

Antibacterial activity of diacetylcurcumin against *Staphylococcus aureus* results in decreased biofilm and cellular adhesion

Janaina de Cássia Orlandi Sardi,¹ Carlos Roberto Polaquini,² Irlan Almeida Freires,¹ Livia Câmara de Carvalho Galvão,¹ Josy Goldoni Lazarini,¹ Guilherme Silva Torrezan,² Luis Octávio Regasini² and Pedro Luiz Rosalen^{1,*}

Abstract

Purpose. *Staphylococcus aureus* infections have contributed to the global healthcare burden, particularly with regard to hospital-acquired methicillin-resistant *S. aureus* (MRSA) infections.

Methodology. This study describes the antibacterial activity of diacetylcurcumin (DAC) against methicillin-susceptible *S. aureus*/MRSA biofilm formation, survival, metabolic activity and structure; its ability to prevent bacterial adhesion to human cells; and toxicity in *Galleria mellonella* larvae.

Results. DAC showed excellent antibacterial activity, with MIC ranging between 17.3 and 34.6 $\mu\text{mol l}^{-1}$, and minimum bactericidal concentration ranging between 69 and 277 $\mu\text{mol l}^{-1}$. It significantly reduced bacterial biofilm survival – by 22–63 % (at MIC, 10 \times MIC or 100 \times MIC) as compared to the 25–42 % reduction by vancomycin ($P < 0.0001$) – and severely affected biofilm cell structures, leading to damaged architecture and the formation of amorphous cell clusters. Treatment with DAC (MIC/4) decreased bacterial adhesion to HaCaT keratinocytes from 1 to 5 h ($P < 0.0001$). Finally, DAC demonstrated low toxicity in *G. mellonella* at its effective anti-biofilm concentrations.

Conclusion. These findings open new avenues for the study of this curcumin derivative as an excellent prototype with anti-MRSA activity.

INTRODUCTION

Staphylococcus aureus is considered to be the main causative agent of community- and hospital-acquired infections [1], most of which occur in asymptomatic carriers. Short- or long-term *S. aureus* colonization becomes an issue when the individual's immune system is compromised [2]. Hence, the outcomes of *S. aureus* infections are seen as a serious public-health issue, particularly in the hospital setting where clones resistant to methicillin and/or to other antibiotics are endemic and may increase the risk of death [3]. Since methicillin-resistant *S. aureus* (MRSA) was first described in the 1960s as a major nosocomial pathogen, its incidence in infections has continued to rise worldwide in healthcare institutions and, more recently, in the community setting. This bacterium has been detected in about 30 to 50 % of

healthy individuals. In the USA, 1/100 individuals are colonized with MRSA, which has raised a serious concern due to its transmissibility by direct contact, predisposing the population to life-threatening infections [4, 5]. MRSA infections have become a major, difficult-to-treat clinical issue, especially in severe cases, due to the limited treatment options currently available [2]. *S. aureus* is commonly associated with artificial surfaces, including prosthetic orthopaedic implants, heart valves, pacemakers and vascular catheters [5, 6]. Hence, an area of primary concern with MRSA biofilm infections is the rapid increase in the use of medical implants and prostheses, and the concomitant rise in device-related infections [5–7].

Biofilm production is an important virulence factor of micro-organisms associated with chronic infections, such

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Author affiliations: ¹Department of Physiological Sciences, Piracicaba Dental School, University of Campinas, 13414-903 Piracicaba, São Paulo, Brazil; ²Department of Chemistry and Environmental Sciences, São Paulo State University Júlio de Mesquita Filho, São Jose do Rio Preto, São Paulo, Brazil.

*Correspondence: Pedro Luiz Rosalen, rosalen@fop.unicamp.br

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Abbreviations: DAC, diacetylcurcumin; MBC, minimum bactericidal concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; SEM, scanning electron microscopy; XTT, sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide inner salt.

as sinusitis, otitis media, cholecystitis, prostatitis, osteomyelitis, skin chronic infections and infections associated with foreign bodies (implants and catheters), with *S. aureus* being frequently identified as an important agent responsible for such infections [8]. Both *S. aureus* and *Staphylococcus epidermidis* are the most clinically significant pathogens among Gram-positive bacteria able to form biofilms [9]. The ability of these bacteria to adhere to the abiotic surfaces of medical devices and form robust biofilms thereon contributes to the pathogenicity of staphylococci infections [10]. After a medical device is installed, its polymer-based material is rapidly covered with plasma proteins and extracellular matrix, thereby enhancing microbial colonization of its surfaces [11]. In addition to indirect bacterial binding to the polymer, direct non-specific binding may also occur, mainly due to electrostatic and hydrophobic interactions promoted by bacterial surface proteins [12]. The rates of infection associated with medical devices in intensive care units of developing countries are higher than those of developed countries [13]. In view of this, there has been a collective effort to find or develop novel molecules with antimicrobial activity in order to increase the arsenal of drugs against infections caused by biofilm-forming resistant strains.

Curcumin is a polyphenol compound isolated from ground rhizomes of the plant *Curcuma longa* L. [14]. This plant has a broad spectrum of biological actions [15, 16], including antioxidant, analgesic, anti-inflammatory, antiseptic, anticancer, antiviral, antibacterial, antifungal and antiplatelet activities [17, 18]. Curcumin has been widely used in Ayurvedic medicine for centuries, with no reports of toxicity [17, 18]. Over the past 50 years, research has shown that polyphenols such as curcumin play an important role in health maintenance and disease prevention [16]. A number of studies have demonstrated that *C. longa* extract and fractions have antibacterial activity against pathogenic bacteria, including *S. aureus* [19, 20]. A study showed that the MICs of curcumin, the main compound isolated from *C. longa*, ranged from 125 to 250 $\mu\text{g ml}^{-1}$ against ten methicillin-susceptible *S. aureus* (MSSA) and MRSA strains. In addition, a checkerboard combinatorial test indicated that curcumin reduced by 2- to 128-fold the MICs of antibiotics commonly used against MRSA, such as oxacillin, ampicillin, ciprofloxacin and norfloxacin, thereby demonstrating the potential clinical effectiveness of curcumin and its derivatives to treat MRSA infections [19]. Many other authors have also studied the action of curcumin on fungal pathogens [21, 22].

Much effort has been made to develop more potent, effective and well-tolerated drugs. Hence, synthetic chemical modification has been prominent in the design of new compounds with enhanced antibacterial activity and fewer toxic effects. Previous studies performed by Changtam *et al.* [23] demonstrated that a derivative molecule of curcumin, named diacetylcurcumin (DAC), was effective against *Mycobacterium tuberculosis*. DAC is a synthetic derivative of curcumin in which the two phenolic hydroxyl (HO-) groups are replaced

by acetyl groups (CH_3COO^-) [24]. Herein, we investigated the antibacterial activity of DAC against MSSA and MRSA biofilm formation, metabolic activity and structure. We further tested the ability of DAC to prevent bacterial adhesion to human cells, and determined its toxicity *in vivo* using the *Galleria mellonella* model.

METHODS

Synthesis of DAC

DAC was synthesized by a curcumin-acetylation reaction according to Changtam *et al.* [23]. Acetic anhydride (25 ml) was added to a solution of curcumin (1.1 g, 3 mmol) in pyridine (25 ml) and the reaction mixture was kept under magnetic stirring at 100 °C. After 96 h, the residue was partitioned with ethyl acetate (3 × 25 ml), washed with H₂O (3 × 25 ml) and dried at room temperature. The crude product was purified over a silica gel column eluted with mixtures of hexanes and ethyl acetate. The molecular structure of DAC was established by ¹H and ¹³C NMR spectral analysis. For the microbiological assays, DAC was diluted following the protocol proposed by Scorzoni *et al.* [25].

Micro-organisms

S. aureus ATCC 25923 (MSSA) and *S. aureus* ATCC 33591 (MRSA) strains were used in this study. Both strains were maintained as frozen stocks at -80 °C until use. For the assays, the strains were subcultured onto trypticase soy agar (TSA), and a single colony was inoculated into trypticase soy broth (TSB) medium and incubated at 37 °C for 24 h.

Determination of MIC and minimum bactericidal concentration (MBC)

The MIC of DAC was determined by the microdilution technique according to the Clinical and Laboratory Standards Institute protocol M07-A9 [26]. DAC was diluted and tested at concentrations ranging from 553 to 1.0 $\mu\text{mol l}^{-1}$. Vancomycin (Sigma-Aldrich) was used as a standard drug, 2 % DMSO (v/v; vehicle) was used as a negative control and culture medium free of any other agent was included to check for sterility. The MIC was defined as the lowest concentration of the molecule that inhibited visible microbial growth.

The MBC was determined by subculturing aliquots from the wells corresponding to the MIC and above onto TSA plates, which were incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration of the molecule that allowed no visible growth on the solid medium.

Effects of DAC on *S. aureus* and MRSA adhesion to HaCaT cells

Cell cultures of human keratinocytes (HaCaT) were obtained from the Bank of Cells of Rio de Janeiro (Rio de Janeiro, Brazil). Cells were maintained in Dulbecco's medium (DMEM) supplemented with 10 % fetal bovine serum (Gibco) plus 100 U penicillin ml^{-1} , 100 μg streptomycin sulfate ml^{-1} and 200 mM L-glutamine at 37 °C, 5 % CO₂. In this assay, the inhibitory effects of DAC against *S. aureus*/

MRSA adhesion to HaCaT cells were investigated. First, an adhesion curve (0, 2, 3, 4 and 5 h) was plotted to determine the initial time of bacterial adhesion to the human cells. The adhesion assay was performed using 24-well plates containing 1×10^5 cells per well, according to Sardi *et al.* [27] with minor modifications. After formation of monolayers of cells in the wells, aliquots of 500 μ l bacterial inocula (5×10^3 c.f.u. ml^{-1}) were added to each well. The plates were incubated at 36.5 °C and 5 % CO_2 for the previously mentioned times. After each incubation time, the cells were washed three times with sterile PBS and then trypsinized. Aliquots of 100 μ l were plated for c.f.u. measurement onto TSA plates and incubated at 37 °C for 24 h. The effects of DAC on bacterial adhesion to HaCaT cells were evaluated by adding the compound at MIC/4 (final concentration) simultaneously with the inoculum. This concentration was chosen as it ensures that bacterial growth and survival are not affected by treatment with the antimicrobial substance [28]. After 1, 2, 3, 4 and 5 h of adhesion the cells were washed three times with sterile PBS, trypsinized and plated for c.f.u. measurement onto TSA plates. The percentage of inhibition of adhesion was calculated based on the final number of adhered bacteria in relation to an untreated group indicating 100 % bacterial adhesion. In order to demonstrate that DAC directly affects the mechanism of bacterial adhesion to keratinocytes instead of decreasing adhesion indirectly by limiting bacterial growth and survival, we included a control with DAC-treated (MIC/4) cells and culture medium.

Effects of DAC on biofilm formation and preformed biofilm survival

Effects on biofilm formation

An aliquot of 100 μ l standardized cell suspension (1×10^8 c.f.u. ml^{-1}) was added to the wells of 96-well microplates. The plates were incubated at 37 °C for 2 h to allow for cell adhesion. Then each well was washed with PBS to remove non-adherent yeasts, and the forming biofilms were treated with DAC at MIC, 10 \times MIC or 100 \times MIC (final concentration) for 24 h at 37 °C. After incubation, biofilms were washed to remove planktonic and killed cells, and then serially diluted and plated for c.f.u. measurement, as described below (in the quantification of c.f.u. section). Vancomycin was used as a standard drug, and a negative control with culture medium alone was also included.

Effects on preformed biofilms

A total of 100 μ l bacterial inoculum (OD_{600} 0.08–1.0) was added to TSB plus 0.1 % glucose (TSB-g). The medium was added to each well of a 96-well cell culture-treated polystyrene microtiter plate with a final inoculum concentration of 1×10^7 c.f.u. per well. The plates were incubated at 37 °C for 24 h to allow for biofilm formation [29]. Following mature biofilm formation, the wells were washed three times with 200 μ l sterile saline (0.85 %) to remove planktonic cells. Then 100 μ l TSB-g containing the DAC (MIC, 10 \times MIC and 100 \times MIC) was added. TSB-g was added to wells with and without bacterial inoculum to serve as controls. The plates were incubated at 37 °C for 24 h. After the incubation

period, the plates were washed once with PBS and processed for semi-quantitative (sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide inner salt (XTT) assay) or quantitative (counting of c.f.u. ml^{-1}) analysis.

XTT assay

The XTT reduction assay was used to determine the metabolic activity of DAC-treated biofilms [30]. After treatment, biofilms were washed once with PBS, and the plates were incubated at 37 °C for 2 h with 100 μ l XTT (1.0 mg XTT ml^{-1} and 1 mmol menadione l^{-1}). Conversion of the XTT substrate to a soluble coloured formazan product correlates with cell viability. The resulting absorbance was read at 490 nm using a microplate reader (ASYS UVM 340).

Quantification of c.f.u.

After treatment with DAC, biofilms were scraped from the bottom of the wells using a 100 μ l micropipette to allow for dissociation of cells. A volume of 100 μ l containing the cell suspension was aspirated from the wells, transferred to a tube containing 900 μ l PBS and vortexed for 3 min. Then serial dilutions were carried out and 100 μ l of each suspension was plated onto TSA plates. The survival (percentage of c.f.u.) was determined comparatively based on the survival of *S. aureus*/MRSA untreated biofilms.

Effects of DAC on biofilm structure

Scanning electron microscopy (SEM) analysis was carried out to evaluate the effects of DAC on the structure and integrity of *S. aureus*/MRSA biofilms. First, biofilms were grown for 24 h at 37 °C on tissue-culture-treated chambered glass slides (Corning BD Falcon), washed three times with PBS to remove planktonic cells, and then treated with DAC at different concentrations (MIC, 10 \times MIC, 100 \times MIC). A negative control group with untreated biofilm was also included. After 24 h, the samples were washed twice and maintained in 2.5 % glutaraldehyde/PBS (v/v, pH 7.4) for 2 h at room temperature. The slides were serially dehydrated with ethanol (from 50 to 100 %) for 5 min, coated with gold at 40 mA (BAL-TEC SCD 050) and observed using a scanning electron microscope (Jeon JSM 5600LV) [31].

Systemic toxicity of DAC in *G. mellonella* larvae

This assay was carried out to evaluate the acute toxicity of DAC in a *G. mellonella* alternative model, as previously described, with modifications [32]. A total of 20 healthy larvae were randomly selected for each group, weighing between 0.2 and 0.3 g. The larvae were chilled on ice for 20 min and had their prolegs cleaned with 70 % ethanol. Five microlitres DAC at different concentrations (MIC, 10 \times MIC, 100 \times MIC) and the vehicle (DMSO) were injected into the haemocoel of each larva through the last left proleg by a trained operator using a 25 μ l Hamilton syringe (Hamilton). After injection, the larvae were incubated at 37 °C and their survival was monitored at selected intervals over 72 h. The larvae unable to move when touched and showing high levels of melanization were counted as dead.

Statistical analysis

All assays were performed in triplicate for three independent experiments. The data concerning the biofilm assays were analysed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, with a significance level of 5%. For the *G. mellonella* toxicity model, Kaplan–Meier killing curves were plotted on GraphPad Prism 5.0 and estimations of differences in survival were compared using the log-rank test.

RESULTS

Antibacterial activity against planktonic cells

DAC was tested for its ability to inhibit bacterial growth of *S. aureus* ATCC 25923 (MSSA) and ATCC 33591 (MRSA) strains. Table 1 shows the MIC and MBC values of DAC and vancomycin (a standard drug) against MSSA and MRSA. The MIC values of DAC ranged from 17.3 to 34.6 $\mu\text{mol l}^{-1}$ and the MBC values from 69.2 to 277.1 $\mu\text{mol l}^{-1}$, suggesting that it is a potent bacterial inhibitor. The MBC/MIC ratio was indicative that DAC has predominantly bacteriostatic activity against these strains. Vancomycin also showed low MIC (0.69 to 1.4 $\mu\text{mol l}^{-1}$) and MBC (5.52 to 11.0 $\mu\text{mol l}^{-1}$) values.

Inhibitory effects of DAC on *S. aureus* and MRSA adhesion to human cells

The inhibitory effects of DAC on bacterial adhesion to HaCaT cells were investigated. As seen in Fig. 1, treatment with DAC at MIC/4 led to a significant decrease of adhesion in both strains from 1 to 5 h ($P < 0.0001$) when compared to the untreated group. The inhibitory effects were more pronounced against MSSA (43 to 66%) than MRSA (34 to 46%). These findings show that DAC (used at a sub-inhibitory concentration) directly affects the mechanism of *S. aureus* adhesion to keratinocytes.

Effects of DAC on biofilm formation and preformed biofilm survival

DAC was tested for its ability to inhibit biofilm formation (Fig. 2) and survival of preformed biofilms (Figs 3 and 4). Treatment with DAC at 10 \times MIC and 100 \times MIC caused a

Table 1. MIC and MBC values of DAC and vancomycin (a standard drug) against *S. aureus* ATCC 25923 (MSSA) and *S. aureus* ATCC 33591 (MRSA)

Values are expressed in $\mu\text{mol l}^{-1}$.

Group	<i>S. aureus</i> ATCC 33591 (MRSA)			<i>S. aureus</i> ATCC 25923 (MSSA)		
	MIC	MBC	MBC/MIC ratio	MIC	MBC	MBC/MIC ratio*
DAC	34.6	277.1	8	17.3	69.2	4
Vancomycin	1.4	11.0	8	0.69	5.52	8

*Bacteriostatic (MBC/MIC ≥ 4) or bactericidal (MC/MIC < 4) activity.

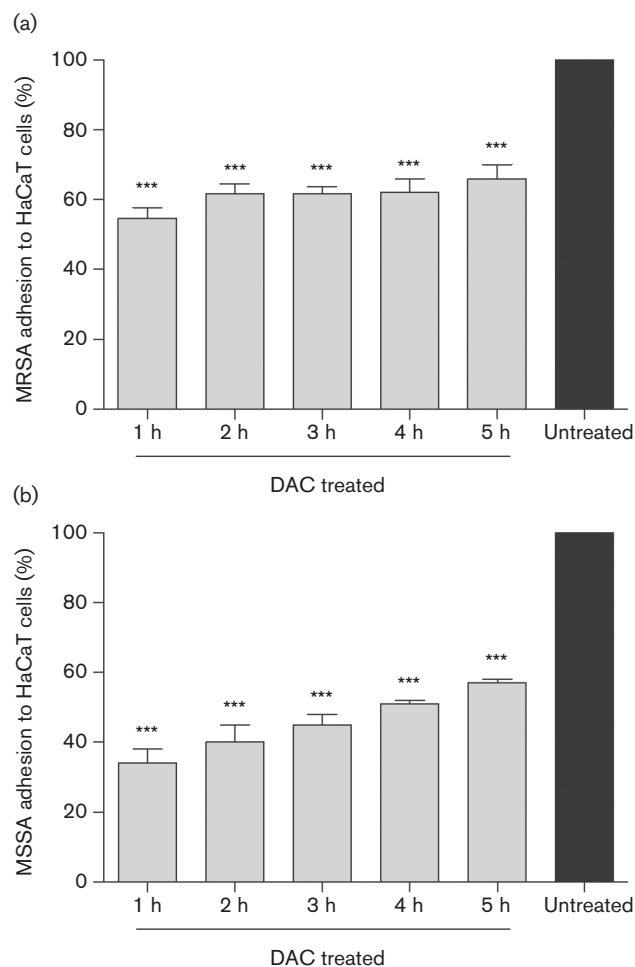


Fig. 1. Percentage inhibitory effects (mean \pm SD) of DAC on MRSA and MSSA adhesion to human keratinocytes (HaCaT). Treatment with DAC at MIC/4 led to a significant decrease of MRSA (a) and MSSA (b) adhesion to HaCaT cells over time as compared to the untreated group (***) $P < 0.0001$, ANOVA with Tukey's post-test).

significant decrease of MSSA and MRSA biofilm formation as compared to the untreated group ($P < 0.0001$). Furthermore, significant differences in biofilm formation were observed upon treatment with vancomycin, and when comparing the effects of DAC and vancomycin on biofilm formation (Fig. 2). As seen in Fig. 3, MSSA and MRSA preformed biofilms treated with DAC at 10 \times MIC and 100 \times MIC showed concentration-dependent reduced metabolic activity in both strains when compared to the untreated group ($P < 0.0001$). However, at MIC it did not affect the biofilm metabolic activity significantly ($P > 0.05$), similarly to vancomycin. In order to confirm these semi-quantitative findings, we carried out a quantitative assay for determination of c.f.u. ml^{-1} in treated biofilms. Fig. 4 shows that all concentrations of DAC led to a significant decrease of biofilm survival ($P < 0.0001$) as compared to the control, with better results for the concentration 100 \times MIC.

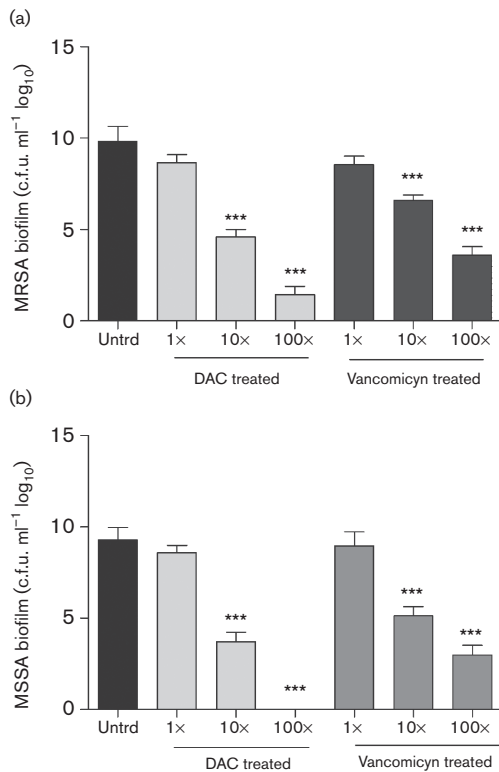


Fig. 2. Quantitative analysis (determination of c.f.u. ml⁻¹) of the inhibitory effects (mean±sd) of DAC and vancomycin on MRSA and MSSA biofilm formation. Treatment with DAC and vancomycin at 10×MIC and 100×MIC caused a significant decrease of MRSA (a) and MSSA (b) biofilm formation as compared to the untreated (untrd) group (***P*<0.0001, ANOVA with Tukey's post-test).

In brief, DAC reduced MSSA preformed biofilm survival by approximately 22 % (MIC), 32 % (10×MIC) and 63.7 % (100×MIC) as compared to 25 % (MIC), 33 % (10×MIC) and 42 % (100×MIC) for vancomycin. DAC-treated MRSA biofilms were reduced by 14 % (MIC), 27 % (10×MIC) and 53 % (100×MIC) as compared to 15 % (MIC), 28 % (10×MIC) and 42 % (100×MIC) for vancomycin. These findings support the view that the inhibitory effects of DAC on biofilm formation and survival are comparable to or more effective than those of the standard drug vancomycin (Figs 2, 3 and 4).

Effects of DAC on biofilm structure

SEM analysis was carried out to evaluate the deleterious effects of DAC on the structure and integrity of mature biofilms of MSSA and MRSA. It was observed that DAC severely affected biofilm cell structures at 10×MIC, and particularly at 100×MIC, leading to damaged architecture and formation of amorphous cell clusters (Fig. 4). The SEM photomicrographs also illustrate the reduced bacterial population in DAC-treated biofilms (Figs 4 and 5), which corroborates the findings of the quantitative analysis shown in Fig. 4.

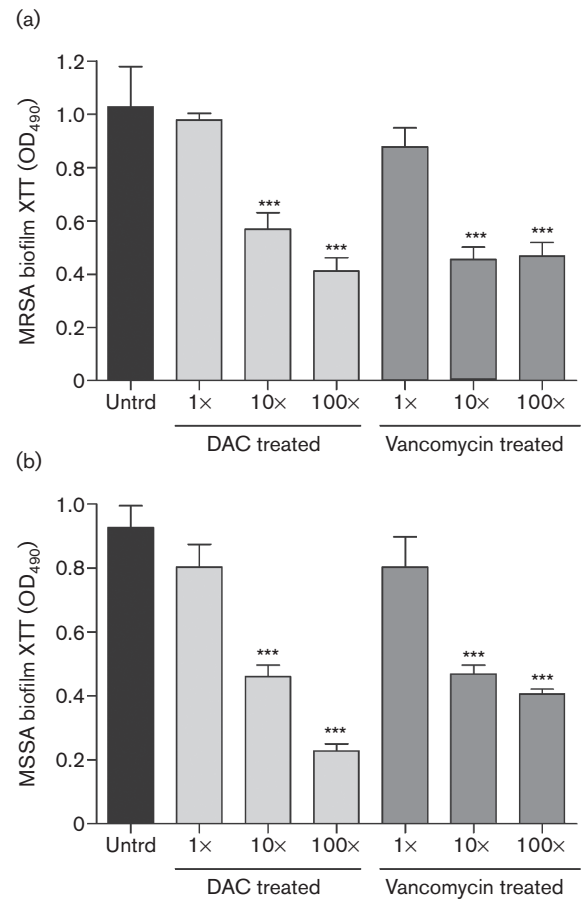


Fig. 3. Semi-quantitative analysis (XTT assay) of the inhibitory effects (mean±sd) of DAC and vancomycin on MRSA and MSSA preformed biofilm metabolic activity. Treatment with DAC and vancomycin at 10×MIC and 100×MIC led to a significant decrease of MRSA (a) and MSSA (b) biofilm metabolic activity as compared to the untreated (untrd) group (***P*<0.0001, ANOVA with Tukey's post-test).

Systemic toxicity of DAC in *G. mellonella* larvae

The *in vivo* toxicity of DAC was assessed using the *G. mellonella* alternative model, as previously described. The larvae were injected with DAC at concentrations of 10×MIC and 100×MIC, which showed significant anti-biofilm activity *in vitro* and correspond to the doses 3.12 and 31.2 mg (kg larvae)⁻¹, respectively. As shown in Fig. 6, intra-haemocoelic administration of DAC did not cause significant acute toxic effects in the larvae over a period of 72 h (*P*>0.05). DMSO was used as a vehicle and did not show statistically significant toxicity either (*P*>0.05).

DISCUSSION

MRSA has been held responsible for an increasing healthcare burden worldwide, with high asymptomatic carriage among healthcare workers and the population in general [5, 33]. To date, vancomycin is the most commonly administered drug in cases of infections associated with MRSA

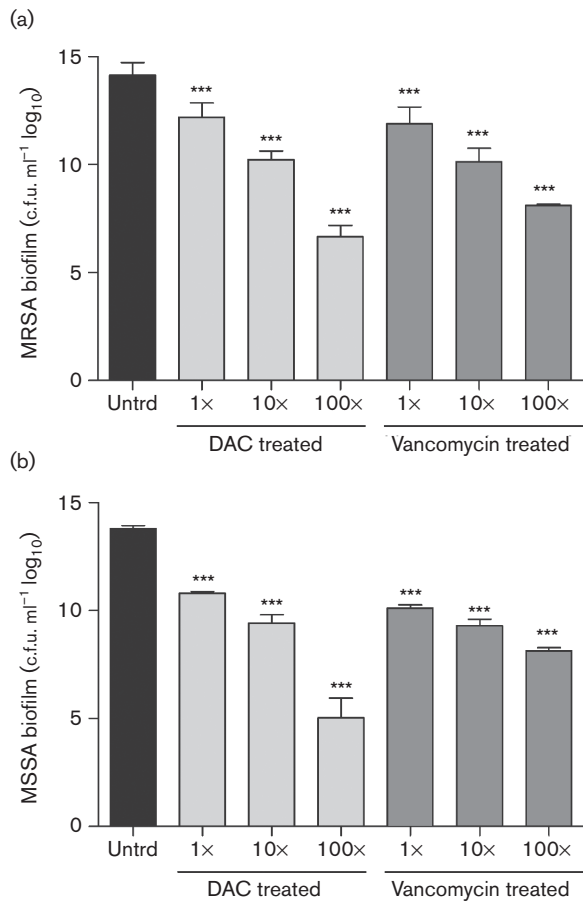


Fig. 4. Quantitative analysis (determination of c.f.u. ml⁻¹) of the inhibitory effects (mean±SD) of DAC and vancomycin on MRSA and MSSA preformed biofilm survival. Treatment with DAC and vancomycin at MIC, 10×MIC and 100×MIC caused a significant decrease of MRSA (a) and MSSA (b) mature biofilm survival as compared to the untreated (untrd) group (***) $P < 0.0001$, ANOVA with Tukey's post-test).

biofilms [34]. However, 9.3% of MRSA strains isolated from healthcare workers were found to be resistant to vancomycin [33]. Therefore, there has been a significant effort from the scientific community to develop novel target-specific drugs with potent antimicrobial activity to be used alternatively against multi-resistant strains. Herein, a DAC compound was synthesized and proved for what is believed to be the first time to have promising anti-MRSA activity comparable to that of vancomycin, as well as low toxicity *in vivo*.

Bacterial attachment to host cells is one of the early strategies for successful establishment of infection [35]. This process can be mediated by a number of components, such as adhesins, pili or fimbriae, and specific exopolysaccharides [35, 36]. As *S. aureus* is a frequent colonizer of skin and mucosal surfaces, we tested the ability of DAC to prevent bacterial adhesion to HaCaT cells. The findings indicated that at sub-inhibitory concentrations DAC significantly

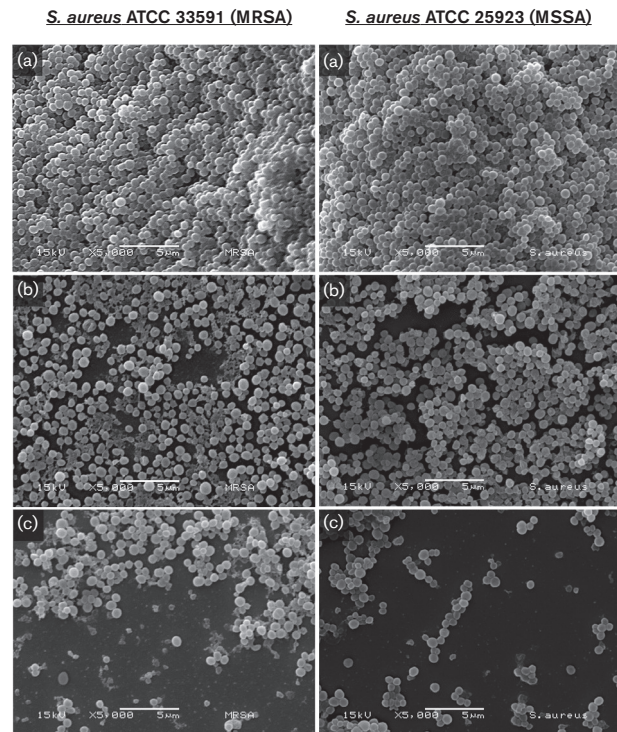


Fig. 5. SEM photomicrographs (×5000) showing MSSA and MRSA biofilm cells untreated (a), and treated with DAC at 10×MIC (b) and 100×MIC (c). Treated biofilms showed deleterious structural changes and reduced microbial population. Bars, 5 μm.

inhibited adhesion of both MSSA and MRSA to keratinocytes over time, which could contribute to the prevention of infection onset. Further studies should clarify the exact structures and mechanisms affected by DAC in the bacterial cell leading to disrupted adhesion.

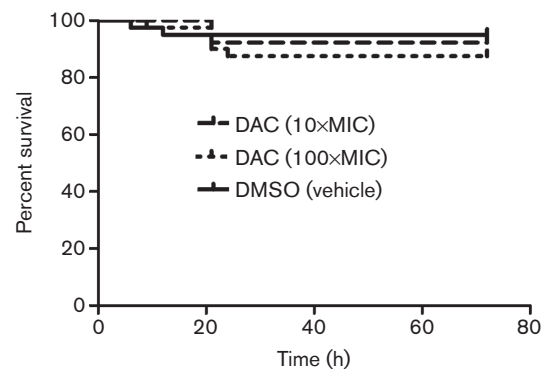


Fig. 6. Percentage survival over time of *G. mellonella* larvae injected with DAC at doses of 3.12 and 31.2 mg (kg larvae)⁻¹, which correspond to their effective anti-biofilm concentrations (10×MIC and 100×MIC, respectively) previously determined *in vitro* ($P > 0.05$, log-rank test).

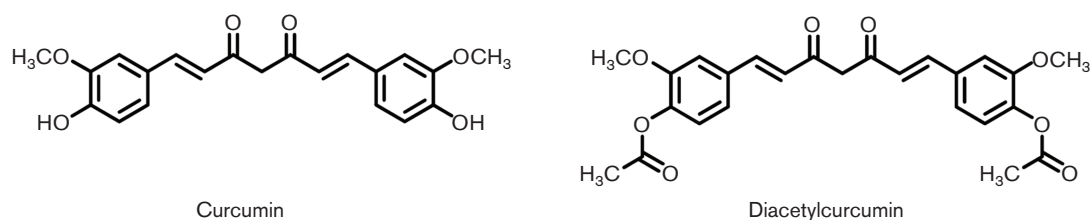


Fig. 7. Structure of curcumin and DAC. Curcumin and DAC have diphenylheptanoid skeletons; however, the substituent groups are different. Curcumin has the methoxy and phenol groups, but in DAC the phenol group is converted into an acetyl group, while the methoxy group is maintained. Physico-chemical data of DAC: yellow solid. Yield: 91 %. ¹H NMR (CDCl₃; 400 MHz) δ_H (mult.; J in Hz): 2.35 (s; 4'-OCOCH₃); 3.90 (s; 3'-OCH₃); 5.88 (s; H-4); 6.59 (d; J=16.0 Hz; H-2); 7.09 (d; J=8.0 Hz; H-5'); 7.15 (d; J=2.0; H-2'); 7.18 (dd; J=2.0 e 8.0 Hz; H-6'); 7.64 (d; J=16.0 Hz; H-1). ¹³C NMR (CDCl₃; 100 MHz) δ_C: 20.7 (4'-OCOCH₃); 55.9 (3'-OCH₃); 101.8 (C-4); 111.0 (C-2'); 121.1 (C-6') 123.3 (C-5'); 124.3 (C-2); 134.0 (C-1'); 140.0 (C-1); 141.3 (C-4') 151.4 (C-3'); 168.8 (4'-OCOCH₃); 183.1 (C-3).

A number of studies have described the potential of curcumin as an antimicrobial agent [37–39], but very little research has focused on its biconjugate analogue DAC. Mishra *et al.* [40] showed that DAC has antibacterial activity against multi-resistant Gram-positive and Gram-negative bacteria, including *S. aureus*, but there are no reports of its anti-biofilm effects. Semi-quantitative and quantitative data suggest that DAC may have clinically significant effects against MSSA and MRSA biofilm formation, and survival of preformed biofilms as the results were as satisfactory as or more effective than those of vancomycin. Mun *et al.* [19] demonstrated that curcumin exhibited inhibitory activity on MRSA strains, with MIC values of 125 to 250 µg ml⁻¹. Our results showed that DAC has action at much lower concentrations (7.8 to 15.6 µg ml⁻¹). Conversion of the phenolic hydroxyl group (HO–) present in the structure of curcumin into the acetyl group (CH₃COO–) in DAC (Fig. 7) resulted in the potentiation of the antibacterial activity of the latter. This change results in increased lipophilicity, which may be related to greater capacity of penetration through the biomembranes [41, 42].

Teow *et al.* [43] suggested that curcumin activity is not altered by the drug-resistance machinery in *S. aureus*, since in other studies it did not show differences in MIC values against MSSA and MRSA [19]. However, our results showed that MSSA was more sensitive to treatment with DAC than MRSA, indicating that the activity of DAC is affected by resistance mechanisms.

It has been well established that bacteria growing in biofilms are considerably less susceptible to the action of antibiotics than those in the planktonic form. During biofilm development, bacteria may evade host defences and become tolerant to high concentrations of antimicrobials, making infections particularly difficult to eradicate [5, 44, 45]. The effective concentration of antimicrobials against biofilms can be up to 10- to 1000-fold higher than that against planktonic cultures [46–48]. Therefore, the concentrations 10×MIC and 100×MIC were selected in this study for the treatment of forming and mature biofilms. A recent study by Manner *et al.* [49] showed that dehydroabietic acid – a

diterpenoid abundant in the resin of coniferous trees – prevents *S. aureus* biofilm formation and that 2- to 4-fold higher concentrations of this compound are needed to significantly reduce the viability and biomass of preformed *S. aureus* biofilms. In this study, we demonstrated that 10- to 100-fold higher concentrations of DAC were able to reduce bacterial population and disrupt the conformation of mature MSSA and MRSA biofilms in a concentration-dependent manner.

In order to provide preliminary evidence on the short-term toxicity of DAC for future clinical use, we carried out an assay with the invertebrate model of *G. mellonella* larvae. This validated model is broadly used in the international literature and indicates the acute toxic effects over time of exogenously administered substances [32]. The results of this study showed that DAC has no significant toxic effects *in vivo* when tested at its effective anti-biofilm concentrations, which is encouraging for clinical use. The *G. mellonella* model has several advantages over other ones, including quick and low-cost data generation, and in particular it is an alternative approach to the use of mammals for primary toxicological assessment, therefore reducing the number of vertebrate animals for experimentation and refining subsequent studies [50].

Conclusion

It may be concluded that DAC has strong anti-biofilm activity against MSSA and MRSA strains comparable to that of vancomycin. In addition, it is able to inhibit bacterial adhesion to human cells and has low toxicity in the *G. mellonella* model. These findings open new avenues for the study of this curcumin derivative as an excellent prototype with anti-MRSA activity. Further studies should focus on its mechanism of action and long-term toxicity in other relevant models.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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