

## POTENTIATION OF CIPROFLOXACIN ACTIVITY BY CHALCONES BY MODULATING EFFLUX PUMP AND BIOFILM REGULATORY GENE IN *STAPHYLOCOCCUS AUREUS*

BHAWANDEEP KAUR<sup>1</sup>, SHASHIKANTA SAU<sup>2</sup>, SUMAN K. JANA<sup>1</sup>, NITIN PAL KALIA<sup>2\*</sup>, GOPAL LAL KATHIK<sup>3</sup>, ASHISH SUTTEE<sup>4</sup>, SARIKA SHARMA<sup>5</sup>, SANDEEP SHARMA<sup>1\*</sup>

<sup>1</sup>Department of Medical Laboratory Sciences, School of Allied Medical Sciences Lovely Professional University Phagwara (Punjab), India.

<sup>2</sup>Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research, Hyderabad, India.

<sup>3</sup>Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research, Raebareli, India. <sup>4</sup>Department of Pharmacognosy, Lovely Professional University, India. <sup>5</sup>Department of Sponsored Research, Lovely Professional University, India

\*Corresponding author: Sandeep Sharma; \*Email: [sandeep.23995@lpu.co.in](mailto:sandeep.23995@lpu.co.in)

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### ABSTRACT

**Objective:** Natural and synthetic compounds with dual activity to inhibit efflux pumps and biofilm production in *Staphylococcus aureus* (*S. aureus*) were investigated in this study. Chalcones emerged as potential compounds with the possibility to inhibit efflux pump and biofilm production at once.

**Methods:** We performed pre-clinical screenings of different chalcones-ciprofloxacin combinations with a NorA overexpressing *S. aureus* strain. Chalcones inhibition of the NorA efflux pump and Agr gene expression, a major regulator of biofilm formation, were tested. In silico modeling was also used to confirm the dual inhibitory activity of chalcones.

**Results:** Chalcones effectively modulated the action of ciprofloxacin by reducing the MIC by 2-8 fold and improving the time kill study, also inhibiting the NorA efflux pump and suppressing Agr gene expression, resulting in increased antibacterial activity and decreased biofilm formation.

**Conclusion:** The present research brings to the limelight chalcones as dual-function inhibitors aiming at efflux-based antibiotic resistance and biofilm formation in *S. aureus* with a potential strategy to repress drug-resistant infections.

**Keywords:** Chalcones, Efflux pump inhibition, Biofilm inhibition, Antibiotic resistance, Agr gene expression, *S. aureus*

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### INTRODUCTION

*Staphylococcus aureus* (*S. aureus*) is a significant human pathogen that causes a variety of infections, ranging from mild skin infection to life-threatening conditions like sepsis and endocarditis [1]. The difficulty of treating this infection has been compounded by the emergence of methicillin-resistant *S. aureus* strains [2-4]. *S. aureus* resistance primarily arises through Horizontal Gene Transfer (HGT) and spontaneous mutation, enabling bacteria to overcome the action of antibiotics [5, 6]. One major resistance mechanism involves efflux pumps, particularly the NorA pump, which actively exports diverse antimicrobial agents from the cell, which contributes to multidrug resistance [7]. Yet another key determinant in *S. aureus* virulence is the Accessory Gene Regulator (Agr) system, a quorum-sensing network that regulates virulence factor expression and biofilm promotion [8]. Biofilm serves to protect from hostile environment, from antibiotics, and sustaining the overexpression of efflux system such as NorA. Both NorA and Agr inhibition constitute a promising line of attack in novel antimicrobial development. Ciprofloxacin, a fluoroquinolone antibiotic used extensively, acts on bacterial DNA gyrase and topoisomerase IV. Ciprofloxacin activity is compromised by resistance mechanisms such as NorA overproduction, target mutations and biofilm formation. For the improvement of ciprofloxacin efficacy, ciprofloxacin is combined with other drugs such as gentamicin, minocycline or plant compounds like rutin [9-11]. *Avicennia marina* and *Salix alba* plant extracts have also revealed promising anti *S. aureus* activity because of their bioactive compounds [12]. Chalcones, a vital class of flavonoids, have garnered significant interest due to their broad-spectrum pharmacological properties, including antibacterial, anti-inflammatory, and antioxidant activities. Their structural versatility enables interactions with various biological targets, making them promising candidates for combating antibiotic resistance [13]. Among these targets, bacterial NorA efflux pumps and Agr quorum

sensing pathways play critical roles in antibiotic resistance and pathogenicity of *S. aureus*. NorA efflux pumps contribute to multidrug resistance by actively extruding antibiotics, while the Agr quorum sensing system regulates virulence factor expression, enhancing bacterial survival and infection potential.

This research study aims to investigate the role of chalcones in combating drug resistance mediated by the NorA efflux pump and the Agr system's role in biofilm formation when used in combination with ciprofloxacin against *S. aureus*. Among the various chalcones explored, chalcone C9 was selected based on its superior inhibitory activity observed in preliminary screenings. A systematic evaluation of multiple chalcone derivatives was conducted to assess their potency against NorA efflux pump inhibition and Agr quorum sensing disruption. Comparative analysis revealed that C9 exhibited the highest efficacy in inhibiting NorA-mediated efflux and significantly attenuated Agr-regulated virulence factor expression. Additionally, its structural attributes, such as enhanced hydrophobicity and favourable molecular interactions with target proteins, supported its selection for detailed analysis.

### MATERIALS AND METHODS

The *S. aureus* ATCC 29213 strain was procured from the American Type Culture Collection (ATCC) located in Manassas, Virginia. Two additional strains of *S. aureus* were generously provided by Dr. Nitin Pal Kalia from the National Institute of Pharmaceutical Education and Research (NIPER) in Hyderabad, India: SA1199B, which overexpresses the NorA efflux pump and its wild-type counterpart, *S. aureus* 1199. All chemicals, including antibiotics, required for this research project were sourced from Hi Media Laboratories, India. Dr. Gopal Kathik from the Department of Medicinal Chemistry at NIPER Raebareli, Uttar Pradesh, synthesized and supplied the fully characterized chalcone derivatives. The primers and real-time PCR (RT-PCR) kit were procured from Invitrogen.

### Time-kill studies of chalcones with ciprofloxacin

Time-kill kinetics approach was executed using the protocol previously released [14]. Ciprofloxacin coupled with chalcone C9 in Mueller Hinton Broth (MHB) was utilized to create a time-kill curve against the *S. aureus* strain SA-1199B. A bacterial suspension was made at a concentration of  $1 \times 10^6$  CFU/ml. Separately, ciprofloxacin at a concentration of 2 mg/l [ $1/4 \times \text{MIC}$ ] was examined separately and in combination with chalcone C9 at 25 mg/l [MEC of chalcone C9 determined earlier]. Moreover, MIC concentration of ciprofloxacin [8 mg/l] was evaluated. The bacterial suspensions were incubated at 37 °C. At set time periods, i. e., 0, 2, 4, 6, and 24 h, aliquots were collected, serial dilutions were made, and then it was spot plated on the media plates for the determination of bacterial colonies in CFU/ml [14]. The concentrations of ciprofloxacin (2 mg/l and 8 mg/l) and chalcone C9 (25 mg/l) were selected based on preliminary MIC assays and previously published studies on similar compounds. These concentrations were chosen to evaluate both the sub-inhibitory and inhibitory effects of ciprofloxacin, as well as potential synergistic interactions with chalcone C9.

### Mutation prevention concentration and rate of mutation frequency

After an 18 h culture on trypticase soy agar, a bacterial solution containing  $10^{10}$  CFU/ml of *S. aureus* ATCC 29213 was created. 100 µl of this suspension was used as an aliquot and plated on MHA with ciprofloxacin at  $4 \times$ ,  $8 \times$ , and  $16 \times$  MIC concentrations. Chalcone and ciprofloxacin at 25 and 12.5 mg/l, respectively, were also examined at the same concentrations. Following a 48-hour incubation period at 37 °C, colonies were enumerated, mutation frequency was computed, and the concentration of mutation prevention was also ascertained [15].

### Post-antibiotic effect (PAE) of effective combination

The PAEs of the ciprofloxacin alone and in combination with the chalcone were measured [16]. Ciprofloxacin was added at their MIC concentration to test tubes containing  $10^6$  CFU/ml each of the strain *S. aureus* 1199B. Chalcone C9 was also added in combination with ciprofloxacin at concentrations of 25 mg/l. Following a two-hour exposure to either chalcone C9 or the antibiotic ciprofloxacin, samples were diluted to a ratio of 1:1,000 in order to efficiently remove the medicines. Every 30 min, samples were collected for the CFU count until visible cloudiness was seen. The calculation of PAE was done with the following formula:

PAE = T – C Where T is the time to grow test culture by  $\log_{10}$  CFU/ml above the count

That was seen right after the drug was removed and C is the time it takes for the count in the untreated control tube to rise by  $\log_{10}$  CFU/ml.

### Biofilm susceptibility of ciprofloxacin with chalcones (C9): biofilm inhibition (96 well plate)

The synergistic anti-biofilm activity of chalcone C9 when combined with ciprofloxacin antibiotic, was investigated with minor

modifications as done in previously published relevant publications [17]. In a 96-well flat-bottom microtiter plate, 100 µl of SA-1199B bacterial culture ( $10^6$  CFU/ml) and 100 µl of ciprofloxacin and chalcone C9 alone and in combination prepared as two-fold serial dilutions in tryptic soy broth supplemented with 2% sucrose, were added to each well and incubated for 24 h at 37 °C. After incubation, each well was rinsed with 200 µl of Phosphate Buffer Saline (PBS) to remove loosely attached, non-viable bacterial cells. Further, the plates were dried and stained with 0.1% Crystal Violet (CV) dye for 30 min under dark conditions at room temperature. Afterwards, the plates were washed three times with sterile PBS to remove unbound CV and finally solubilized in 33% glacial acetic acid. The absorbance at 595 nm was determined using a microplate spectrophotometer reader. The experiment was performed three times. The mean absorbance of each ciprofloxacin-chalcone combination was measured and percentage biofilm inhibition was calculated by using the formula,

$$\text{Percent biofilm inhibition} = \frac{\text{Abs (Control)} - \text{Abs (Treatment)}}{\text{Abs (Control)}} \times 100\%$$

### Scanning electron microscopy (SEM) for biofilm inhibition

SEM was performed to observe the extent of biofilm inhibition and morphological changes induced within the bacterial cells upon treatment with ciprofloxacin and C9 alone as well as in combination. *S. aureus* SA-1199B cells were treated with C9 at concentration of 25 mg/l and ciprofloxacin at  $1/4 \times \text{MIC}$  concentration alone and in combination for 24h in 6-well plates containing thin films onto which biofilms were allowed to grow. After 24h incubation, the films were washed with sterile PBS solution (pH=7.4). Later fixation was done with 2.5% glutaraldehyde for a period of 24h by placing the plates containing the films at 4 °C. Next day, films were washed thrice with PBS and subjected to second fixation by 1% osmium tetroxide for 1h at 4 °C under dark conditions. Afterward, dehydration was done by employing ethanol at sequential concentrations of 10%, 50%, 70%, 80%, 90%, 95%, and 100% for 20 min each at a time. Final dehydration was carried out with isoamyl acetate and acetone (1:1) for 30 min followed by incubation with pure isoamyl acetate for 1h. Lastly, the films were kept in the desiccator until they were completely dry and finally, samples were mounted with gold before observation under the microscope [18].

### Quantitative real-time study: expression studies to establish relation between AgrA and NorA genes in *S. aureus*: primer designing for qRT-PCR analysis

The sequences of all primers used in this study together with their amplicon length are listed in table 1. The Open Reading Frame (ORF) sequences of NorA, AgrA gene and 16S gene were taken from the genome sequence of *S. aureus* ATCC 29213. Primer pairs were designed using PRIMER3 software and synthesized by Eurofin. The housekeeping gene 16S was taken as an internal control for the normalization of mRNA levels in the samples as well as control RNA isolated from *S. aureus* ATCC 29213 grown in cation adjusted by MHB.

**Table 1: List of the sequences of all primers with their amplicon length**

Gene	Primer sequences	Annealing temp
16s	F: TGAGTAACACGTGGATAACCTAC R: CGGATCCATCTATAAGTGACAG	62 °C
norA	F: CAGCTATTAAACCTGTACACACC R: AGCTATTAAACCTGTACACACCAG	62 °C
agrA	F: TGTCTACAAAGTTGCAGCGATG R: TAAATGGGCAATGAGTCTGTGAG	62 °C

### RNA extraction

MHB was used to cultivate overnight cultures of *S. aureus* ATCC 29213, for planktonic and biofilm formation (described in biofilm section), which were harvested by centrifugation into 10 ml aliquots. Following centrifugation, the cell pellets were transferred to tubes holding a mixture of 0.2 ml glass beads, 0.2 ml chloroform, and 1 ml

TRIZOL Reagent (Invitrogen, CA, USA). The tubes were vortexed and violently shaken for 15 to 30 seconds before being incubated for 5 to 15 min at room temperature. Following that, the samples were centrifuged for 15 min at 4 °C at  $12,000 \times g$ . After carefully removing the top aqueous phase containing the total RNA and transferring it to a fresh 1.5 ml centrifuge tube, each sample received 0.5 ml of isopropyl alcohol. After 10 min of room temperature incubation, the

tubes were centrifuged for 8 min at 4 °C at 12,000 × g. After twice washing the pellet with 1 cc of Merck's 75% ethanol, it was centrifuged at 12,000 × g for 5 min at 4 °C. The RNA pellet was dissolved in water that was free from DNase and RNase contamination, it was heated to 55 °C for 10 min.

#### Quantification of RNA

Quantification of RNA was done using nanodrop. The sterile Milli Q Water (MQ water) was used to set blank. A ratio of 2.1 of 260/280 wavelengths is an indication of good quality of RNA. All the RNA quantification was done using the same procedure.

#### First-strand cDNA synthesis

Takara Bio Inc.'s PrimeScript 1st Strand cDNA synthesis kit was used to synthesis cDNA. To successfully eliminate contaminating genomic DNA, 1 µg of pure sample of RNA was quickly treated in gDNA wipeout buffer at 42 °C for 2 min. The RNA sample was processed for reverse transcription using a master mix made from Primescript Reverse Transcriptase, oligo dT and random 6-mer primers included in the kit after genomic DNA was removed. After 15 min of incubation at 42 °C, the reaction tubes were inactivated for 3 min at 95 °C. The same procedure was followed for all cDNA synthesis process.

#### Relative expression studies

Two sets of primer pairs amplifying portions of the *NorA* and *agrA* ORF were used in SYBR Green quantitative PCR on the resultant cDNA and the negative control (without cDNA) in a Bio-Rad CFX Opus 96 System. The critical Threshold Cycle (CT) is the cycle number at which fluorescence exceeds background levels and is inversely proportional to the logarithm of the initial template concentration. A standard curve was plotted for the 16S rRNA gene as previously mentioned. In a 20 µl reaction volume, a two-step real-time PCR was conducted.

10 pmol of each primer (2 µl), 10 µl of SYBR Green I master mix, 2 µl of cDNA (1:10 dilution of cDNA from 1 µg of total RNA), and a reaction volume made up of nuclease-free water comprised the RT-PCR mixture. The methodology for the real-time PCR run included a 45-cycle amplification process (10 s at 95 °C, 20 s at 62 °C, and 20 s at 72 °C), with a single fluorescence reading acquired at the conclusion of each cycle. The PCR was activated at 95 °C for 10 min. The 16S rRNA gene was used as an endogenous reference to normalize the quantitative data for *NorA* and *AgrA*. The expression of both genes was measured in *S. aureus* ATCC 29213 planktonic cell relative to Biofilm. Each experiment was performed in triplicate. The effect of proposed Chalcone (a known *NorA* EPI) on expression of *NorA* and *agrA* genes was compared.

#### In silico studies

##### Preparation of the *NorA* target via homology modelling

To better comprehend the binding relationship with a *NorA* MFS protein, a 3D homology model was constructed using Uniport Q5HHX4 protein sequence with UniParc-UPI00000522D0 and contrasted to EmrD MFS from *E. coli* with pdb ID: 2G as described in previous studies with slight modifications [19, 20]. It was further assessed employing the Swiss Model [20], and the binding site was chosen as described in two research publications [21, 22].

##### Molecular docking C9 compared with capsaicin for *NorA* and *Agr* inhibition

Autodock vina (a molecular docking software) was utilized for investigating the interaction of modeled *NorA* protein with Capsaicin and C9. Likewise, both the ligands were evaluated on *Agr*, a gene cascade that governs biofilm development in *S. aureus*. An accessible pdb 4bxi was downloaded from RCSB and made ready for the docking analysis.

#### Statistical analysis

For every experiment, three duplicates of each experiment were carried out. The data is shown as mean±SD. Statistical comparisons between two group means were performed using the student's t-test, whereas one-way analysis of variance (ANOVA) was employed for multiple group comparisons, with a significance threshold set at  $P < 0.05$ .

## RESULTS

#### Time-kill studies of chalcones with ciprofloxacin

The time-kill curve analysis of *S. aureus* mutant SA-1199B was used to evaluate the bactericidal efficacy of ciprofloxacin alone and in combination with chalcone C9. Ciprofloxacin had no inhibitory effect on cell growth when employed at a sub-inhibitory concentration of 2 mg/l ( $1/4 \times \text{MIC}$ ), but at 8 mg/l, it showed bactericidal efficacy (99.9% kill) within 6 h. Moreover, bactericidal activity was reported when the combination of ciprofloxacin at 2 mg/l and chalcone C9 at 25 mg/l was evaluated. The combined bactericidal activity was comparable to that obtained with 8 mg/l ciprofloxacin. Despite the pathogen recovery after 24h in all the test groups, the combination of ciprofloxacin and Chalcone C9 maintained its bacteriostatic action, holding the final  $\log_{10}$  CFU below the initial inoculum at the time of commencement of the experiment (0h) (fig. 1). These results imply that chalcone C9 can enhance ciprofloxacin action against mutant *S. aureus* strain 1199B, thus providing a novel strategy for combating antibiotic resistance in this bacterium.

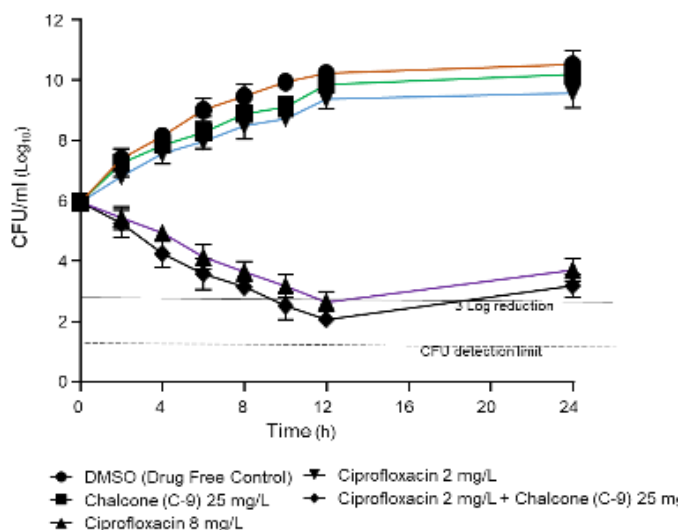


Fig. 1: Time-kill curves of *S. aureus* SA-1199B demonstrating the bactericidal action of ciprofloxacin ( $1/4 \times \text{MIC}$ , 2 mg/l) combined with chalcone C9 (25 mg/l). The individual time point was the average  $\log_{10} \pm \text{SD}$  of the three readings

### Mutation prevention concentration and rate of mutation frequency

The probability of mutant development against ciprofloxacin and its combination with C9 was investigated in *S. aureus*. *S. aureus* ATCC 29213, a wild-type strain lacking any known mutations in the NorA regulatory domain or drug target domain (which included DNA gyrase and topoisomerase IV), was used in a mutant selection experiment. Since ciprofloxacin did not cause any mutant formation

at a minimal concentration of 4 mg/l ( $16 \times \text{MIC}$ ), this value is known as the Mutant Prevention Concentration (MPC).

However, when ciprofloxacin was combined with chalcone C9 at doses of 12.5 mg/l and 25 mg/l, the MPC values dropped to 2 and 1 mg/l, respectively. Moreover, the combination's MPC was lower than ciprofloxacin's maximum concentration (i. e.,  $C_{\text{max}}$  value of 4 mg/l), implying that these combinations could be clinically significant in preventing the evolution of resistant bacterial mutants (table 2).

Table 2: Mutation frequency of *S. aureus* ATCC 29213

Chalcone (C-9) (mg/l)	Mutation frequency with ciprofloxacin			
	2 $\times$ MIC (0.5 mg/l)	4 $\times$ MIC (1.0 mg/l)	8 $\times$ MIC (2.0 mg/l)	16 $\times$ MIC (4.0 mg/l)
0	$4 \times 10^{-8}$	$1 \times 10^{-8}$	$2 \times 10^{-9}$	$<10^{-9}$
12.5	$8 \times 10^{-8}$	$4.5 \times 10^{-9}$	$<10^{-9}$	$<10^{-9}$
25	$2.0 \times 10^{-9}$	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$

### PAE of effective combinations

PAE studies revealed that in conjunction with chalcone C9, ciprofloxacin was able to suppress the pathogen growth for longer periods even after the removal of the antibiotic with longer suppression observed as the doses of antibiotic employed increased (table 3).

### Biofilm susceptibility of ciprofloxacin in combination with chalcones (C9) biofilm inhibition (96 well plate)

The study investigated the impact of chalcone C9 on the Minimum Biofilm Inhibitory Concentration (MBIC<sub>50</sub>) of ciprofloxacin against *S.*

*aureus* ATCC 29213. The results confirm a notable influence of C9 on the efficacy of ciprofloxacin in inhibiting biofilm formation (fig. 2). Although the chalcone on its own had no significant effect in biofilm inhibition at concentrations of 50 mg/l, 25 mg/l, and 12.5 mg/l, respectively, but in conjunction with ciprofloxacin, it was observed that lower dose concentration of C9 of 12.5 mg/l produced almost negligible potentiation of ciprofloxacin's efficacy. At 25 mg/l of C9, MBIC<sub>50</sub> of ciprofloxacin enhanced by 13%, whereas with 50 mg/l concentration of C9, most pronounced reduction in MBIC<sub>50</sub> was obtained, potentiating the antibiotic's biofilm inhibition potential by almost 58% (Data represented in table 4, fig. 3).

Table 3: Post-antibiotic effect with chalcone C-9 and ciprofloxacin

Chalcone (EPI mg/l)	PAE's(h)		
	$\frac{1}{4} \times \text{MIC}$ (2 mg/l)	$\frac{1}{2} \times \text{MIC}$ (4 mg/l)	MIC (8 mg/l)
Without C-9	$0.4 \pm 0.1$	$1.25 \pm 0.5$	$1.45 \pm 0.5$
C-9 (25 mg/l)	$1.0 \pm 0.3$	$1.45 \pm 1$	$2.5 \pm 0.25$

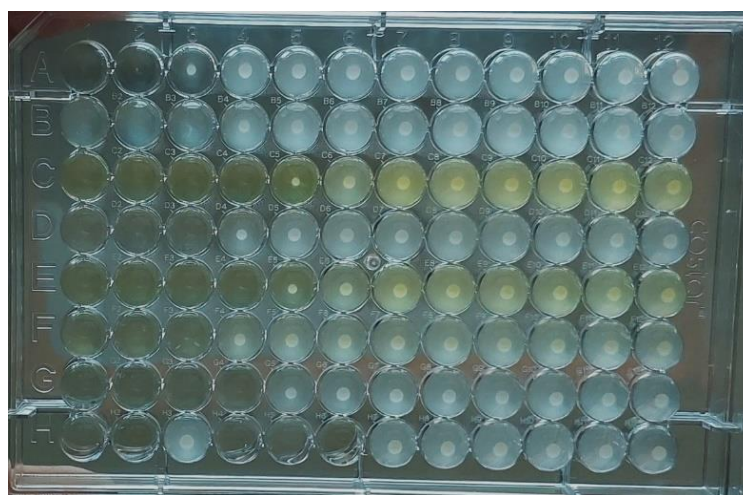


Fig. 2: 96 well plate showing a synergistic effect

Table 4: MBIC<sub>50</sub> of ciprofloxacin against *S. aureus* ATCC 29213 when used in combination with Chalcone C9

Chalcone C-9 (mg/l)	MBIC <sub>50</sub> of ciprofloxacin against <i>S. aureus</i> ATCC 29213 in combination with C9
0	0.33
12.5	0.32
25	0.29
50	0.14



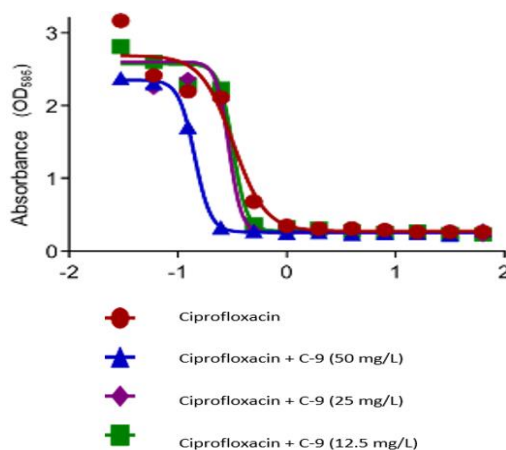


Fig. 3: MBIC<sub>50</sub> of ciprofloxacin against *S. aureus* ATCC 29213 when used in combination with chalcone C9 (represented in terms of OD)

#### SEM for biofilm inhibition

The SEM micrographs were obtained to demonstrate that chalcone C9 at the sub-inhibitory dose of 25 mg/l had no discernible effect on suppressing the *S. aureus* SA-1199B bacterial biofilms (fig. 4C), but treatment with  $1/4 \times \text{MIC}$  concentration of ciprofloxacin resulted in

a moderate reduction in biofilm cell mass (fig. 4A). However, when a combination of C9 (25 mg/l) and ciprofloxacin ( $1/4 \times \text{MIC}$ ) was evaluated, the SEM pictures revealed a significant inhibitory effect of this synergistic combination (fig. 4B) on *S. aureus* SA-1199B biofilm development compared to biofilms produced by untreated control (fig. 4D).

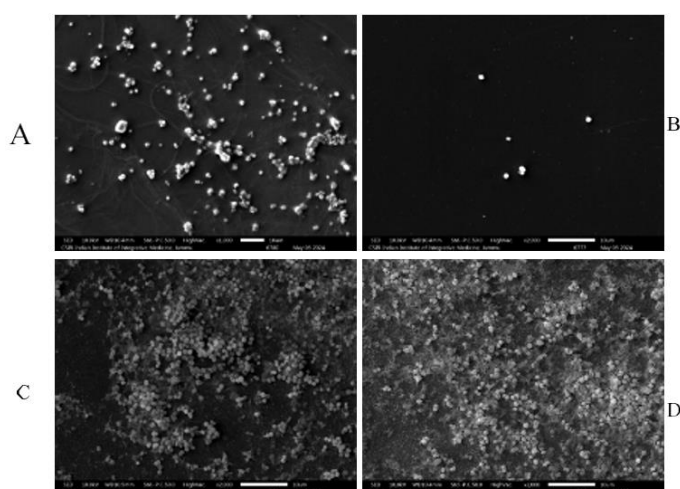


Fig. 4: SEM images demonstrating the antibiofilm effect induced on the *S. aureus* ATCC 1199B treated with ciprofloxacin at 2 mg/l, (A) *S. aureus* ATCC 1199B treated with ciprofloxacin at  $1/4 \times \text{MIC}$  (2 mg/l) (Reduced Biofilm Cell mass), (B) Combination [Ciprofloxacin (2 mg/l) + Chalcone C9 (25 mg/l)] (Significant Biofilm Inhibition), (C) Chalcone C9 at 25 mg/l alone (No Noticeable effect), (D) Untreated controlled (Dense biofilm formation)

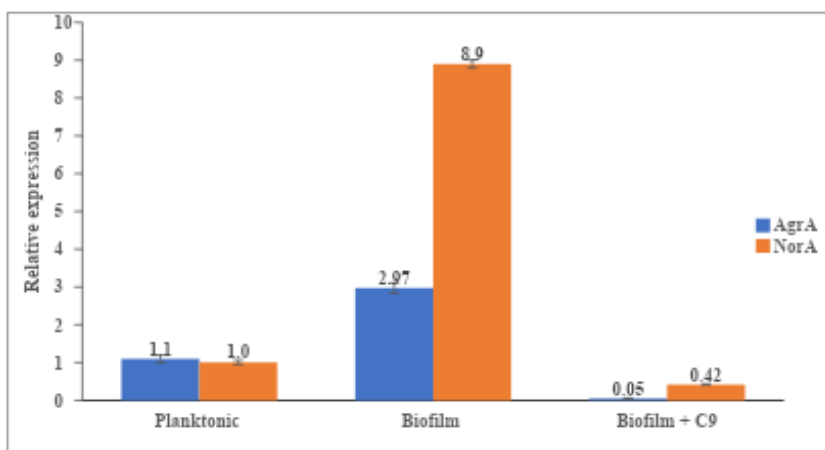


Fig. 5: Biofilm induced co-overexpression of AgrA and NorA and impact of chalcones on expression of both the genes

### qRT PCR analysis

For the *NorA* and *AgrA* gene, *S. aureus* ATCC 29213 was grown in the presence and absence of C9 (at MEC). The results of the qRT-PCR research showed that *AgrA* and *NorA* expression considerably increased in biofilm-associated to 2.97 fold and 8.9 fold respectively, when compared to planktonic cells. However, the expression of both *AgrA* and *NorA* was significantly downregulated to decimal points in biofilm-associated cells in the presence of C9 when compared with planktonic cells (fig. 5).

### In silico studies

#### Molecular docking C9 compared with capsaicin for NorA and Agr inhibition

Finally, the interaction between capsaicin and C9 was analyzed for the NorA protein and the Agr gene of *S. aureus*. Autodock vina

results showed that the binding site residues in NorA were Phe16, Ile19, Ile23, Gln61, Met109, Glu222, Ile244, Phe303, Arg 310, Asn340, and Phe341. Capsaicin and C9 showed binding affinities of -6.7 kcal/mol and 7.4 kcal/mol, respectively, in the NorA protein (table 5) C9 is involved in hydrophobic interactions at amino acid residues such as Phe140, Ile141, and Ile244 and shows  $\pi$ -stacking of the aryl ring with Phe140 (fig. 6a and 6b).

The binding site residues observed in the Agr gene are Ile285, Glu286, Leu288, Lys294, Ile297, Thr298, and Ile313. The binding affinities of capsaicin and C9 to the Agr gene were 6.3 and -6.3 kcal/mol, respectively. C9 molecules were observed to have hydrophobic interactions with Ile285, Leu288, Ile297, and Ile313. It was also found to form a hydrogen bond with Thr298 and its hydroxyl functional group. Furthermore, a strong cation- $\pi$  interaction was observed between the aryl ring and Lys294 (fig. 6d and 6c).

Table 5: Docking results using Autodock vina

S. No.	Molecule	NorA	Binding affinity (kcal/mol)	Agr	Binding affinity (kcal/mol)
		Binding residues		Binding residues	
1	Capsaicin	Phe16, Ile19, Ile23, Gln61, Met109, Ile244, Phe303, and Asn340	-6.7	His379, Phe382, Phe386, Lys401	6.3
2	C9	Asn137, Phe140, Ile141, Ile244, and Phe303	-7.4	Ile285, Glu286, Leu288, Lys294, Ile297, Thr298, and Ile313	-6.3

C9 is possibly a promising inhibitor of the NorA and Agr genes compared to capsaicin, reflecting its potential to mitigate biofilm-assisted drug resistance in microbes.

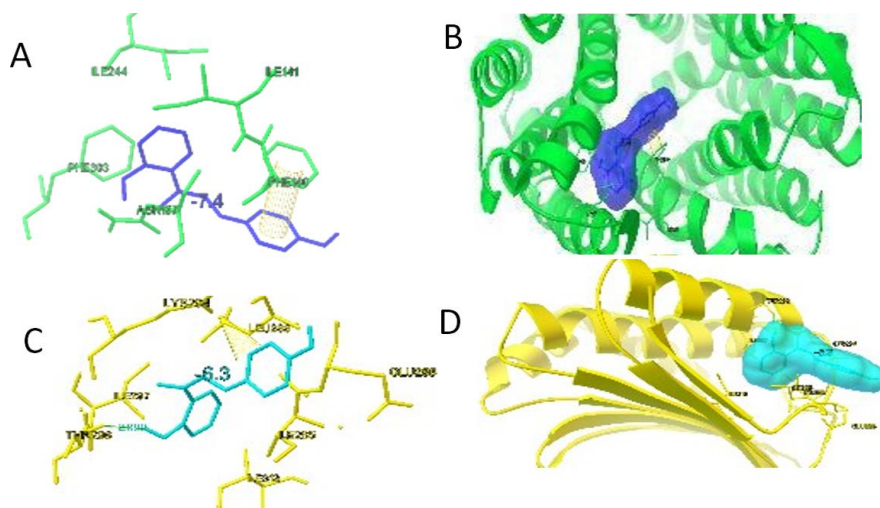


Fig. 6: Binding interaction of C9 (a) ball and stick model NorA (green), C9 (blue), (b) ribbon structure NorA (green), C9 (blue), (c) ball and stick model Agr (yellow), C9 (blue), (d) ribbon structure Agr (yellow), C9 (blue)

### DISCUSSION

The bacterial pathogen *S. aureus*, is popular for causing infectious illnesses of varying severity in animals and humans combined. The search for potent molecules, regardless of their source of origin (natural or synthetic), that can assist researchers to combat the spread of silent AMR pandemic by reversing the resistance is a daunting task [23, 24].

Chalcones are natural molecules containing two aromatic rings linked by an  $\alpha$ ,  $\beta$ -unsaturated carbonyl group. They exhibit numerous biologically essential properties, including antimalarial, antibacterial, anticancer, antifungal, antioxidant, neuroprotective action, anti-inflammatory, and much more [25]. Chalcones exert their antibacterial effect by obstructing the efflux pumps, which are in control of removing chemicals and drugs from the bacterial cells that are detrimental to them in nature [26]. Inhibiting the efflux pumps might boost antibiotic efficacy whilst reducing the issues of

multidrug resistance. Moreover, the Chalcones have been shown to block NorA, an efflux pump found in *S. aureus*, a g-positive bacterium that is a causative agent for numerous illnesses in humans and animals. NorA is responsible for providing resistance to pathogens against numerous clinically important antibiotic classes, including fluoroquinolones, phenothiazines, quaternary ammonium compounds, and dyes [27]. Hence, chalcones offers promising prospects as an antibacterial agent against MRSA. Several studies have focused on the synthesis and assessment of chalcone derivatives as NorA inhibitors, as well as their modulatory activities towards ciprofloxacin since this drug is widely used to treat *S. aureus*-associated bacterial infections [27-29].

The investigation into the antibacterial properties of chalcone derivatives against *S. aureus* revealed significant findings regarding their potential as antibiotic adjuncts. The initial agar well diffusion assay results revealed that various chalcone derivatives that we

tested in our study, i. e., Chalcone C1, C3, C4, C5, C7-11, C13, C15, and C16, did not show any Zone Of Inhibition (ZOI) when tested alone. This lack of intrinsic antibacterial activity suggests that these compounds may not be effective in combating *S. aureus*. This is consistent with previous research showing that many chalcones exhibit variable antibacterial activity depending on their structural characteristics and bacterial strains used [27, 30].

However, further testing of chalcones in conjunction with ciprofloxacin demonstrated a significant increase in antibacterial activity, particularly for chalcone C9. With ciprofloxacin at a dose concentration of 5 mg/l, C9 showed potentiation forming ZOI equal to 16.5 mm, 18 mm, and 20 mm at employed concentrations of 12.5 mg/l, 25 mg/l, and 50 mg/l, respectively. This synergistic impact highlights the ability of chalcone C9 to improve ciprofloxacin's efficacy, particularly in the context of MRSA as reported earlier [28].

Moreover, the in vitro checkerboard results in our research work demonstrated that upon combining chalcone C9 with ciprofloxacin, the minimum inhibitory concentration of ciprofloxacin reduced 2-8 folds in the two strains of *S. aureus*, i. e., *S. aureus* ATCC 1199, and *S. aureus* ATCC 1199B. This synergistic interaction appears to be caused by chalcone C9's capacity to inhibit NorA, consistent with the findings [31], which also reported enhanced antibacterial activity upon NorA inhibition.

The time-kill curve assay confirmed the synergistic bactericidal action of Chalcone C9 in conjunction with ciprofloxacin. This combination had a 99.9% kill rate at sub-inhibitory concentrations, indicating that C9 can improve ciprofloxacin efficacy even at lower dosage concentrations, potentially reducing the life-threatening risks of toxicity associated when higher dosage concentrations are involved. Furthermore, the combination displayed a much longer PAE than ciprofloxacin alone, indicating persistent suppression of pathogen growth, which is very critical for optimizing the dosage regimens and minimizing the chances of resistance development.

Another critical feature of this study was the assessment of chalcone C9's effect on biofilm development by *S. aureus*. Biofilms are complex bacterial populations concealed in a self-generated extracellular matrix that provide resistance to antimicrobial agents and immune responses and protecting against bacterial pathogens [32]. The development of bacterial biofilms is influenced by several factors, with quorum sensing being a critical one. This complex communication network allows bacteria to modulate gene expression in response to change in cell density, facilitating coordinated behaviour and adaptation. In the case of *S. aureus*, QS is mediated via the agr system, which regulates the production of bacteria's virulence factors such as Alpha-Hemolysin (Hla) and biofilm-associated genes like *icaA* [33]. Biofilm inhibition studies predicted that Chalcone C9 possesses the potential to enhance ciprofloxacin's capacity to penetrate or disrupt biofilm formation or it is likely that it may have direct role in the bacteria's ability to form biofilms. The prediction was made based on the fact that with increasing concentrations of chalcone C9 used, the ciprofloxacin's ability to inhibit biofilms produced by *S. aureus* ATCC 29213 strengthened with most pronounced effect produced at 50 mg/l concentration of C9. The association of *agrA* and *norA* gene was revealed during biofilm formation. The overexpression of *norA* and *agrA* in biofilm-associated cells made it clear the dependency of both the genes on expression of each other. Further, C9 as a proposed NorA inhibitor, inhibited the expression of both the genes. (as depicted in fig. 5). Subsequently, the SEM imaging revealed that C9 when used at sub-inhibitory concentration of 25 mg/l had no obvious impact in suppressing *S. aureus* SA-1199B bacterial biofilms, whereas treatment with  $\frac{1}{4} \times$  MIC concentration of ciprofloxacin induced mild reduction in biofilm cell mass. However, in case combination of C9 (25 mg/l) with ciprofloxacin ( $\frac{1}{4} \times$  MIC) was tested, SEM images visually confirmed substantial inhibitory effect of this synergistic combination on the formation of *S. aureus* SA-1199B biofilms. These findings could have significant clinical implications, potentially allowing for lower doses of ciprofloxacin to be used in treating *S. aureus* biofilm-associated infections when combined with Chalcone C9.

Importantly, Chalcone C9 demonstrated inadequate potential for generating resistant mutants in *S. aureus*. Ciprofloxacin's MPC was

reduced by 2-4 folds in the presence of chalcone C9, indicating that the combination may successfully prevent resistance in vitro. This discovery highlights the chalcone C9's potential as an adjuvant for fighting MRSA infections and emerging antibiotic resistance.

The interaction of ligand capsaicin and C9 was analysed on NorA protein and Agr gene of *S. aureus*. The Autodock vina results showed the binding site residues at NorA are Phe16, Ile19, Ile23, Gln61, Met109, Glu222, Ile244, Phe303, Arg 310Asn340, and Phe341. Capsaicin, and C9 showed binding affinities of -6.7 kcal/mol, and -7.4 kcal/mol, respectively on NorA protein (table 5). C9 is involved in the hydrophobic interaction at the amino acid residues like Phe140, Ile141, Ile244 and also showed the  $\pi$ -stacking of the aryl ring with Phe140 (fig. 6a and 6b).

The binding site residues observed in the Agr gene are Ile285, Glu286, Leu288, Lys294, Ile297, Thr298 and Ile313. The binding affinities of Capsaicin, and C9 on the Agr gene were -6.3 kcal/mol, and -6.3 kcal/mol, respectively. C9 molecules were observed to have hydrophobic interaction with Ile285, Leu288, Ile297, and Ile313. It was also found to have a hydrogen bond with Thr298 and its hydroxyl functional group. Further a strong cation -  $\pi$  interaction was noted between aryl ring and Lys294 (fig. 6b and 6 c).

To summarize, the findings of this study emphasize the potential of chalcone C9 as a beneficial adjuvant in the treatment of MRSA infections, particularly through its ability to improve ciprofloxacin efficacy and regulate the resistance pathways. Further research, especially in vivo studies, is needed to fully understand the clinical significance of these findings and to investigate the therapeutic potential of the chalcone derivatives in combating antibiotic resistance.

## CONCLUSION

The research indicates Chalcone C9 as a potential adjuvant to fight MRSA infection by maximizing ciprofloxacin potency and addressing the mechanisms of bacterial resistance. C9 had no direct antibacterial effect, yet it greatly improves bacterial susceptibility upon combination with ciprofloxacin, indicated by agar well diffusion, checkerboard and time-kill assays. Specifically, C9 blocked the NorA efflux pump and inhibited biofilm growth, lowering the MIC of ciprofloxacin and increasing bacterial eradication. Molecular docking validated high binding affinities of C9 to NorA and Agr genes, which also justified its use in overcoming antibiotic resistance. Moreover, C9 was able to suppress biofilm-associated gene expression and exhibited reduced potential for resistance development. These results highlight the therapeutic potential of chalcones derivatives in the treatment of MDR *S. aureus* infection.

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## CONFLICT OF INTERESTS

Declared none

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