is effective against the biofilm formation of MRSA and MSSA.

This study determined that *C. zedoaria* rhizome ethanolic extract is effective against the biofilm-forming ability of MRSA and MSSA by reducing eDNA release and cell-surface attachment *in vitro*. Further investigations on the underlying mechanism of CZ extract destroying biofilm formation should provide greater insight into the role of this extract, and a study of the combination of CZ extract and antibiotics could be enhanced the efficiency of antibiofilm. These findings provide a baseline for

using CZ extract in the development of effective antibiofilm approaches to improve the treatment of biofilm-related infections by MRSA and MSSA.

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Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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