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Chalcone derivatives as efflux pump inhibitor of *Staphylococcus aureus*

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Abstract: *Staphylococcus aureus* (S. aureus) is the most important gram-positive nosocomial pathogen. Emerging drug resistance among *S. aureus* isolates is a global concern and the mechanism developed by *S. aureus* to become resistant to existing antibiotics makes it even more difficult to treat in clinical settings in immunocompromised patients. Efflux pumps are involved in low levels of drug resistance and a combination of drugs with efflux pump inhibitors showed promising reduction in MIC values in many studies. In present studies a series of Chalcones were screened as efflux pump inhibitors and antibiotic modulators in combination with ciprofloxacin using *S. aureus* strains and the results obtained are promising.

Keywords: *Staphylococcus aureus*, NorA, Efflux pump inhibition, Chalcones.

Introduction:

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterium that belongs to the *Staphylococcus* genus. It is a highly versatile and prevalent bacterium found in various environments and commonly inhabits the skin and mucous membranes of humans and animals. It is a major cause of skin and soft tissue infections, as well as more serious infections such as pneumonia, bloodstream infections, and endocarditis (Abebe and Birnahu, 2023). One of the ways that *S. aureus* can cause infections is through the formation of biofilms and another major concern is development of resistance to multiple antibiotics, which has become a significant challenge in the treatment of infections caused by this pathogen, especially the emergence of methicillin-resistant *S. aureus* (MRSA) (Kaur et al., 2021). The development of drug resistance in *S. aureus* is primarily due to the acquisition of resistance genes through horizontal gene transfer, a process in which bacteria can exchange genetic material with other bacteria in their environment. Resistance genes can also arise through mutations in the bacterial genome (Reygaert, 2018).

Antibiotic resistance mediated by efflux pumps has emerged as one of the most significant mechanisms of resistance in *S. aureus* (Adefisoye and Olaniran, 2023). The NorA efflux pump plays a pivotal role in conferring multidrug resistance by actively expelling various structurally unrelated antibiotics from the bacterial cell, thereby reducing intracellular drug concentrations to sublethal levels (Santos et al., 2023).

The NorA efflux pump, a member of the multidrug and toxic compound extrusion (MATE) family, is one of the most well-studied efflux pumps in *S. aureus*. Nor A actively pumps out structurally diverse antimicrobial compounds from the bacterial cell, conferring resistance to a wide range of antibiotics, including fluoroquinolones, tetracyclines, and beta-lactams (Stephen, 2023; Costa, 2011). Additionally, *S. aureus* possesses a global regulatory system known as the accessory gene regulator (Agr), which plays a pivotal role in coordinating virulence factor expression and pathogenicity. This quorum-sensing system also contributes to antibiotic resistance by promoting biofilm formation, which provides a protective environment for bacterial survival and fosters horizontal gene transfer of resistance genes (Hajhamed et al., 2023). Interestingly, there is evidence suggesting that Agr may also contribute to antibiotic resistance by modulating the expression of various efflux pumps, including NorA. The overexpression of NorA and Agr is a major mechanism of antibiotic resistance in *S. aureus*, and inhibitors of these efflux pumps and quorum-sensing systems have been identified as potential targets for the development of new antimicrobial agents (Hajhamed, 2023; Kaur, 2021).

In this study the potentiating effect of chalcones derivatives was studied in combination with ciprofloxacin using agar diffusion assay and in vitro combination studies against *S. aureus*,

followed by its putative role as an antibiotic modulator and efflux pump inhibitor.

Material and methods:

Bacterial cultures and chemicals used:

The bacterial culture *Staphylococcus aureus* ATCC 6538 used in the current research was obtained from the American Type Culture Collection (Manassas. Va.). In addition, NorA over-expressing *S. aureus* strain SA-1199B was generously provided by Dr Nitin Pal Kalia of NIPER Hyderabad, India. All other chemicals including antibiotics used during this study were purchased from Hi Media Labs India. The chalcones derivatives were provided by Dr Gopal Kathik from pharmaceutical department of Lovely Professional University, India.

Growth conditions and media:

Mueller-Hinton Broth (MHB) (HiMedia Labs India) was used for all screening, minimum inhibitory concentration (MIC) determination, and checkerboard studies. Mueller- Hinton Agar (MHA) (HiMedia Labs, India) was used for the conduct of agar well diffusion assay and Trypticase Soya Agar (TSA) (HiMedia) was used for maintaining bacterial cultures. Growth conditions were optimized for 18–24-hour period at 37°C.

Agar diffusion assay:

Disk diffusion assay was performed as per CLSI guidelines. The overnight grown culture of *S. aureus* ATCC 6538 was adjusted to 0.5 McFarland and then final bacterial inoculum of 10^6 cfu/Ml was mixed with molten MHA. Different chalcone derivatives designated as **C1, C3, C4, C5, C7-C11, C13, C15,** and **C16** at 12.5, 25, 50 µg/mL concentration were dissolved in DMSO. The 50µl of different concentrations of chalcone derivatives were loaded in wells to check for individual antimicrobial activity, while for combination 25µl of different concentrations of chalcone derivatives and 25µl of ciprofloxacin at 5µg/ml were used. Plates were incubated over night at 37°C. The zone of inhibition (ZOI) observed if any, was measured in mm (Atef et al, 2019).

In-vitro combination studies of Chalcone derivatives:

The broth chequerboard microdilution method is the most frequently used technique for in vitro combination studies. The ciprofloxacin and chalcone derivatives as combination were tested in MHB (pH 7.0) against *S. aureus* SA-1199B (NorA overproducing). The experiment was performed in 96-well U-bottomed plates (Tarson, India). Ten 2-fold serial dilutions of ciprofloxacin, ranging from 0.03 to 64 mg/L, were prepared in the presence of chalcone derivatives (25 µg/ml). Bacteria grown overnight on TSA plates were suspended in normal saline (0.85%) and the turbidity was

adjusted so that it was equivalent to that of a 0.5 McFarland standard, corresponding to 1.5×10^8 cfu/mL. Further dilution of the inoculum in MHB was done in such a manner that each well contained 5×10^5 cfu/mL as a final bacterial inoculum and the plates were then incubated at 37℃ for 18 h. Piperine (a known efflux pump blocker) was used as the control in this study. The MEC of chalcone derivatives that produced the maximal reduction in the MIC of ciprofloxacin was determined (Kumar et al., 2008).

Results and Discussion:

Agar Diffusion Assay:

Staphylococcus aureus is notorious for causing variety of infections among humans and animals. The hunt for potent compounds irrespective of their nature of origin (natural or synthetic) that can help fight the spread of silent pandemic of AMR by reversing resistance is quite a challenge (Costa et al, 2016). Recent research has shown that a new class of active compounds know as chalcones owning to their simple structure and diverse pharmacological properties is gaining rapid popularity to be explored for their antimicrobial potential (Da Silva et al., 2021). Keeping this in mind, we employed agar well diffusion assays to test the anti-staphylococcal activity of chalcone derivates. It was revealed that when used alone, various chalcone derivatives **C1, C3, C4, C5, C7-C11, C13, C15 and C16** did not exhibit any zone of inhibition (ZOI) against *S. aureus* ATCC 6538. The next objective was to evaluate the chalcones for their antibiotic modulating activities. Hence, agar diffusion method was performed using various chalcone derivatives and ciprofloxacin in combination. Results showed that with Ciprofloxacin (5 µg/ml), only C9 at concentrations of 12.5, 25, and 50 µg/ml displayed 16.5mm, 18mm, and 20mm ZOI respectively against S. *aureus* ATCC 6538 (Figure 1)

In vitro checkerboard studies for MEC determination:

The antibiotic modulating activity of chalcone derivatives was further evaluated employing in vitro checkerboard method. In this experiment, instead of employing wild type *S. aureus* 1199, NorA overpressing mutant *S.aureus* SA-1199B was used to screen the chalcones as antibiotic modulators. As per the results obtained in our study, the MIC of ciprofloxacin, with and without chalcone derivatives, are listed in Table 1. A 2- to 8-fold decrease in ciprofloxacin MIC was observed in the presence of chalcones tested at Minimum effective concentration of 25 mg/L (Table 1). It is also important to note that no further reductions in MICs of ciprofloxacin by chalcones were observed at concentrations above 25mg/L. Moreover, a similar study (Leal et al., 2021) indicates that efflux pump inhibition could be one of the likely mechanisms through which chalcones exert antimicrobial effects and the above results are reconfirmation of the same. Hence, it is safe to say that all the

chalcones exhibited antibiotic modulating activities, with Chalcone "C9" exerting the most prominent antibacterial activity against *S. aureus* in broth microdilution assays followed by the possibility of acting as a NorA inhibitor as evidenced in our study against NorA overexpressing *S. aureus* strain.

Conclusion:

The purpose of the study was to assess the antibacterial activity of chalcone derivatives in wild-type and mutant *S. aureus* strains, and further analysing their ability to modulate ciprofloxacin's activity and inhibition of NorA efflux by targeting only the mutant strain with over expressive NorA efflux pump. All the chalcones considered in the study considerably reduced the Minimum Inhibitory Concentrations (MICs) of ciprofloxacin in the *S. aureus* strain SA-1199B by 2-8-fold, which can be considered as a noteworthy finding, with most potent activity being shown by Chalcone "C9". Furthermore, these results are indicative of the fact that Chalcone "C9" possesses a great potential for efflux inhibition in NorA, with superior modulation and efflux inhibition results. Hence, we can conclude that Chalcone "C9" examined in this work may be utilized in conjugation with ciprofloxacin as an adjuvant to treat infections caused by NorA overproducing *S. aureus* strains. Although further investigations are needed to assess the safety and toxicity profiles of C9 prior to its application in the clinical settings.

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Table 1: In vitro checkerboard study to screen Chalcones as Antibiotic Modulators at single concentration (25µg/ml) in combination with ciprofloxacin against *S. aureus* SA 1199B.

S.No	Test Compound (25µg/ml)	MIC of Ciprofloxacin (µg/ml)
1	Ciprofloxacin	8
2	Ciprofloxacin + C-1	2
3	Ciprofloxacin + C-3	4
4	Ciprofloxacin + C-4	4
5	Ciprofloxacin + C-5	4
6	Ciprofloxacin + C-7	4
7	Ciprofloxacin + C-8	4
8	Ciprofloxacin + C-9	1
9	Ciprofloxacin + C-10	4
10	Ciprofloxacin + C-11	2
11	Ciprofloxacin + C-13	4
12	Ciprofloxacin + C-15	2
13	Ciprofloxacin + C-16	4
14	Ciprofloxacin + Piperine	2

Figure 1: Agar diffusion assay: showing increase in zone of inhibition of ciprofloxacin in presence of chalcone (C9) at different concentrations. Agar well diffusion result with Cipro (5µg/ml)- 15.5mm, C9 (50µg/ml)- No ZOI, C9(25µg/ml)- No ZOI, C9 (12.5µg/ml)- No ZOI, C9 12.5 +C- 16.5mm, C9 25 +C- 18mm, C9 50 +C- 20mm