CHALCONES EFFECT ON COMBATING ANTIBIOTIC RESISTANCE DUE TO NOR A AND AGR IN *S. AUREUS*

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DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Chalcones effect on combating antibiotic resistance due to Nor A and Agr in *S. aureus*.**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of **Dr. Sandeep Sharma**, working as **Professor of Clinical Microbiology** in the **Department of Medical Laboratory Sciences** of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "**Chalcones effect on combating antibiotic resistance due to Nor A and Agr in *S. aureus*** submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in **Clinical Microbiology** in the Department of Medical Laboratory Sciences, is a research work carried out by **Bhawandeep Kaur, Registration No. 41800434**, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Antimicrobial resistance (AMR) is an emerging concern in clinical settings and threatens the health and well-being of people everywhere with growing intensity. Bacterial pathogens have developed an effective workaround to antibiotic resistance leading to the emergence of multidrug-resistant strains. In this collection of persistent pathogens, *Staphylococcus aureus* stands out as a highly troublesome pathogen, implicated in a wide range of infections that span from mild skin conditions to severe and potentially high-mortality illnesses like sepsis and endocarditis.

Staphylococcus aureus (*S. aureus*) is a highly adaptable gram-positive bacterium commonly found on human and animal skin and mucous membranes. It has a remarkable versitality to trigger a wide range of infectious diseases, spanning from mild skin and soft tissue infections to severe and life-threatening conditions such as pneumonia, sepsis, and endocarditis, thereby posing a substantial risk to public health. The bacterium's capacity to form biofilms and develop resistance to multiple antibiotics, including the increasing incidence of MRSA has become a formidable challenge in the treatment of *S. aureus* infections. The emergence of drug-resistant *S. aureus* is largely facilitated by the bacterium's ability to acquire resistance genes through horizontal gene transfer, a mechanism where bacteria share genetic material with neighboring bacteria. This genetic exchange enables the spread of resistance traits among bacterial populations. Additionally, random mutations within the bacterial genome can also give rise to new resistance genes, further complicating efforts to combat these tough microbes.

In *Staphylococcus aureus*, efflux pumps have become a major contributor to antibiotic resistance, with the NorA efflux pump being a key player in the bacterium's ability to resist multiple antibiotics. By vigorously expelling a broad range of antibiotics with diverse structures, NorA significantly reduces the concentration of drugs within the bacterial cell, rendering them ineffective at lethal levels.

The NorA efflux pump, a prominent representative of the MATE family, plays a pivotal role in conferring antibiotic resistance in *S. aureus*, renowned for its

exceptional capacity to extrude a wide array of antimicrobial agents from the cellular structure of bacterial cells. By actively extruding structurally diverse compounds, NorA confers resistance to a wide range of antibiotic classes, encompassing fluoroquinolones, tetracyclines, and beta-lactams, thereby significantly limiting the effectiveness of these drugs.

Recent findings indicate that Agr may have an additional role in promoting antibiotic resistance by modulating the expression of efflux pumps, such as NorA. The synergistic overexpression of NorA and Agr has been implicated as a key factor contributing to the insensitivity of *S. aureus* to antimicrobial agents. The discovery of efflux pumps and quorum-sensing systems has opened up new possibilities for the creation of innovative antimicrobial therapies, with the potential to combat antibiotic resistance by targeting these mechanisms.

The broad-spectrum antibiotic ciprofloxacin, a fluoroquinolone, is effective against a wide range of bacterial pathogens, including Gram-positive (e.g., *Staphylococcus aureus*, *Streptococcus pneumoniae*) and Gram-negative species (e.g., *Escherichia coli*, *Pseudomonas aeruginosa)*. Its mode of action involves the disruption of DNA gyrase and topoisomerase IV function. Despite its efficacy, bacteria have developed resistance to ciprofloxacin through multiple mechanisms. To overcome this resistance, combination therapy with other antibiotics is often employed. Additionally, various natural and synthetic compounds have been identified as potential adjuvants to enhance the antibacterial activity of ciprofloxacin, offering new strategies to combat resistant bacterial infections.

Chalcones are a ubiquitous class of flavonoids found throughout the plant kingdom, with their name derived from the Greek word "chalcos," meaning bronze. They are commonly produced by plants of the genera Angelica, Glycyrrhiza, Humulus, and Scutellaria, where they serve as foundational compounds in the biosynthetic pathways of flavonoids and isoflavonoids or as intermediates in the production of heterocyclic compounds.

The unique chemical architecture of chalcones, featuring two aromatic rings connected by a three-carbon α, β-unsaturated carbonyl system, has enabled these naturally occurring plant compounds to possess a diverse array of bioactive properties. As a result, chalcones have become increasingly important in medicinal chemistry, leveraging their non-toxic profile and diverse pharmacological properties to drive innovations in medicine, agriculture, and industrial applications.

Chalcones have been found to possess potent antibacterial properties, exerting their effects through various mechanisms, including the inhibition of cell wall synthesis, enzyme function, and disruption of cell membrane integrity. Additionally, they have been shown to target bacterial DNA replication and transcription processes, leading to the inhibition of bacterial growth. These findings highlight the potential of chalcones as a new generation of antibacterial agents. Notably, recent research has demonstrated that chalcones and their derivatives, exhibit considerable potential in enhancing the efficacy of current antimicrobial agents, such as oxacillin, and reversing the trend of antimicrobial resistance, including the notorious MRSA.

Research has confirmed chalcones possess significant efflux inhibition activity, effectively targeting a wide range of bacterial species, including both Gram-positive and Gram-negative pathogens. Specifically, chalcones have been shown to be highly effective against certain bacterial species, such as *Staphylococcus aureus*, E*scherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*, making them a promising adjunct to traditional antibiotic therapies.

The rise of antibiotic-resistant pathogens has prompted researchers to seek innovative solutions to treat infections. An alternative strategy involves creating innovative antibiotics with novel modes of action, however, the prolonged development process often enables pathogens to develop resistance before the drug reaches the market. Combination therapy, where multiple drugs are used together, is another strategy to combat resistance. Many combination regimens are currently used to treat severe infections. Furthermore, researchers have explored the use of natural or synthetic molecules that can alter drug resistance mechanisms and enhance the effectiveness of

existing antibiotics. While efflux pump and enzyme inhibitors have shown promise, bacteria have adapted by forming biofilms, which can prevent drug entry into the cell, highlighting the need for continued innovation in this field.

This study aimed to investigate natural and synthetic molecules with dual inhibitory activity against efflux pumps and biofilm formation. Chalcones emerged as promising candidates, demonstrating potential to inhibit both mechanisms simultaneously. When combined with ciprofloxacin, these molecules significantly enhanced antibiotic efficacy. We conducted pre-clinical evaluations of various chalcone-ciprofloxacin combinations and validated our hypothesis using a NorA overexpressing strain, where chalcones modulated ciprofloxacin's effect by inhibiting the NorA efflux pump and Agr gene expression, a key regulator of biofilm formation. Additionally, we employed *in- silico* modeling to further confirm our findings, providing a comprehensive understanding of the dual inhibitory activity of chalcones.

In this study Ciprofloxacin (an inhibitor of gry a and topoisomerase IV) were used alone and in combination with a series of chalcones derivatives were screened as an EPI's using checkerboard method. During the experiment, the presence of chalcone C9 reduced the MIC of ciprofloxacin by 2-8-fold, with an optimal reduction of 8-fold observed among all chalcones tested. The kill kinetics study also showed increased bactericidal effects of ciprofloxacin when used in combination with C9 compared to ciprofloxacin alone. The same combination provides significant results in mutation prevention concentration experiments and reduces the emergence of ciprofloxacin resistance. Furthermore, during PAE studies ciprofloxacin and C9 combination showed extended effects compared to ciprofloxacin alone. This PAE is critically important for pharmacological studies which help in deciding the dose of antibiotics during treatment. Furthermore, the synergistic effect of the combination was evaluated for its potential to prevent biofilm formation, and the results revealed that the ciprofloxacin and C9 at (MEC) concentration effectively inhibited biofilm growth and results for SEM also support the finding.

Agr and NorA, by their respective roles in efflux pump and biofilm production, are

implicated in *S. aureus* resistance. Using qRT-PCR, a study was done to determine how C9 affected the regulation of Agr and NorA. Moreover, the docking results from the *in silico* investigation using the protein database showed that the binding affinity C9 is higher than that of capsaicin, a well-known efflux pump inhibitor.

This research demonstrates the promising potential of chalcone derivatives as dual inhibitors of key bacterial resistance mechanisms, including biofilm formation and efflux pumps. By enhancing the efficacy of ciprofloxacin, these compounds offer a valuable strategy to combat antibiotic resistance. To fully realize their potential, we recommend further research to identify and characterize additional dual-activity compounds using *in silico* approaches. Ultimately, comprehensive wet lab studies are necessary to validate these findings and pave the way for clinical applications.

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Date: Bhawandeep Kaur

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S. aureus	*Staphylococcus aureus*
AMR	Antimicrobial resistance
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
MATE	Multidrug and toxic compound extrusion
Agr	Accessory gene regulator
DNA	Deoxyribonucleic acid
CAPD	Continuous ambulatory peritoneal dialysis
TSST-1	toxic shock syndrome toxin-1
PVL	Panton–Valentine leucocidin
MSA	Mannitol Salt Agar
SCCmec	Staphylococcal chromosomal cassette mec
PBP-2a	Penicillin-binding protein 2a
Mep	Multidrug and Toxin Extrusion
EPS	Extracellular polymeric substances
MICs	Minimum inhibitory concentrations
MBECs	Minimum biofilm eradication concentrations
SMR	Small multidrug resistance family
MFS	Major facilitator superfamily
RND	Resistance-nodulation cell division superfamily
ATP	Adenosine-triphosphate
ABC	Adenosine-triphosphate (ATP)-binding cassette superfamily
mRNA	Messenger RNA
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
PIA	Polysaccharide intercellular adhesion
AIP	Auto inducing peptide
PSMs	Phenol-soluble modulins
QRDRs	quinolone resistance-determining regions

PPAR	Peroxisome proliferator-activated receptors
EPI	Efflux pump inhibition
PTPs	Protein tyrosine phosphatases
E. coli	*Escherichia coli*
FtsZ	Filamentous temperature-sensitive mutant Z
PMQR	Plasmid-Mediated Quinolone Resistance
NT*Hi*	Nontypeable *Haemophilus influenza*
CIP·QUE	ciprofloxacin with quercetin
anti-HIV	Anti-Human Immunodeficiency Virus
DPP4	dipeptidyl peptidase-4
PTP1B	protein tyrosine phosphatase
PPAR	peroxisome proliferator-activated receptors
MHB	Mueller-Hinton Broth
TSA	Trypticase Soya Agar
DMSO	Dimethyl sulfoxide
MEC	Minimum Effective Concentration
CLSI	Clinical & Laboratory Standards Institute
ZOI	Zone of inhibition
MHA	Mueller-Hinton Agar
PAE	Post Antibiotic Effect
PBS	Phosphate Buffered saline
EtBr	Ethidium bromide
qRT-PCR	Quantitative real-time reverse-transcription PCR
QS	Quorum sensing
SEM	Scanning electron microscopy
MPC	Mutant prevention concentration

CHAPTER-1 INTRODUCTION

1. Introduction

Antimicrobial resistance (AMR) poses a significant and escalating threat to human health worldwide. This phenomenon occurs when microorganisms evolve and begin to resist antimicrobials, including antibiotics and antivirals (Ranjbar & Alam, 2023). Bacterial pathogens have demonstrated remarkable adaptability in developing strategies to evade antibiotic efficacy, resulting in the emergence of broad-spectrum resistant strains. Among these resistant pathogens, *Staphylococcus aureus* stands out as a notorious offender, responsible for a diverse range of infections that span from relatively benign skin conditions to potentially life-threatening complications, such as sepsis and endocarditis (Taylor & Unakal 2023).

Staphylococcus aureus (S. aureus) is a Gram-positive bacterium belonging 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 (Masalha et al., 2001). This bacterium is a significant contributor to various types of infections, from mild skin and soft tissue infections to more severe and critical conditions, such as pulmonary infections, bloodstream infections, and endocarditis. One-way *S. aureus* causes infections is through the formation of biofilms. Moreover, 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, particularly the emergence of methicillin-resistant *S. aureus* (MRSA) (Karmakar et al., 2016). The development of drug resistance in *S. aureus* is predominantly associated with the acquisition of resistance genes through horizontal gene transfer, a process where bacteria exchange genetic material with other bacteria in their environment. Resistance genes can also arise through mutations in the bacterial genome.

Antibiotic resistance mediated by efflux pumps has emerged as one of the most significant mechanisms of resistance in *S. aureus*. The NorA efflux pump plays a pivotal role in conferring multidrug resistance by actively extrudes various

structurally unrelated antimicrobial agents out of the bacterial cell, thereby reducing intracellular drug concentrations to sublethal levels.

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*. NorA actively pumps out structurally diverse antimicrobial compounds from the bacterial cell, conferring resistance to numerous antibiotic classes, including fluoroquinolones, tetracyclines, and beta-lactams (Costa et al., 2013). 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 (Sionov et al., 2022). 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. Furthermore, there is evidence suggesting that Agr may also contribute to antibiotic resistance by modulating the activation of various efflux mechanisms, such as the NorA efflux pump. 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 to develop novel antimicrobial agents (Kaatz *et al.*, 2004).

The emergence and dissemination of antibiotic resistance in *S. aureus* have substantially compromised the efficacy of conventional antimicrobial therapies, necessitating the exploration of novel strategies to combat this growing threat (Samreen *et al.*, 2021). Natural products have emerged as promising antimicrobial agents, garnering substantial attention for their diverse bioactivities and minimal toxicity. Compounds, such as chalcones and capsaicin, have shown promise in inhibiting efflux pumps, disrupting biofilms, or enhancing antibiotic efficacy, offering potential solutions to overcome antibiotic resistance.

Ciprofloxacin of the class fluoroquinolones display broad-antibacterial spectrum, extensively covering Gram-positive bacteria (e.g., *Staphylococcus aureus*, *Streptococcus pneumoniae*) Gram-negative bacteria (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*), and works by targeting bacterial DNA gyrase and

topoisomerase IV. Unfortunately, bacteria develop resistance to ciprofloxacin by any of mechanism viz target site mutation, drug inactivation, over expression of efflux pump and biofilm formation. To encounter the resistant bacteria, ciprofloxacin in combination with other antibiotics are in clinical use. Many natural/synthetic molecules when used in conjunction increase the efficacy of ciprofloxacin and have been reported in the past (Kalia *et al.,* 2012).

Chalcones are among the most common flavonoids across the plant kingdom, with the name originating from the Greek literature where "chalcos" means bronze. Plant species belonging to Genera *Angelica*, *Glycyrrhiza*, *Humulus*, and *Scutellaria*, typically synthesize them as precursors in the biosynthetic pathways of flavonoids and isoflavonoids, or as intermediates in the synthesis of compounds that are heterocyclic in nature (Kamei *et al.*, 1997; Abbas *et al.*, 2014).

Chalcones have widespread applications in medicinal chemistry since they occur naturally in some plants and are non-toxic. Their unique chemical structure, consisting of two aromatic rings connected by a three-carbon α, β-unsaturated carbonyl system, has imparted chalcones with diverse biological properties and extended usage in the field of medicine, agriculture, and industry (Salehi et al., 2021).

They exert their antibacterial activity through multiple modes of action, including disruption of bacterial cell wall formation, inhibiting essential enzymes and compromising the structural integrity of the cell membrane. Additionally, chalcones have been shown to interfere with bacterial DNA replication and transcription processes, thereby inhibiting bacterial growth. The unique characteristics of chalcones make them attractive prospects for the creation of novel antibacterial therapeutics. Recent research reveals that chalcone and its derivatives when used alone or as an adjunct with antibiotics (e.g. Oxacillin) have produced satisfactory results in containing the transmission of multidrug resistant infectious agents, including methicillin-resistant *Staphylococcus aureus* (MRSA) (Talia et al., 2011).

Chalcones efflux inhibition activity has been investigated and proven effective against a variety of bacterial species, including Gram-positive and Gram-negative bacteria. Some of the most susceptible bacterial species include *S. aureus*, *E. coli*, *P.*

aeruginosa, and Salmonella typhimurium. (Dan and Dai., 2020; Holler *et al*., 2012; Thai *et al*., 2015).

In this study, we invertigated the role of chalcone in combating the drug resistance due to NorA efflux pump and Agr gene's role in biofilm formation when tested in combination with ciprofloxacin against *S. aureus*. The study was further extended to establish the possible association between efflux pump and biofilm in *S. aureus* resistance.

CHAPTER -2 REVIEW OF LITERATURE

2.1. *Staphylococcus aureus*

Staphylococcus is a type of bacterium that stains purple in the Gram staining process, indicating it is gram-positive. It is characterized by spherical cells, known as cocci, which typically range from 0.5 to 1.5 micrometers in size. These cocci divide in a single plane, resulting in clusters resembling grapes. *Staphylococci* are non-motile and do not form spores. They are facultative anaerobes, meaning they can grow in both aerobic and anaerobic conditions, utilizing aerobic respiration or fermentation for growth. (Masalha et al., 2001). Surgeon Alexander Ogston initially identified *Staphylococcus aureus* in 1880 while treating patients with ulcerated wounds in Aberdeen, Scotland (Guo et al., 2020). *Staphylococci* need organic sources of nitrogen to meet their nutritional needs, these can be found in B vitamins and important amino acids like arginine and valine. Catalase-positive and oxidase-negative characteristics set s*taphylococci* apart from catalase-negative *streptococci*. Additionally, they can withstand high salt concentrations and temperatures. 33 species and 8 subspecies make up the genus *Staphylococcus*; 17 of these species are mostly found in human specimens and colonize the human body. Among these *Staphylococcus epidermidis* and *Staphylococcus aureus* are the extensively researched strains (Medveďová & Valík., 2012).

Pathogenic *staphylococci* are characterized by their ability to produce coagulase. This characteristic is the basis for the differentiation of coagulase-positive *S. aureus* in human and *S. intermedius* in animals from other staphylococcal species such as *S. epidermidis*, which are coagulase negative (Cheng et al., 2010).

2.2. *Staphylococcus aureus* Subsp. *aureus*

Staphylococcus aureus bacteria are Gram-positive, cocci-shaped, and typically seen in clusters that are referred to as "grape-like.". Aureus, which means golden or yellow, refers to the color of the colonies of these bacteria, these organisms grow at temperatures ranging from 18°C to 40°C, either facultatively or anaerobically. It is a normal resident of the human body and can be found in the nasal passages of 20 to 40% of individuals. It can lead to a broad range of infections, from mild cutaneous

problems to serious, sometimes fatal systemic illnesses. Skin infections include deeper infections of subcutaneous tissue, such as furuncles and carbuncles, as well as more superficial disorders like folliculitis and impetigo. Systemic infections can result from invasive occurrences where *S. aureus* gets into wounds from burns, surgery, or accidents. *S. aureus* is linked to hospital-acquired pneumonia in patients suffering from obstructive lung disorders in healthcare settings.

However, soon after β-lactam antibiotics were introduced in 1940's, bacteria became resistant to such antibiotics by producing β-lactamases. The subsequent introduction of methicillin in 1960 was intended to overcome this resistance; despite this effort, methicillin-resistant strains emerged within a decade, and currently, approximately 60-80% of clinical *S. aureus* isolates exhibit methicillin (oxacillin) resistance. Methicillin resistant *Staphylococcus aureus* (MRSA) can cause chronic nosocomial infections which are due to its ability to adhere to medical devices such as catheters and form a biofilm (Bhattacharya et al., 2015).

People who have a history of cancer are more likely to develop bacteremia. Bacteremia can spread to other locations and cause endocarditis, osteomyelitis, septic arthritis, and abscesses in the skin, lungs, liver, kidneys, and brain. Patients with anomalies of the central nervous system resulting from trauma, surgery, cancer, or hydrocephalus may develop staphylococcal meningitis. Additionally, *S. aureus* is linked to peritonitis in patients receiving continuous ambulatory peritoneal dialysis (CAPD) (Taylor & Unakal, 2023).

Staphylococcus aureus (*S. aureus*), is a highly versatile microorganism that opportunistically infect humans, causing a range of skin infections with varying severity and potentially high mortality rates. While *S. aureus* is not always disease-causing, it is a frequent cause of skin infections (e.g. boils) and food poisoning (Tucker ME., 2005). Strains associated with disease often employ virulence mechanisms, including the production of potent protein toxins and the expression of cell-surface proteins that can neutralize antibodies by binding and inactivating them. *Staphylococcal* infections can turn deadly if the bacteria invade deeper into the human body, entering bloodstream, joints, bones, lungs or heart (Thomer et al.,

2016). Also, people are developing life-threatening staphylococcal infections and majority of these infections are no longer respond to common antibiotics is use. The growing threat of antibiotic-resistant *S. aureus strains*, including MRSA, has become a major hurdle in the global effort to combat bacterial infections, with significant implications for public health (Achek et al., 2018).

Toxic shock syndrome, food poisoning, and epidermal necrolysis are all linked to the toxins that *S. aureus* produces. Numerous virulence factors that are released by the bacteria and are linked to its cell surface enable it to adhere to both host cell surfaces and injured tissue. Certain virulence factors help evade the immune system by binding to blood proteins. Extracellular enzymes like lipases, hyaluronidases, proteases, and nucleases are secreted and cause tissue damage, which facilitates the organism's spread. Superantigens exacerbate the symptoms of septic shock, while *S. aureus*'s membrane-damaging toxins cause cytolytic effects on host cells (Fisher et al., 2018).

S. aureus is a bacterium that poses a serious clinical risk because of its propensity to cause a wide range of illnesses as well as its exceptional capacity to become resistant to antibiotics Past data indicates that *S. aureus* quickly acquired antibiotic resistance soon after they were first introduced. *S. aureus* is a common bacterium that is regularly found on the skin and nasal passages of healthy people and causes normal skin infections to deadly ailments like sepsis, pneumonia, and endocarditis (Tong et al., 2015)

2.3. Morphology of *S. aureus*

2.3.1 Shape and Arrangement: As described above, morphologically *S. aureus* is spherical (cocci) in shape and typically found in clusters when observed under a microscope. These clusters are a result of cell division occurring in multiple planes, giving rise to irregular arrangements (Taylor & Unakal, 2023).

2.3.2 Cell Wall Structure: The cell wall of *S. aureus* is an important factor of its morphology. It is majorly composed of peptidoglycan, which provides structural support and protection to the bacterium. Additionally, the cell wall contains teichoic acids, which help to stabilize the cell wall structure and are involved in interactions

with host cells during infection. (Sutton et al., 2021)

2.3.3 Capsule: A polysaccharide capsule can be produced by specific strains of *Staphylococcus aureus,* constituting the outermost component of their cell wall. This capsule enhances bacteria's ability to elude the host's immune system and contributes to its virulence by preventing phagocytosis. (Kuipers et al., 2016)

2.3.4 Flagella and Motility: *S. aureus* typically lacks flagella and is considered non-motile. However, there are exceptions, as some strains may possess flagella and exhibit motility, although this is relatively rare compared to other bacteria. (Pollitt et al., 2017)

2.3.5 Biofilm Formation: Biofilm formation is a capability of *S. aureus*, involving the accumulation of surface-associated cells surrounded by an extracellular polymeric scaffold produced by the bacteria themselves. The ability of biofilms to adhere to surfaces, including medical equipment and host tissues, enhances the bacteria's resistance to antimicrobial agents and immune responses, thereby playing a pivotal role in the pathogenicity of *S. aureus* and significantly contributing to disease development and progression. (Idrees *et al.,* 2021).

2.3.6 Pigmentation: *S. aureus* can exhibit various pigmentations, including golden-yellow (due to the production of the carotenoid pigment staphyloxanthin) or white. The synthesis of pigments, as reported by Liu et al. (2020), is a valuable characteristic for identifying and classifying strains, and may also have implications for the bacteria's virulence and survival strategies.

2.3.7 Size and Growth Characteristics: Under optimal conditions, *S. aureus* cells typically measure around 0.5 to 1.0 micrometers in diameter. It is a facultative anaerobe, meaning it can grow in the presence or absence of oxygen, although it prefers aerobic conditions. *S. aureus* colonies grown on agar plates are typically smooth, round, and creamy white to golden in color (Missiakas et al., 2013)

2.3.8 Biochemical Characteristics of *S. aureus*

The catalase test is a fundamental step in identifying *S. aureus*. This test relies on the detection of the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen. When a colony of *S. aureus* is exposed to hydrogen peroxide, bubbling

or effervescence occurs due to the release of oxygen gas, indicating a positive catalase reaction. Another crucial test is the coagulase test, which detects the production of coagulase, a key enzyme that triggers blood clotting by converting fibrinogen to fibrin. *S. aureus* typically exhibits rapid clot formation, indicating a positive coagulase reaction. Mannitol Salt Agar (MSA) is a selective and differential medium commonly used to differentiate *S. aureus* from other staphylococci. *S. aureus* can ferment mannitol present in the medium, leading to acid production, which lowers the pH and causes the colonies to appear yellow. Hemolysis on blood agar is another distinguishing characteristic of *S. aureus*. It typically exhibits beta-hemolysis, resulting in the complete lysis of red blood cells around colonies, forming clear zones. The API *Staphylococcus aureus* test is a commercial kit containing various biochemical tests in strip format, providing a profile of reactions to aid in identification. Additionally, the nitrate reduction test evaluates the ability of bacteria to reduce nitrate to nitrite or other reduced products under anaerobic conditions, providing further insights into the metabolic capabilities of *S. aureus*. These biochemical tests, along with others, collectively contribute to the accurate identification and differentiation of *Staphylococcus aureus* in clinical and laboratory settings (Karmakar et al., 2016).

Table 2.1: Major Biochemical Characteristics of *S. aureus*

Biochemical Characteristics	Description
Gram Stain Reaction	Gram-positive cocci in clusters
Catalase Test	Positive (bubbling or effervescence)
Coagulase Test	Positive (rapid clot formation)
Mannitol Fermentation	Positive (acid production, yellow colonies on MSA)
Hemolysis on Blood Agar	Beta-hemolysis (complete lysis of RBCs, clear zone around colonies)
Growth on Selective Media	Growth on Mannitol Salt Agar (MSA)
Enzyme Production	Produces lipases, proteases, nucleases
Fermentation of Sugars	Ferments glucose, maltose, lactose, sucrose
Nitrate Reduction	Positive for nitrate reduction
Acid Production from Glucose	Positive (acid production from glucose fermentation)

2.4 *S. aureus* infection and disease

Among bacterial pathogens, *Staphylococcus aureus* is the leading cause of human infection with wide range of clinical symptoms. These infections are common in hospital as well as in community. The rise of multi-drug resistance strains like MRSA makes the treatment of this pathogen more difficult. For the most part, healthy individuals have *S. aureus* on their skin and mucous membranes (typically the nose area). It is also found in the natural flora of humans and the surroundings. When *S. aureus* goes into the circulation or internal tissues, it can lead to a variety of potentially deadly infections, but it usually does not cause sickness on healthy skin. Transmission usually occurs through direct contact. In contrast, various diseases have distinct modes of transmission (Tong et al., 2015).

2.4.1 Epidemiology

Staphylococcus aureus is primarily found on human skin and mucosal membranes, making humans its main reservoir. Additionally, this bacterium may be drug resistant. Approximately half of the population is believed to be colonized with *Staphylococcus aureus*, while around 15% are persistent carriers, harboring the bacteria in their anterior nares. Certain populations such as healthcare professionals, diabetics and IV drug users have higher colonization probability (as high as 80%). Moreover, Transmission primarily occurs through direct contact, although alternate routes exist in certain cases (Tong et al., 2015; Rasigade et al., 2014).

2.4.2 Pathophysiology

S. aureus stands out as one of the most prevalent bacterial pathogens afflicting humans, accountable for a myriad of infections. These encompass a spectrum of conditions ranging from bacteremia, infective endocarditis, to skin and soft tissue infections like impetigo, folliculitis, and more (Tong et al., 2015). *S. aureus* can cause toxin-mediated disorders or invasive diseases, depending on the strain and infection site. The pathogenesis of these infections varies greatly, involving host immune response evasion mechanisms such as biofilm formation, antibody sequestration, and capsule synthesis.

In infectious endocarditis, binding to extracellular matrix proteins is facilitated by cell wall-associated proteins while super antigens like TSST-1 play crucial roles in endocarditis and toxic shock syndrome (Salgado-Pabón et al., 2013). Pneumonia associated infections involve virulence factors like PVL, Protein A, and alpha-hemolysin, particularly prevalent post-influenza or in Cystic Fibrosis patients. Prosthetic device infections often stem from biofilm formation and quorum sensing (Le et al., 2015).

S. aureus, Because of its ubiquity in both community and hospital settings presents a significant problem in clinical care, compounded by the rise of multi-drug resistant strains such as MRSA. While typically residing in the environment and human flora, *S. aureus* doesn't usually cause issues unless it breaches the skin or mucous membranes, potentially leading to severe infections. (Rasigade et al., 2014).

2.4.3 Etiology

Staphylococcus aureus exhibits a distinctive Gram-positive profile, characterized by a purple appearance in Gram staining, and typically displays a cocci-shaped morphology, often forming clusters that resemble grapes. The bacteria can thrive on a media containing 10% salt concentration, and bacterial colonies are typically characterized by golden or yellow coloured hue. They exhibit facultative anaerobic growth and flourish at temperatures ranging between 18℃ and 40℃. MRSA strains harbor a mec gene on their chromosome, responsible for impeding resistance to various antibiotics. This gene encodes for a protein PBP-2a (Penicillin binding protein 2a), which catalyzes synthesis of peptidoglycan in bacterial cell wall. PBP-2a has weaker binding affinity for beta-lactam antibiotic class (and other penicillin-derived antibiotics) in contrast to other PBPs, enabling continuous bacterial cell wall synthesis even if antibiotics are present, rendering resistance in MRSA strains (Rasigade et al., 2014).

2.5. Drug resistance mechanism of *S. aureus*

The overuse of antibiotics and bacterial adaptability have led to a progressive rise in *S. aureus* drug resistance. Many anti-*staphylococcus* medications are available, however because the bacteria have developed resistance mechanisms, they quickly

lose their therapeutic efficacy. According to Mlynarczyk-Bonikowska et al., 2020, the three main basic mechanisms of antimicrobial resistance include alterations in bacterial proteins targeted by, enzymatic degradation of antibacterial medicines, and modifications in membrane permeability to antibiotics. By acquiring the staphylococcus cassette chromosome (SCC mec), a genomic island that contains the methicillin resistance determinant mecA, *S. aureus* acquires resistance to beta-lactamase (Hiramatsu et al., 2013). Antibiotic resistance in *S. aureus* has been demonstrated through biofilm development and quorum sensing.

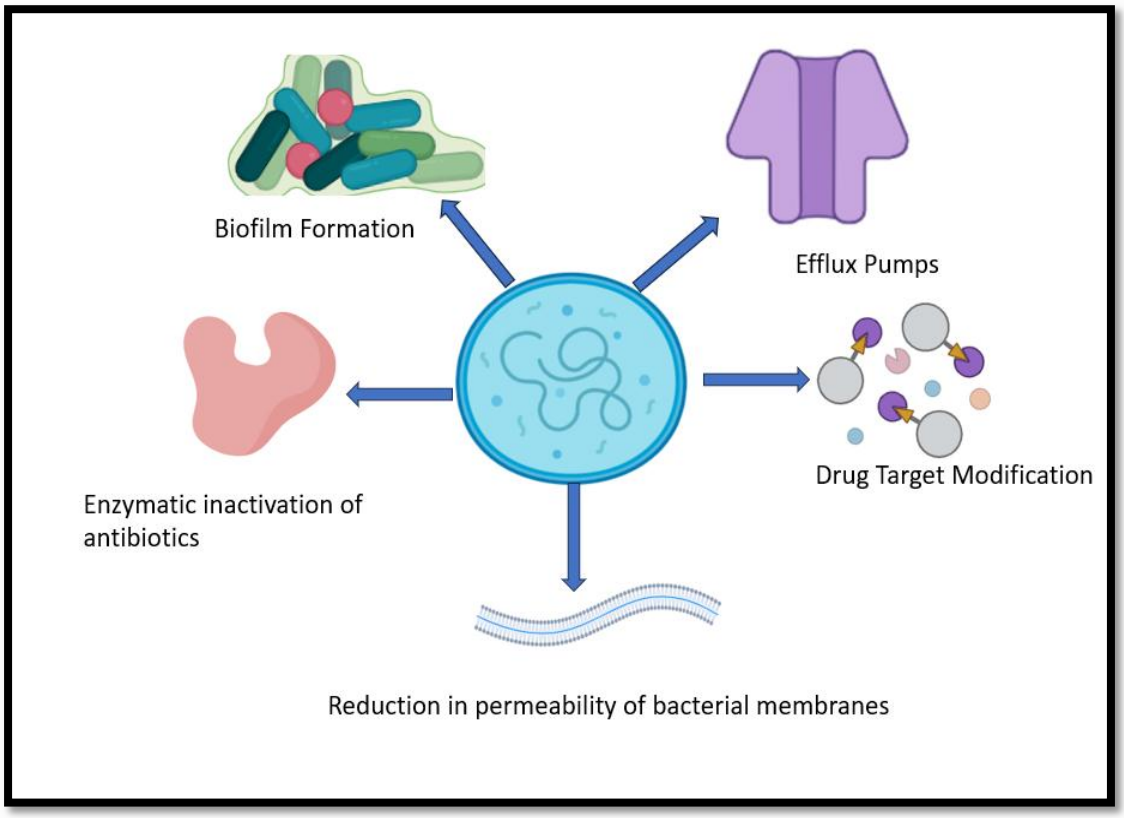


Figure 2.1: Different drug resistance mechanisms of *S. aureus*

2.5.1 Structural alteration of a biological target

A mechanism of resistance to antibiotics of the β-lactam and glycopeptide types is the modification of the biological target. The resistance of certain bacterial strains, such as MRSA, to β-lactam antibiotics is attributed to the acquisition of a gene encoding a mutated form of transpeptidase, specifically PBP2a. Kinetic studies have revealed that the binding affinity of PBP2a to β-lactam antibiotics is significantly reduced,

with a binding rate constant that is three to four orders of magnitude lower than that of other transpeptidase. The structure of PBP2a, which shows considerable conformational changes prior to covalent binding to a β-lactam, is responsible for this substantial drop. Through their binding to the D-alanine-D-alanine terminal segment of the peptidoglycan precursor, glycopeptides prevent the development of bacterial cell walls. The majority of bacteria in this group may be resistant to antibiotics due to the acquisition of genes encoding enzymes that collaboratively produce a modified peptidoglycan precursor, featuring a terminal D-alanine-D-lactate fragment. Compared to the normal form of the peptidoglycan, glycopeptides have a substantially lower affinity for this modified form (Zeng et al., 2016).

2.5.2 Enzymatic inactivation of antibiotics

inactivation of enzymes is a common mechanism of resistance in bacteria including *S. aureus*. Penicillin and cephalosporins are examples of beta-lactam antibiotics that are vulnerable to enzymatic breakdown by beta-lactamases, which hydrolyze the beta-lactam ring and cause the antibiotic to become inactive. Beta-lactamases can be produced by *S. aureus*. An example is the production of penicillinase by some strains of *S. aureus*, which hydrolyzes penicillin, making it ineffective against these bacteria (Bush & Bradford, 2016).

2.5.3 Reduction in permeability of bacterial membranes

S. aureus can develop mechanisms to reduce the entrance of antibiotics in bacterial cell, thereby reducing their effectiveness. This can be achieved through various means, such as thickening of the cell wall or outer membrane, which acts as a barrier to antibiotic penetration. Additionally, changes in porin proteins, which are channels that facilitate the passage of molecules across the cell membrane, can reduce the antibiotic acceptance. For example, some strains of *S. aureus* may down-regulate the expression of porins, thereby restricting the penetration of antibiotics into the bacterial cell (Reygaert 2018).

2.5.4 The activity of efflux pumps

One of the key mechanisms contributing to multidrug resistance phenotype is the expression and activity of multidrug efflux pumps. These transport proteins are capable of extruding a variety of structurally unrelated antimicrobial compounds,

thereby reducing their intracellular accumulation and conferring resistance to the bacterial cell. Various efflux pump mechanisms are identified and characterized in this clinically significant pathogen, includes the Nor, Mep, NorA, NorB, NorC, MdeA, SepA, and SdrM pumps (Costa et al., 2013).

The Nor efflux pumps, which include NorA, NorB, and NorC, are among the most well-studied multidrug transporters in *S. aureus*. These pumps can extrude a diverse array of antimicrobial agents, including fluoroquinolones, antiseptics, and dyes. Nor efflux system expression is frequently elevated in clinically significant strains of *Staphylococcus aureus*, playing a key role in the emergence of multidrug resistance (Costa et al., 2013).

Another important efflux system is the Mep (Multidrug and Toxin Extrusion) family of pumps, which can confer resistance to a wide range of antibiotics, biocides, and dyes. The MepA pump, in particular, has been extensively studied and shown to play a significant role in the intrinsic and acquired resistance of *S. aureus* to various antimicrobial agents. Other efflux systems identified in *S. aureus*, such as the MdeA and SepA pumps. The MdeA efflux pump plays a pivotal role in conferring resistance to antibiotics, antiseptics, and dyes, while the SepA pump has been associated with increased efflux of fluoroquinolones and ethidium bromide (Sharma et al., 2019).

The differential expression of various efflux transporters in clinically relevant strains of *S. aureus* is a crucial factor in determining the overall multidrug resistance phenotype of the pathogen. The regulation of efflux pump expression is a complex process that involves the interplay of various transcriptional regulators, environmental signals, and stress responses.

Advancements in the understanding of the structural features and molecular mechanisms of efflux pump function in *S. aureus* have shed new light on the complex modalities of these versatile drug transport systems, revealing novel targets for antibiotic development. Studies have revealed the presence of specific substrate-binding sites, the importance of proton-motive force for pump activity, and the role of conformational changes in the efflux process. In *S. aureus*, beyond their impact on AMR. These transporters have been implicated in the extrusion of various

metabolites, toxins, and signaling molecules, suggesting their involvement in cellular homeostasis, virulence, and quorum sensing (Kim et al., 2021).

2.5.5 Biofilm formation

The capacity to form biofilms is considered a pivotal virulence attribute in *Staphylococcus aureus*, significantly contributing to its pathogenicity. In literal terms, biofilms are bacterial cell communities that synthesize their own extracellular polymeric matrix [comprising of polysaccharides, proteins, DNA, and lipids]. The biofilm matrix serves as a protective barrier to the bacteria from host immune defenses and antimicrobial agents, making biofilm-associated infections extremely difficult to treat. The biofilm formation process involves several stages: initial attachment to a surface, extracellular matrix production and bacterial proliferation, biofilm maturation, and final dispersal of cells (Yamazaki et al., 2024). Key genes and regulatory systems like the Agr quorum sensing system coordinate this complex process. Biofilms are characterized by well-organized specialized configurations regulating intimate relationships by interactions (Kolenbrander & Palmer, 2000). Self-sufficient, social lifestyle of bacteria derived by their coordinated communication has led researchers to ascertain more details about these well-designed communities (Nadell et al., 2008). Microbiologists continuously keep on discovering the coordinated behavior of microorganisms involved in biofilm formation (Watnick & Kolter 2000). The *Staphylococcal* biofilms show remarkable resistance to host immune system and other chemotherapies currently used in clinical settings (Ricciardi et al., 2018). These virulent microbial communities are significantly characterized by specific proteins, polysaccharides, and extracellular DNA which are supposed to be paramount components (Otto, 2008). These drug resistant microbial communities adhere to a suitable surface by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), adhesion proteins and other non -proteinaceous adhesions like polysaccharide intracellular adhesion (PIA). Ica operon (icaA, icaD, icaB and icaC) is responsible for synthesizing PIA whereas oleic acid, glucose, urea and glucosamine have been found to be influential factors for PIA expression (Yu et al., 2012). Disparaging of *S. aureus* biofilm formation is also attributed by decreased level of PIA. Accumulation includes cell multiplication, fusion of slime (PNAG: Poly-s (1,6)-Nacetyl-D-glucosamine),

surface proteins and extracellular DNA. Maturation leads to carbohydrate consumption and nutrition depletion that leads to emergence of persister cells (Fig. 2.2). These persister cells also involve into the reoccurrence of Staphylococcal infection. It was also reported that the process of biofilm dispersal is mediated by agr system in *S. aureus* (Tan et al., 2018).

A significant problem with *S. aureus* biofilm infections is the high tolerance of biofilm-embedded cells to antibiotics compared to planktonic bacteria. The minimum inhibitory concentrations (MICs) and minimum biofilm eradication concentrations (MBECs) can differ by up to 1000-fold. Some of the mechanisms underlying this increased antibiotic tolerance include changes in cell permeability, up-regulation of antibiotic-modifying enzymes, and the presence of persister cells in the biofilm (Peng et al., 2022).

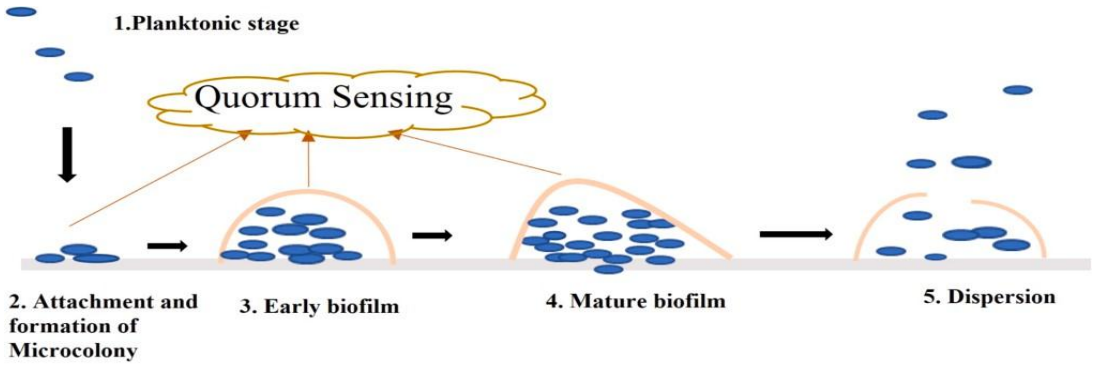


Figure 2.2: Quorum sensing allows individual bacteria in colonies to coordinate and carry out colonial functions such as: sporulation, virulence, conjugation, and biofilm formation

Glycopeptides like vancomycin, which are commonly used to treat MRSA infections, have poor penetration into biofilms and require extremely high concentrations to be effective. Penicillins, cephalosporins, and other cell wall-active agents also show limited activity. More promising results have been seen with antibiotics like rifampicin, aminoglycosides, daptomycin, and fosfomycin. Rifampicin in particular demonstrates robust anti-biofilm activity, but should not be used as monotherapy due to rapid resistance development. Combination antibiotic regimens, such as vancomycin plus rifampicin or daptomycin plus fosfomycin, have shown synergistic effects in some in vitro and *in vivo* studies (Tuon *et al.*, 2023).

S. aureus biofilm infections, especially those associated with implanted medical devices, remain a significant clinical problem attributed to the high biofilm tolerance towards conventional antibiotic therapies.

2.6 Efflux pumps

Embedded in the membrane of pathogens, actively ejecting drugs causing reduction of drug accumulation and causing drug resistance (Figure 2.3). Physiologically their role in bacteria is the extrusion of noxious metabolites and production of virulence determinants, providing a view that the drugs are sudden substrates of these exporters (Forrellad et al., 2013). Microbial efflux system can either transport a single class of drug or antibiotic or more than one class of chemotherapeutic agents, so they named as multidrug resistance efflux pumps. Active efflux of antibiotics is one of the supreme mechanisms of drug resistance in *Staphylococcus aureus*. Though these pumps alone are not resistance conferring yet plays a major role in hampering efficient antimicrobial therapy (Bhardwaj & Mohanty., 2012). *In Staphylococcus aureus*, more than ten MDR efflux pumps are known to exist, with their genes located on either the chromosome or plasmids (Table 2.2).

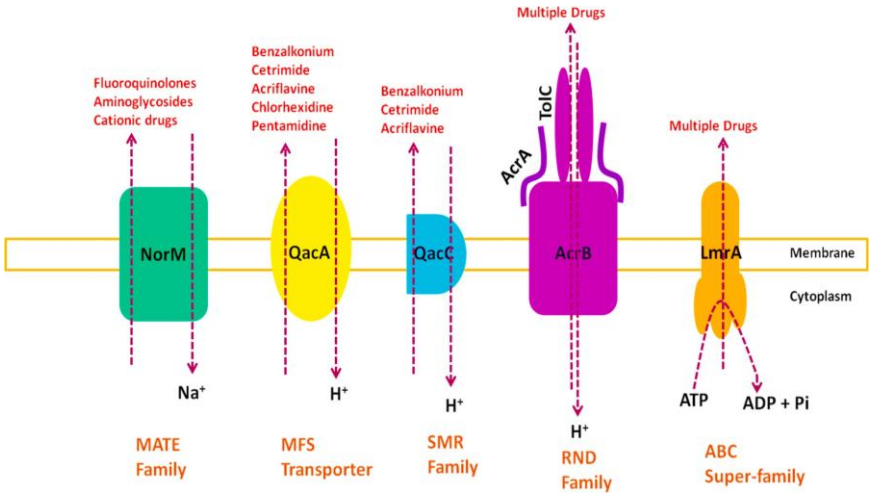


Figure 2.3: Bacterial efflux pumps. The Figure shows diagrammatic representation of the five structural classes of antibiotic transporters. Abbreviations: ABC: ATB- binding cassettes, MFS: major facilitator superfamily, RND: resistance-nodulation-division, SMR: small multi-drug resistance, MATE: multidrug and toxic compound extrusion.

Table 2.2: MDR efflux pumps and their identified substrates.

S. No	Efflux Pump	Substrate	Family
1	QacA	Benzalkonium chloride, dequalinium, Diamidines, Biguanidines, tetraphenylphosphonium, ethidium bromide, rhodamine	MFS
2	Smr	Cetrimide, benzalkonium chloride, ethidium bromide	SMR
3	QacG	Benzalkonium chloride, cetyltrimethylammonium, ethidium bromide	SMR
4	QacJ	Benzalkonium chloride, cetyltrimethyl ammonium, ethidium bromide	SMR
5	NorA	Ciprofloxacin, norfloxacin, tetraphenylphosphonium, benzalkonium chloride, ethidium bromide, rhodamine	MFS
6	NorB	Ciprofloxacin, norfloxacin, moxifloxacin, sparfloxacin, tetracycline, tetraphenyl phosphonium, cetrimide, ethidium bromide	MFS
7	NorC	Ciprofloxacin, moxifloxacin, rhodamine	MFS
8	SdrM	Norfloxacin, ethidium bromide, acriflavine	MFS
9	MdeA	Ciprofloxacin, mupirocin, norfloxacin, fusidic acid, Virginiamycin, novobiocin, tetraphenylphosphonium, benzalkonium chloride, dequalinium, ethidium bromide	MFS
10	Tet38	Tetracyclines	MFS
11	MepA	Fluoroquinolones (e.g.hydrophillic: ciprofloxacin, sparfloxacin, norfloxacin, moxifloxacin, tigecycline), tetraphenylphosphonium, benzalkonium chloride, cetrimide, ethidium bromide	MATE
12	LmrS	Erythromycin, linezolid, chloramphenicol, fusidic acid, kanamycin, florfenicol, trimethoprim, tetraphenylphosphonium, sodium docecyl sulphate, ethidium bromide	MFS

2.6 .1 Type of Multidrug efflux pumps in *S. aureus*

The multidrug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), the resistance-nodulation cell division (RND) superfamily, the small multidrug resistance (SMR) family, and the adenosine-triphosphate (ATP)-binding cassette (ABC) superfamily are the five families that make up antimicrobial efflux systems of *S. aureus*. While the other four are second-order transporters that use the proton gradient, the ABC family's transporters use energy from the hydrolysis of ATP (Zhang et al., 2023).

The most thoroughly researched multidrug efflux pump in *S. aurues* is the MFS, which consists of NorA, NorB, NorC, MdeA, SdrM, LmrS, QacA, and QacB Of them, NorA, NorB, NorC, MdeA, SdrM, and LmrS are found on chromosomes, and QacA and QacB are plasmid encoded. Plasmids harbor the SMR transporters Smr (QacC, QacD, and Fbr), QacG, QacH, and QacJ, which are smaller in size than other *S. aureus* efflux proteins. MepA is the sole chromosomally encoded protein that is a member of the MATE family of efflux pumps in *S. aureus*. The ABC efflux pump family in *S. aureus* includes the chromosomally encoded SepA, AbcA and Sav1866, while MsrA, VgaA, Vga (A) LC and VgaB are present on a plasmid. In the RND family, FarE is a homolog of the *E. coli* AcrB protein and is responsible for drug resistance in *S. aureus* (Lekshmi et al., 2018).

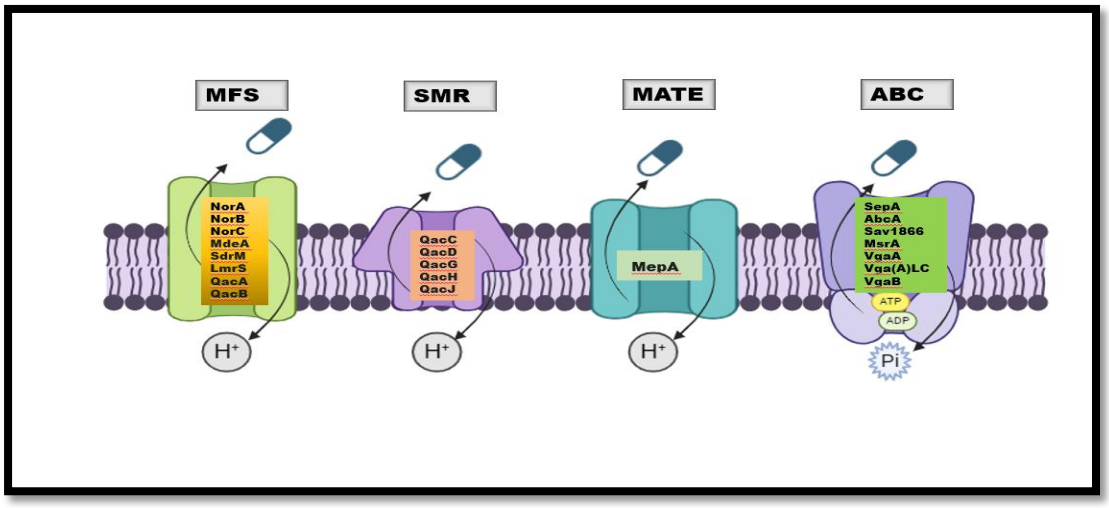


Figure 2.4: Different multidrug efflux pumps in *S. aureus*

2.6 .2 Efflux pump inhibitors(EPIs)

With the arrival of novel resistance paradigms, drug resistance in pathogens continues to heighten globally. Exploring new promising therapeutic agents or upgrading the molecular motif of conventional antibiotics that circumvent resistance mechanisms is a blueprint to withstand drug resistance. To combat this drug resistance, efflux pump inhibition appears to be a captivating approach for enhancing drug efficacy by its accumulation inside the cell. A series of efflux pump inhibiting compounds are nominated for their vital role in interfering with efflux activity. These structurally diverse efflux interrupting derivatives act synergistically with most frequently used antibiotics, influencing their substantially increased activity. Several molecules either isolated from a natural source or synthesized/modified com- pounds have been used to potentiate the efficacy of antibiotics against pathogens in past few decades (Cheesman et al., 2017). Huge number of EPIs has been isolated from natural sources but many of these do not find uses in the clinical settings due to menacing toxicity, as is the case with reserpine. Studies had reported number of structurally manifold inhibitors of NorA, a multi-drug transporter of *S. aureus*, that pursue an activity in a synergistic manner with the most widely used antibiotics and can increases their activity against both wild type and NorA over-expressing *S. aureus* isolates (Markham et al., 1999). In-vitro evaluation of numerous compounds of various groups has emphasized novel efflux pump inhibitors of NorA (Markham et al., 1999); Ambrus et al., 2008).

2.6.3 Plant alkaloid

Plant alkaloid reserpine, an antihypertensive mammalian multidrug efflux inhibitor, originally extracted from the roots of Rauwolfia vomitoria, has been found to enhance antibiotic efficacy by blocking bacteria efflux pump, which are a key mechanism of drug resistance. Notably, combining reserpine, a potent inhibitor of multidrug efflux pumps, with antibiotics significantly lowers the minimum inhibitory concentration (MIC) required to combat methicillin-resistant Staphylococcus aureus (MRSA), as demonstrated by Gibbons and Udo (2000). Based on this precedents Mullin and his co-workers in 2004 evaluated, biricodar (VX-710) and timcodar (VX-853), two novel mammalian multiple drug efflux pump inhibitors for activity against *S. aureus* drug resistance, suggesting that VX-710 and VX-853 are an emerging category of bacterial

efflux inhibitors with the capability to improve the therapeutic effectiveness of medications (Mullin et al., 2004). Stermitz et al, had identified berberine a plant alkaloid as a substrate for bacterial MDR pump. In addition to these Flavonolignan and flavone inhibitors, methoxylated flavones (Stermitz et al., 2002) and isoflavones (Morel et al., 2003) flavones chrysosplenol-D and chrysoplenetin isolated from Artemisia annua (Asteraceae) (Liu et al., 1992), chalcone, catechin gallates and spinosan A and isoflavone extracted from 'smoke tree' (Belofsky et al., 2006), are the plant molecules which act significantly either alone or synergistically with other molecules, against NorA over-expressing *S. aureus* strains. Kaempferol rhamnoside extracted from Persea lingue Nees (Holler et al., 2012) and Caffeoylquinic acids isolated from Artemisia absinthium (Fiamegos *et al.,* 2011) act effectively as efflux pump inhibitor against pathogenic gram-positive bacteria. A study with Lawsoniainermis extract had demonstrated its bactericidal activity for *S. aureus* and other pathogenic Gram-positive and Gram-negative microbes (Baskaran & Suruthi., 2016) and in recent studies it has been assessed as potent efflux pump inhibitor of fluoroquinolones (Nakaminami et al., 2010) and has profound probability for assessing further as efflux pump inhibiting potential. Verapamil a calcium channel blocker, another P-glycoprotein inhibitor, was found to be an efficient intracellular activity modulator of ciprofloxacin and azithromycin against *Staphylococcus aureus* by increasing its cellular concentration but has reduced potency as compared to reserpine (Thota et al., 2008). Piperine, another alkaloid extracted from Piper longum (black pepper) of great significance, assessed as an efficient putative multidrug inhibitor of P-glycoprotein i.e., ATP dependent drug efflux pump (Sharma et al., 2014). The nano liposomal formulations of piperine were also found effective against MRSA and act via efflux inhibitory activity (Khameneh, et al., 2015). This resistance obstructing molecule reduced inflammation and improved digestion as well as an array of disorders. A study with piperine expands the existing comprehensive concept of efflux inhibition in *S. aureus* that revert the drug resistance (Mirza et al., 2011). A clinical trial elucidates the potential of piperine to enhance the pharmacokinetics of Omeprazole, a proton pump inhibitor, designed as gastro-retentive microsphere by emulsification- solvent evaporation method and therefore, improving the drug potential by demolition of drug extrusion maintaining the required plasma level of

drug (Hashem et al., 2013). Milk thistle seed contains a flavonolignan component, Silybin, which is effective in inhibiting the efflux system of MRSA by targeting NorA and disrupts its resistance to antibiotics (Wang et al., 2018).

2.6.4 Synthetic and modified inhibitors

Synthetic or modified inhibitors are also effective against drug resistant pathogenic bacteria. Evaluation of new synthetic efflux pump inhibitors that could be used in conjugation with novel antibiotics can restore the drug efficacy by suppressing the resistant phenotype that might emerge during chemotherapeutic or antibiotic treatment. Various documentations had signified progress of medicinal chemistry gadgets competent in search for efflux pump inhibitors of pathogenic gram-positive bacteria (Durães et al., 2018). A study with ofloxacin based efflux pump inhibitor validated that chemical alteration by inserting a functional motif of an efflux pump inhibitor is a worthwhile approach in a direction to revert drug resistance. GG918 mammalian efflux hampering agents, have been assessed against efflux derived resistant *S. aureus* and found to be comparable to reserpine in intensifying the affectivity of ciprofloxacin and norfloxacin, therefore, confer enhanced drug sensitivity (German et al., 2008). Butyrophenones and fractionalized 2-aryl-5-nitro-1H-indoles were also assessed as potent inhibitor of *S. aureus* NorA efflux pump (Zilla et al., 2013). By modifying the flavones nucleus of known efflux pump inhibitors, a series of 2-phenyl-4(1H)-quinolone and 2-phenyl-4-hydroxyquinoline derivatives have been synthesized with dominant EPI activity (Sabatini et al., 2011). Fabrication of (E)-3-(1-chloro-3,4-dihydronaphthalen-2-yl) acrylic acid (Rath et al., 2019) and new aryl benzo[b]thiophene and diarylthiophene derivatives and their synergistic evaluation with ciprofloxacin against *S. aureus* results in 2-16 fold reduction in MIC of antibiotic in an ethidium bromide efflux inhibition assay. These modified EPIs in combination with ciprofloxacin display a potent inhibitory action against NorA overexpressing *Staphylococcus aureus* strain (Sabatini et al., 2011). Citral amide-derivatives were evaluated as most potent efflux pumps inhibitors, that have been found to be highly effective to potentiate minimum inhibitory concentration of ciprofloxacin for *S. aureus* over expressing NorA pump as compared to well-known EPIs like verapamil, reserpine, piperine etc. (Li et al., 2019);Thota et

al., 2008). Phenylpiperidine selective serotonin reuptake inhibitors (PSSRIs) are capable of obstructing the performance of some MDR efflux pumps. Paroxetine was one of the first identified PSSRI that inhibits both NorA and MepA efflux pumps. On this basis, additional chemical modifications were performed in order to find out more potent EPIs (German et al., 2008). Piperine analogs were synthesized and their quantitative structure activity relationship (QSAR) was studied and further evaluated for its efflux pump inhibition activity. Ethidium bromide assay confirm their potency to inhibit efflux pump activity and surprisingly they were found to be more potent than other EPIs such as reserpine and verapamil (Kalia et al., 2012). Ferulic acid derivatives 4-((E)-2-(diethylcarbamoyl)vinyl)-2-methoxyphenyl acetate and (E)-methyl 3-(4-((p-tolylcarbamoyl)methoxy)-3- methoxyphenyl) acrylate have been screened for their putative efflux pump inhibitory activity and were found to be effective by inhibiting Nor type efflux pumps by both *in-silico* docking studies and in-vitro in clinical isolates of MRSA. The latter one also showed synergistic activity with Ciprofloxacin (Sundaramoorthy et al., 2018). Cinnamaldehyde derivatives were also found to be effective against NorA overexpressing *S. aureus* (Radix et al., 2018). These synthetic and modified EPIs provide broader spectrum and are highly potent inhibitors of *S. aureus* MDR efflux pumps. Previous studies have analyzed a synergistic effect of a proton pump inhibitor omeprazole and an antibiotic in human clinical trials for the growth of human tumor cells (Ikemura et al., 2017). Further three β-lactam antibiotics carbapenem, meropenem, and imipenem have been combined with clavulanic acid in clinical trial and explored for its efficacy to restore the drug activity against *Mycobacterium tuberculosis*. This recent study had demonstrated that the clavulanic acid in synergy with meropenem exhibits bactericidal bustle (Sharma et al., 2020).

2.7 Efflux pump NorA

One of the *S. aureus* efflux mechanisms that has been explored the most is the NorA multidrug efflux pump. In 1986, a fluoroquinolone-resistant strain from a Japanese hospital was found to carry the norA gene. Genetic studies have shown significant variability in NorA alleles, with three distinct variants identified so far, exhibiting up to 10% difference in their nucleotide sequences (Yu et al., 2002).

The NorA protein consists of 388 amino acids, is part of the Major Facilitator Superfamily (MFS), and features 12 transmembrane segments and have 24% sequence identity with the efflux pump Bmr from *B. subtilis* and 44% with the tetracycline efflux pump Tet (A) from *E. coli*. Studies reveal that NorA is capable of eliminating a broad variety of chemically and structurally distinct substances from the bacterial cell. These substances include hydrophilic fluoroquinolones, like ciprofloxacin and norfloxacin, dyes, like ethidium bromide(EtBr), and biocides, like quaternary ammonium compounds (Yoshida *et al.,* 1990; Kaatz *et al.,* 1993)

Increased expression may be constitutive, resulting from mutations in the NorA promoter region, or inducible, controlled by regulatory proteins, as per Kaatz et al. The first mutations in the NorA promoter region were found to be point mutations that happened inside the 5'-UTR region, 89 base pairs upstream of the initiation codon and downstream of the -10 motif (Kaatz et al., 2004). By changing its secondary structure and decreasing its susceptibility to RNase activity, these alterations may improve the stability of NorA mRNA. On the other hand, research has also shown that strains with these point mutations exhibit increased rates of NorA transcription, which has led to an increase in NorA transcripts (Kaatz et al., 2004). In this similar area, more recent studies have found deletions and insertions that might enhance NorA mRNA stability. Nevertheless, mutations within the NorA coding area have not been linked to antimicrobial resistance thus far. A distinct regulatory mechanism has not yet been found, however, a number of regulatory systems may be able to regulate the synthesis of NorA in addition to modifications in the NorA promoter region. Similar to other MFS efflux pumps, NorA uses an H^+ drug antiport mechanism to use the proton motive force to help move antimicrobial drugs across the cell membrane. Studies have demonstrated that protonophores, like carbonyl m-chlorophenylhydrazone (CCCP), can affect the efflux of ethidium bromide (EtBr) and norfloxacin via the NorA-mediated pathway by upsetting the membrane proton gradient (Costa et al., 2019).

2.8 Quorum sensing and Accessory gene regulator (Agr)

Biofilm formation represents a collective behavior among bacterial communities. Each phase, spanning from adhesion to mature biofilm dispersal and transmission, is

intricately regulated by a network of multiple regulatory systems or factors. Bacteria synchronize diverse physiological activities to perpetuate communications through a unique mechanism known as quorum sensing (Li et al., 2019);Li et al., 2018).This system allows bacteria to detect fluctuations in cell density or the presence of other bacterial populations in their vicinity by monitoring changes in the concentration of specific signal molecules known as autoinducers. Upon reaching a critical threshold, these signal molecules trigger the activation of pertinent genes, enabling bacteria to adapt to environmental changes. (Lu *et al.*, 2019).

Production, perception and response to tiny diffusible signaling molecules are versatile and unique features of quorum sensing (Antunes et al., 2010). Pertinently, it has long been known that quorum sensing-derived activities in any microbe need a maximum cell density in biofilm to invade the host immunity and communicate virulence (Pasmore &Costerton., 2003). *S. aureus* virulence factors include muster of adhesion molecules, toxins, and assorted molecules which present challenge to host immune system. Infections such as endocarditis and osteomyelitis are not caused by biofilm associated *S. aureus*. The investigation of social behavior of *S. aureus* in biofilm represents a substantial force in inventing preventive strategies to treat such biofilm associated MRSA infections (Pollitt et al., 2014). *S. aureus* quorum sensing includes a framework, unrelated to *Pseudomonas aeruginosa* AHL system and other pathogens, where autoinducer proteins (AIP) play an important role in virulence expression (Sully et al., 2014). Virulence is also regulated via accessory gene regulator (agr) system which is facilitated by virulence accelerating factors such as hemolysin, toxins, surface associated adhesions and autolysin. The agr system of *S. aureus* comprises of 3 Kbp loci consisting of 4 genes (agrA, agrC, agrD, and agrB) and transcribes genes for two effector molecules of the agr system RNAII and RNAIII. These two divergent transcripts are driven by P2 & P3 promoters. Two-component system includes agrA (cytoplasmic regulator) and agrC (transmembrane receptor-histidine kinase) which is encoded by P2 operon. Two component systems respond to AIP encoded by agrD (propeptide) when processed through agrB (integral membrane protein). On reaching a threshold accumulation of AIP in extracellular environment, two-component system gets phosphorylated and P3 promoter initiates transcription (Felden et al., 2011). P3 transcript RNAIII, mediate up-regulation of

virulence factors secretion as well as encodes the gene for d-toxin (hld) that acts as strong surfactant and prevent microbial adhesion to the surface (Queck et al., 2008). This indicates the dependence of biofilm dispersion on activated agr system (Fig. 2.5).

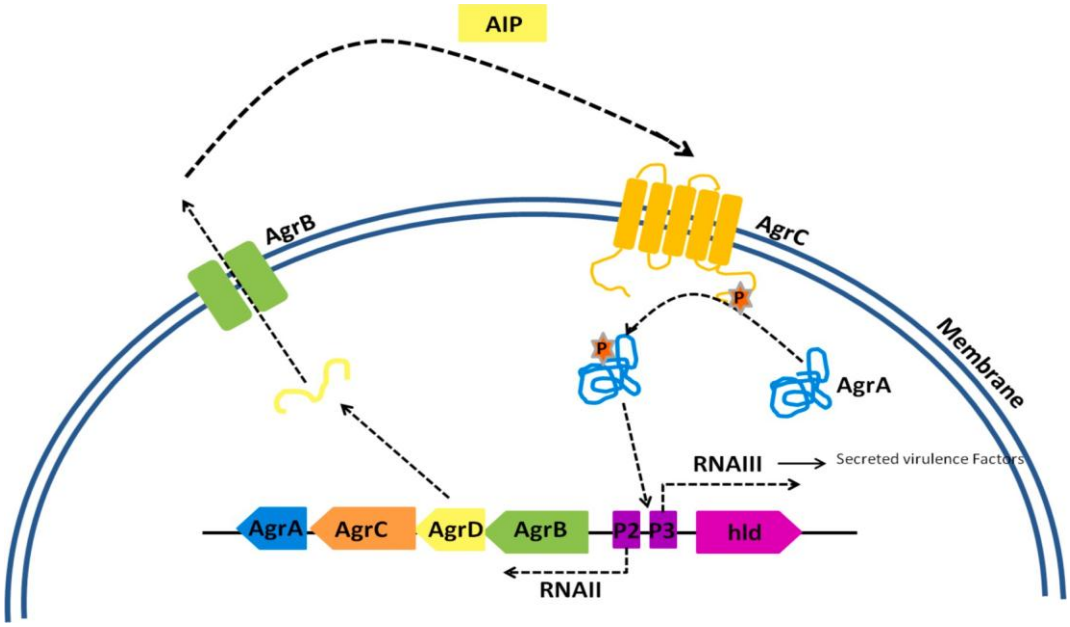


Figure 2.5: Diagrammatic representation of agr system in *Staphylococcus aureus*. AgrB (integral membrane protein) involved in secretion of autoinducer proteins. AgrD (propeptide) processed by AgrB into the AIP. AgrC (transmembrane receptor-histidine kinase) is the sensor part of a two-component system agr locus. AgrA is the cytoplasmic regulator of AgrC, and acts on the P2 or P3 promoter to upregulate *agr* and RNAIII expression. RNA molecule acts on several gene transcripts and through post-transcriptional control modulate gene expression through.

Studies investigating the relationship between agr expression and *S. aureus* biofilm formation has revealed that the agr gene plays a significant role in the heightened severity of patient illness and death. Schweizer in 2011 had reported that agr QS system mediates biofilm dispersal in *S. aureus*. This evidence has brought QS signal transduction system forward as an inexpensive approach to combat biofilm associated MRSA infections (Schweizer et al., 2011). MDR pumps, deriving transportation of quorum sensing signaling molecules also plays specific role in biofilm formation. However, "How these two junctures (QS system and Efflux

pumps) interplay?" this question still needs extensive investigation.

Quorum sensing pathways typically govern various aspects of bacterial behavior, including biofilm formation, virulence, adhesion, antibiotic resistance, motility, and sporulation. In *S. aureus*, the quorum sensing system comprises two key components: the accessory regulatory factor (Agr) system and the LuxS/AI-2 system. These systems modulate biofilm production through distinct mechanisms. The Agr system disrupts bacterial biofilms by up-regulating RNAIII transcription, while the LuxS/AI-2 system decreases the expression of polysaccharide intercellular adhesin (PIA) (Sionov et al., 2022).

The agr locus, a complex polygenic structure, governs the Agr system's activity in response to density of bacterial cells. It controls the expression of adhesion of *S. aureus* to the surface and extracellular proteins, exerting multifaceted regulation over biofilm formation stages. Utilizing an auto-inducing peptide (AIP) as the cell density signal molecule. The agr locus regulated by two-component quorum sensing system made up of two separate transcription units the P2 and P3 promoters (Peng et al., 2022).

The four open reading frames agrA, agrB, agrC, and agrD are encoded by RNAII transcription, which is started by the P2 promoter. Target gene regulation, signal sensing, transport, and AIP biosynthesis are all carried out by these genes. AgrA is a response regulator, AgrD is an AIP precursor, AgrB is an oligopeptide transporter that facilitates AIP secretion and serves as a protease for AgrD processing. AgrC is a sensor histidine protein kinase. AIP attaches to AgrC and activates it when its concentration reaches a certain point, causing autophosphorylation and subsequent signal transduction. AgrC phosphorylates AgrA, which causes the P3 promoter to start RNAIII transcription. RNAIII serves as the primary effector molecule of the quorum sensing system, positively regulating toxin gene expression, preventing toxin repressor translation, and reducing surface adhesin expression, which negatively impacts biofilm formation (Zhao et al., 2022). Elevated AIP levels can enhance *S. aureus* biofilm depolymerization by increasing extracellular protease secretion.

Additionally, up-regulation of agr promotes the production of phenol-soluble modulins (PSMs), facilitating biofilm maturation and spreading. Nutrients like glucose can influence biofilm development through the Agr system; glucose inhibits P3 promoter expression but activates the Agr system in established biofilms, promoting biofilm dispersal. Furthermore, several regulatory systems in *S. aureus* are interconnected with the Agr system, collectively modulating responses to environmental changes and biofilm development (Peng et al., 2022).

2.8.1 Relation between biofilm, efflux pump and quorum sensing

'Biofilms' as exceptionally drug resistant microbial territories have highly energetic efflux pumps which extrude antibiotics as well as transport cell signaling molecules. So, efflux pump prohibition by EPI can also simultaneously interrupt biofilm formation and cell signalling (QS) pathway (Alav et al., 2018). Resistance to quorum quenching molecules is simultaneous to unregulated efflux mechanism. On the other hand, efflux pump inhibiting agents also interact with quorum sensing system. Many studies demonstrated that NorA is core part of *S. aureus* genome and its expression were induced in many *S. aureus* strains (Costa et al., 2019). The role of the NorA efflux pump in biofilm development was investigated using the SA 199B strain, which exhibits elevated expression of NorA and it was found that using NorA (EPI) inhibitors, biofilm formation was also inhibited (Sabatini et al., 2017). Moreover, Kalia et al., 2012 explained that over-expression of NorA increase the intracellular invasion of *S. aureus* in macrophages. These evidences conclude that efflux pumps and quorum sensing system simultaneously derive each other and hindrance with one will upregulate or downregulate another one. Earlier investigations had revealed that that multidrug efflux pumps, biofilm formation and quorum sensing are significantly related to each other (Varga et al., 2011). Mechanisms that play an important contribution to the development of antimicrobial resistance in biofilm-associated microorganisms involve the upregulation or hyperexpression of multiple genes responsible for encoding efflux pump proteins. Studies showed that plasmid exchanges at a very higher rate in biofilms compare to planktonic microbes,

increasing the chances of developing resistance naturally as well as antibiotic-induced (Percival et al., 2015). It is also being demonstrated that inactivation of bacterial efflux pumps led to deficiencies in secretion of quorum sensing signals and biofilm production. The signals of quorum sensing produced by bacterium to inhibit competing bacterial growth can also be considered as foreign molecules and expelled out promoting biofilm formation. Efflux pump functions are required for responding to quorum sensing signals as the secretion of these signals are dependent on bacterial main efflux pump system and any noxious agent produced internally are expelled out through them (Pumbwe et al., 2008). Furthermore, in mixed cultures efflux pumps acts as a defense system when quorum sensing inhibitors are utilized and do not allow the transfer of inapt message from other species. Therefore, efflux pumps and quorum sensing systems have a reciprocal impact on each other's functions, extending beyond their individual reactions to the external environment.

2.8.2 Quorum sensing inhibitors

Substances that disrupt quorum sensing are often called quorum quenchers. Quorum sensing inhibition by enzymatic degradation of AIPs is emerging as a vital approach to address antibiotic resistance. AIPs can be different molecules like oligosaccharides ranging from low to high molecular weight, of which most common are N-acyl homoserine lactones (AHLs). Several organisms produce enzymes like laconases which can degrade AHL and inhibit numerous phases of quorum sensing controlled pathogenicity. A lactonase isolated from *B. thuringiensis* diminishes *Erwinia carotova* pathogenesis by quorum sensing inhibition (Liu et al., 2008). Porcine acylase-I had been demonstrated to degrade AHLs of *Microbacterium* and *Pseudomonas spp.* and also reduces their biofilm formation (Xu et al., 2003). However, the therapeutic application of these enzymes is limited by the fact that they can also hydrolyze some non-AHL molecules and can be toxic to the host, therefore careful examinations are required. The *S. aureus* virulence can also be constrained by inhibiting quorum sensing (Table 2.3). Various toxins like delta-toxin, *Staphylococcal* enterotoxin C and Panton-Valentine leucocidin whose production is

itself modulated by quorum sensing are potent enough to kill this pathogen however less selective pressure must be experienced by the bacteria to drive through this route is facilitating to the emergence of resistance to traditional antimicrobial agents (Silva et al., 2016). Wealth of research data had been devoted to the discovery of quorum quenchers as an alternative strategy for anti- efficacious compound. Of these some showed much effectiveness with 98% inhibition of hemolysis in rabbit erythrocyte by MRSA at 1 μg/ml concentration or against MRSA infection model of Galleria mellonella larva (Kuo et al., 2015). Savirin another synthetic molecule was also found to inhibit the activation of *agr*-P3 in *S. aureus* with reduced production of RNAIII (Sully et al., 2014). It also inhibited the transcription of agr-specific major virulence factors for skin and soft tissue infections (SSTIs) (Cisar *et al.,* 2009). Research revealed that oxacillin (synthetic antibiotic) employed at sub-MIC, was effectively down regulate the agr system and attenuate MRSA virulence but increases MRSA methicillin resistance (Waters et al., 2017). Two antisense oligonucleotides have been synthesized targeting most conserved agrA. These oligonucleotides were modified ribonucleotides (RNA) where an extra bridge was present between 4′ carbon and 2′ oxygen of ribose sugar and were thus called as locked nucleic acid (LNA). They were conjugated with (KFF) 3K, a cell-penetrating peptide and were found to reduce transcription of agrA and RNAIII (Da et al., 2017). RNAIII-inhibiting peptide (RIP) is a naturally occurring peptide produced by *S. aureus* that can block quorum sensing. RIP derivatives like a tetrapeptide FS10 have been screened to break quorum sensing in *S. aureus* but were however found to be less effective (Simonetti et al., 2016). Natural products isolated from both plants and fungi have been screened for their inhibitory effect on quorum sensing in *S. aureus* (Quave et al., 2011);Wang et al., 2019) (Fig. 5). Xenobiotics derived from fungus were found to potentiate the activities of antibiotics. Polyhydroxy anthraquinones isolated from fungus like Penicillium restrictum were found to be very effective (Kumar et al., 2018). ω-hydroxyemodin (OHM) has been shown to potentially inhibit agr signalling in MRSA. Molecular docking studies demonstrated that OHM inhibits binding of agr AC to agr promoter and reduced expression of all four types of agr (Daly et al.,

2015). In-vivo studies revealed that OHM treatment in mouse, whose skin was infected with MRSA, reduced the bacterial burden in abscess, reduced the size of ulcer and also increased the in-vitro clearance of bacteria by both human polymorphonuclear leukocytes (PMNs) and mouse macrophages. Treatment of OHM in agr null *S. aureus*, showed no effect highlighting that agr system is the target of OHM (Malachowa et al., 2013). Another metabolite of fungal origin: ambuic acid was found to inhibit AIP synthesis by targeting agrB. Furthermore, it inhibited production of RNAIII and alpha-toxin by MRSA in dose-dependent manner. A single intradermal 25-μg injection of ambuic acid completely abolished the formation of skin ulcers in mouse model with MRSA infected skin (Todd et al., 2017). Leaf extract of *Castanea sativa* and berry extract of *Schinus terebinthifolia*, both were found to inhibit *agr* expression (IC_{50} ranges 1.52-32 μg/ml) and production of delta-toxin by *S. aureus*. They also reduced morbidity and abolished skin ulcer formation on MRSA infected mouse models (Muhs et al., 2017).

2.8.3 Efflux pump inhibitors and quorum sensing inhibition

As multidrug resistant biofilm associated MRSA bacteria, it is crucial to assess recent advancements in the development of innovative strategies to overcome these resistant (Otto, 2008). Drug extrusion, impermeability of drug inside the biofilm matrix, quorum sensing and virulence factors such as bacterial adhesion factors & toxin are prime resistance contributors in biofilms. Efflux pumps play a dual role in both antibiotic efflux and cell signaling, contributing to quorum sensing processes. They have been found to be AIP secretor and therefore appear to be a crucial target for antibiotic development (Moore et al., 2014). Molecules that target this track way could be applicable as new therapeutic agent.

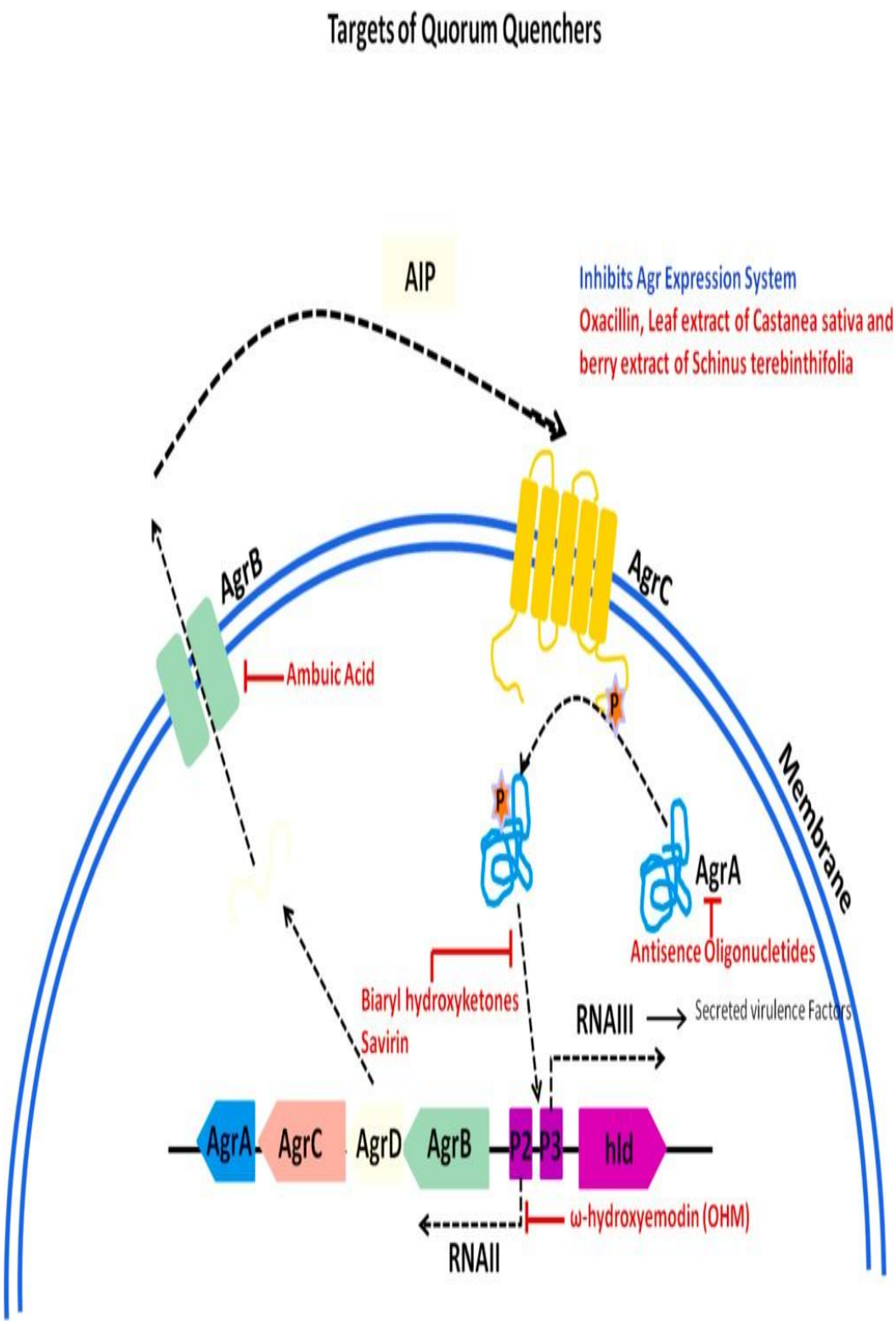


Figure 2.6: Targets of Quorum quenching during bacterial biofilm formation

Table 2.3: Molecules found to interfere with *Staphylococcus aureus* quorum sensing.

Sr. No	Molecule	In Vitro Effects	Mechanism of Action
1	(5Z)-4-bromo-5-(bromo-nethylene)-3-butyl-2(5H)-furanone	Inhibits RNAIII expression, enhances biofilm formation	Leads to increased PIA production enhancing biofilm formation
2	(S)-3-Decanoyl-5-(2-hydroxyethyl)tetramic acid (5-HE-C10-TMA)	Inhibits P3 Activity	Prevents AgrC receptor dimerization or interference with the interactions AgrC-AgrA (Not confirmed)
3	2-(4-Methyl-phenyl)-1,3-thiazole-4-carboxylic acid	Unknown	Interferes with AgrA DNA binding activity
4	3-Dodecanoyltetronic acid (C12-TOA,16)	Inhibits P3 activity	Prevents AgrC receptor dimerization or interference with interactions between AgrC-AgrA (Not confirmed)
5	3-Tetradecanoyltetronic acid (C14-TOA, 17)	Inhibits P3 activity	Prevents AgrC receptor dimerization or interference with the interactions between AgrC-AgrA (Not confirmed)
6	9H-Xanthene-9-carboxylic acid	Unknown	Interferes with AgrA DNA binding activity
7	Alpha-cyperone	Inhibits hla and agrA expression	Unknown
8	Ambuic acid	Inhibits hemolytic activity, RNAIII transcription, AIP-I production	Prevents AIP biosynthesis (Not confirmed)

Sr. No	Molecule	In Vitro Effects	Mechanism of Action
9	Avellanin	Unknown	From sponges; functions via competitive inhibition of AgrC
10	Chromatography eluates	Inhibits P3 activity and hemolytic activity	Unknown
11	Cochinmicin	Unknown	From actinomycetes, functions via competitive inhibition of AgrC
12	Cyclic dipeptides: cyclo (L-Phe-L-Pro) and cyclo (L-Tyr-L-Pro)	Inhibits TSST-1, downregulates RNAII and RNAIII, SarA and SaeRS	Competitive inhibitors of AIP-mediated P3 promoter activation, affecting sarA and saeRS
13	Furanone 202	Inhibits biofilm formation by *S. epidermidis*	Unknown
14	Licochalcone A	Inhibits sea, seb, and agrA expression	Unknown
15	Luteolin	Inhibits hla and agrA expression	Unknown
16	N-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL)	Inhibits production of exotoxins and fibronectin-binding proteins but enhances protein A expression	May affect SarA functionality; potentially antagonizes membrane-associated regulators (e.g., sensor components of ArlRS, SaeRS and SsAB)
17	Naphtalene and biaryl compounds	Inhibits hla, psmo, RNAIII expression	Targets AgrA (N) phosphoryl binding pocket; may interfere with AgrA DNA binding activity
18	Naringenin	Unknown	Reduces agrA and hla transcript levels

Sr. No	Molecule	In Vitro Effects	Mechanism of Action
19	Oxacillin	Pleiotropic effect on toxin expression	Unknown
20	PLNA34	Unknown	Specifically and significantly reduces agrA mRNA levels
21	Polyhydroxyanthraquinones	Inhibits hld expression and P3 activity	Unknown
22	RIP	Unknown	Inhibits synthesis of agr transcripts RNAII and RNAIII
23	RIP derivatives (16P-AC)	Unknown	Inhibits the expression of biofilm-related genes in *S. aureus*
24	RIP-V, RIP-L	Unknown	Down-regulates RNAIII expression and hemolysin production
25	Savarin	Inhibits RNAIII expression (only in *S. aureus*, not *S. epidermidis*), psma promoter	Interferes with AgrA DNA binding activity
26	Solonamide A/B, Ngercheumicins	Reduces hla, rnalll and increases spa transcripts, reduces *S. aureus* mediated killing of human neutrophils	Competitive inhibitor of AgrC
27	Staphylococcus schleiferi	Unknown	Functions by cross-inhibition of the pathogenic agr system
28	Thymol	Inhibits sea, seb, hla and agrA expression	Unknown

Sr. No	Molecule	In Vitro Effects	Mechanism of Action
29	Truncated AIP-I, II, III	Unknown	Inhibits autoinduction of all four *S. aureus* subgroups
30	Vaccination with hapten-linked AIP IV	Unknown	Provides passive immunity and reduces the pathology of agr IV strains
31	w-Hydroxyemodin (OHM)	Unknown	Prevents agr activity by all four *S. aureus* agr group strains

The existence of anti-resistance strategies, such as an efflux pump inhibitor (Holler et al., 2012) and quorum sensing inhibitor (O'Loughlin et al., 2013), with their impact on biofilm inhibition, is now being considered a crucial factor in identifying novel antimicrobial therapies to combat biofilm-derived infections. For this reason, correlation of efflux pump inhibitors and quorum sensing system can possibly be a significant approach to abolish biofilms (Fleeman et al., 2018). These groups of molecules represent the most significant antibacterial arsenals in order to restore the drug efficacy (efflux pump inhibition) and biofilm destruction (Table 2.4). A cooperative endeavour is underway to find out a productive molecule that may revert the mechanism of biofilm formation. Previously interaction of quorum sensing signals and efflux pump inhibitors in biofilm associated infections has been reported (Szabó et al., 2010). Some compounds which are having inhibitory activity against efflux pump has also been found interactive to quorum sensing signalling pathway which results in suppression of virulence gene expression (Szabó et al., 2010);Szemerédi et al., 2020). Most recently, seleno compounds and benzene derivative selenoester moieties showed strong inhibition of bacterial Acr-AB and TolC system in *E. coli* AG 100 strain (Mosolygó et al., 2019). These results can be exploited for rational drug development to inhibit biofilm formation in diseases resulting from MRSA, which can help combat the rise of antibiotic-resistant strains.

Though therapeutic potential of such vital and efficient compounds is very much attractive and should inspire a huge research but unfortunately to date, no EPI has reached the clinical settings for anti-infective purpose antagonistic toward bacterial infections in human beings or veterinary settings. Likewise, agr dysfunction with the aid of EPI is still under experimentation. Moreover, we are lacking with the evidences that how virulence factors are regulated during agr dysfunction.

Table 2.4: List and structure of molecules having efflux pump and biofilm inhibition activity.

Name	Reference
Chlorpromazine	Alav et al, 2018,Chan et al,2007, Baugh et al, 2014
1-(1-Napthylmethyl) piperazine (NMP)	Alav et al., 2018, Kvist et al., 2008
CCCP	Mahamoud et al., 2007
Phenylalaninearginine β-naphthylamide (PABN)	Baugh et al., 2014 , Liu et al., 2010
4′,5′-O-dicaffeoylquinic acid	Fiamegos et al., 2011
Thioridazine	Kvist et al, 2008., Nzakizwanayo et al., 2007
Reserpine	Alav et al., 2018
Piperine	Mirza et al., 2011

2.9 Ciprofloxacin

Ciprofloxacin, a synthetic antibiotic belonging to the fluoroquinolone class, was first introduced in the 1980s. It inhibits essential bacterial enzymes involved in mechanisms of DNA replication, repair, and recombination. Apart from its broad antibacterial spectrum, the antibiotic's ability to penetrate tissues and achieve high

concentrations in various body fluids makes it effective against both systemic and localized infections (Bush et al., 2020).

2.9.1 Resistance to Ciprofloxacin

The extensive use of ciprofloxacin, although effective, has driven the development of resistant bacterial strains, compromising its long-term utility. Ciprofloxacin resistance in bacteria arises through multiple mechanisms that work synergistically to reduce drug efficacy. The primary mechanisms include target-site modifications, enhanced efflux, decreased permeability, and plasmid-mediated resistance (Shariati et al., 2022).

2.9.1.1 Target-site modifications

The most common mechanism involves mutations in genes encoding DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE), which are primary targets of ciprofloxacin. These mutations typically occur in quinolone resistance-determining regions (QRDRs) of these genes. The most frequent mutations were observed in gyrA: codons 83 and 87 (E. coli numbering), and parC: codons 80 and 84. In *Pseudomonas aeruginosa*, the D87G mutation in gyrA significantly contributes to ciprofloxacin resistance. Accumulation of multiple mutations in these target genes can lead to high resistance (Weigel et al., 2022).

2.9.1.2 Enhanced Efflux

The emergence of ciprofloxacin resistance is closely tied to the enhanced activity of efflux pumps, which enable bacteria to efficiently expel the antibiotic, mostly from the resistance-nodulation-division (RND) family. Mutations in regulatory genes can lead to the constitutive overexpression of these pumps. For instance, an F7S mutation in MexS has shown to raise ciprofloxacin's resistance through the up-regulation of the MexEF-OprN efflux system. Efflux pumps' contribution to resistance can be quantified using efflux pump inhibitors such as carbonyl cyanide m-chlorophenylhydrazone (CCCP). Studies have shown that efflux activity contributes to resistance in approximately 18.3% of ciprofloxacin resistant isolates (Shariati et al., 2022).

2.9.1.3 Decreased Permeability

Alterations in the outer membrane proteins, particularly porins, can reduce ciprofloxacin uptake. For example, downregulation of the OmpF porin expression in *E. coli*, often associated with mutations in regulatory genes, such as marA, can contribute to ciprofloxacin resistance (Shariati et al., 2022).

2.9.1.4 Plasmid-Mediated Quinolone Resistance (PMQR)

PMQR genes, which generally confer low-level resistance, can facilitate the development of high-level resistance. Common PMQR determinants include qnr genes (e.g., qnrA, qnrB, and qnrS), that translate into proteins that shield DNA gyrase and topoisomerase IV from ciprofloxacin inhibition. The "aac(6')-Ib-cr" variant aminoglycoside acetyltransferase can acetylate ciprofloxacin, reducing its activity. Gene qepA and oqxAB encode efflux pumps that can extrude ciprofloxacin. A study on gram-negative bacteria from cancer affected people showed that 56.8% harbored PMQR genes, with aac(6')-Ib-cr being the most prevalent (42.6%), followed by qnrS (26.6%), and qnrB (6.5%). The dynamic interaction between these mechanisms often culminates in a progressive escalation of resistance. For example, the presence of PMQR genes can facilitate the selection of target-site mutations by allowing bacteria to survive at lower ciprofloxacin concentrations. The minimum inhibitory concentration (MIC) for highly resistant strains can exceed 32 μg/mL compared with susceptible strains with MICs ≤ 1 μg/mL. Understanding these resistance mechanisms is essential for developing countermeasures to combat ciprofloxacin resistance, such as the use of efflux pump inhibitors, development of new quinolone derivatives that can overcome target site mutations, or combination therapies that target multiple resistance mechanisms simultaneously (Shariati et al., 2022).

The interplay between these mechanisms often results in a stepwise increase in the resistance. For example, the presence of PMQR genes can facilitate the selection of target-site mutations by allowing bacteria to survive at lower ciprofloxacin concentrations. The minimum inhibitory concentration (MIC) for highly resistant strains can exceed 32 μg/mL, compared to susceptible strains with MICs ≤ 1 μg/mL (Hamed et al., 2018)

2.9.2 Combination of Ciprofloxacin with Natural Synthetic Molecules

Combining ciprofloxacin with natural molecules has shown promise as a novel approach to boost antibacterial efficacy and potentially combat antibiotic resistance. Recent studies have explored various natural compounds, particularly those derived from plants that exhibit synergistic effects when used in combination with ciprofloxacin (Seo et al., 2024). Phenazine is a notable class of natural compounds showing synergistic potential with ciprofloxacin. A study published in Nature reported that three phenazines isolated from *Streptomyces luteireticuli* NIIST-D75 - 1-methoxyphenazine, methyl-6-methoxyphenazine-1-carboxylate, and 1, 6-dimethoxyphenazine demonstrated significant synergistic effects when combined with ciprofloxacin against various bacterial strains, including *Staphylococcus aureus*. The combinations resulted in substantially lower minimum inhibitory concentrations (MICs) ranging from 0.02 to 1.37 μg/ml, indicating enhanced antibacterial activity (Jesmina *et al.*, 2023). Flavonoids, a class of plant-derived polyphenolic compounds, have shown promise in combination with ciprofloxacin. A study investigated co-crystals of ciprofloxacin with quercetin (CIP·QUE) and demonstrated improved antimicrobial activity against *Helicobacter pylori* compared to ciprofloxacin alone. The co-crystal formulation reduced the required dose of ciprofloxacin while maintaining an equivalent antimicrobial efficacy (Fiore et al., 2024).

Other natural products that show synergistic effects with ciprofloxacin include *Thymbra spicata L*. extracts demonstrated additive activity *against Klebsiella pneumoniae* strains when combined with ciprofloxacin (Haroun & Kayali, 2016) n-Butanolic When coupled with ciprofloxacin, cyclamen coum extract shown a synergistic impact against Pseudomonas aeruginosa biofilms (Shafiei et al., 2014). When combined with ciprofloxacin, extracts from *Plumbago zeylanica* and *Holarrhena antidysenterica* demonstrated synergism against both methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus* (MRSA).

(Aqil & Ahmad 2007). The mechanisms underlying these synergistic effects have not been fully elucidated; however, they may involve the enhanced penetration of ciprofloxacin into bacterial cells, Inhibition of efflux pumps that contribute to

ciprofloxacin resistance, Disruption of bacterial cell membranes, as observed in scanning electron microscopy (SEM) studies, Modulation of bacterial stress responses

2.10 Chalcones

Chalcones are a type of chemical compounds that are members of the flavonoid family with unique structure consisting of two aromatic rings and an unsaturated carbonyl system described below in detail.

Aromatic Rings: Chalcones typically contain two aromatic rings, often referred to as **Ring A and Ring B**.

Ring A: This ring is usually a benzene ring, which is a six-membered carbon ring with alternating double and single bonds. It's represented as a hexagon with alternating double bonds between the carbon atoms. Ring B: Similar to Ring A, Ring B is also a benzene ring, connected to Ring A.

α,β-Unsaturated Carbonyl System: This is the defining feature of chalcone molecules. It consists of a three-carbon chain (sometimes referred to as the "propenone chain") that connects the two aromatic rings. The carbonyl group (C=O) is attached to the β-carbon, and the α-carbon is typically a carbon that's part of the aromatic ring.

α-Carbon: This carbon atom is directly attached to the Ring A, forming a double bond with one of its carbon atoms.

β-Carbon: This carbon atom is directly attached to the carbonyl group (C=O), forming a double bond with oxygen.

1. Carbonyl Group (C=O): Two carbon atoms are doubly linked to one oxygen atom to form this functional group. It is β to the α-carbon since it is at the end of the three-carbon chain. (Zhuang and others, 2017). Natural chalcones are found in the heartwood, leaves, bark, fruits, and roots of many different plants and botanicals. They are mainly visible in petalcolors (Chtourou &Trabelsi, 2021).

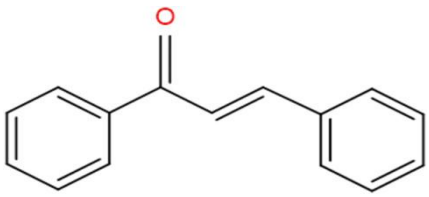


Figure 2.7: Chemical Structure of Chalcone

2.10.1 Pharmacological Applications of Chalcones

Even in the twenty-first century, Chalcones and their derivatives are extremely important in medicinal chemistry due to their broad spectrum of pharmacological properties and therapeutic applications. (Rammohan et al., 2020).

2.10.1.1 Antioxidant Activity:

Chalcones possess antioxidant activities due to the presence of phenolic groups in their molecular structure. They have the capacity to Absorb free radicals, which in turn helps to protect cellular and tissue structures from oxidative stress. This antioxidative activity positions chalcones as promising candidates for combating diseases associated with oxidative stress, such as cancer, cardiovascular diseases, and neurodegenerative disorders (González et al., 2022).

A penta-oxygenated chalcone with strong DPPH radical activity was discovered from *Glycyrrhiza uralensis* (Leguminosae), a plant that is historically utilized in northeastern China, Strong superoxide scavenging activities were exhibited by cedredipronone, another chalcone that was isolated from fruit and seed extracts of *Cedrelopsis grevei* (Ptaeroxylaceae) (Koorbanally et al., 2003). Furthermore, a prenylated chalcone glycoside that was separated from *Maclura tinctoria* (Moraceae) bark exhibited robust antioxidant activity, neutralizing free radicals through multiple mechanisms.

2.10.1.2 Anti-inflammatory Activity

Chalcones exhibit anti-inflammatory properties by suppressing the production of pro-inflammatory mediators such as cytokines, prostaglandins, and leukotrienes. They modulate inflammatory signaling pathways, alleviating inflammation in various disease conditions including arthritis, asthma, and inflammatory bowel diseases (Ysrafil et al., 2023). Interestingly, a naturally occurring substance called naringenin-chalcone exhibits anti-inflammatory properties by preventing the synthesis of cytokines. Significant anti-inflammatory effect is shown by isoliquiritigenin extracted from *Nepalese propolis* and butein from *Rhus verniciflua* which decrease the expression of COX-2 and iNOS. In murine microphage-like cell lines (Salehi et al., 2021). Zhao et al, 2003) showed chalcone as an anti-inflammatory drug by blocking

NO generation generated by LPS and INF-alpha. It has been discovered that synthetic hetero chalcones are effective cytokine inhibitors for treating inflammatory conditions.

2.10.1.3 Anticancer Activity

Chalcones demonstrate promising anticancer activity through various mechanisms including cell proliferation inhibition, apoptosis induction, and tumor angiogenesis suppression. Some chalcones exhibit selective cytotoxicity against cancer cells while sparing normal cells, making them attractive for cancer therapy. Millepachine from *Millettia pachycarpa*, which exhibits anticancer activity. Licochalcone A, isolated from *Glycyrrhiza inflate*, shows toxicity towards leukemia and melanoma cells. Butein, another natural chalcone, suppresses various human cancers including breast cancer, colon carcinoma, and osteosarcoma. Additionally, novel chalcones have been identified as anti-mitotic agents (Ouyang et al., 2021).

2.10.1.4 Antimicrobial Activity

Chalcones possess wide antimicrobial activity against bacteria, fungi, and parasites. Their antimicrobial activity is attributed to the α, β-unsaturated carbonyl function. They disrupt microbial cell membranes, compromising their integrity, disrupting critical metabolic pathways, interfering with microbial DNA replication and protein synthesis, ultimately stifling microbial growth and proliferation. Isobavachalcone and bavachalcone from *Psoralea corylifolia* exhibit antibacterial activity. Synthetic scaffold 3-(Carboxyalkyl) rhodanine displays potent antimicrobial activity against human pathogens. Ring-fused chalcones have been proposed as antimicrobial agents for treating oral infections. A hybrid chalcone comprising fluconazole pharmacophore shows potent inhibition against *Candida albicans* (Rajendran et al., 2022).

2.10.1.5 Antidiabetic Activity

Chalcones show potential in treating diabetes by affecting various metabolic pathways that regulate glucose balance and insulin sensitivity. They act by inhibiting essential enzymes involved in carbohydrate metabolism, boosting insulin secretion, and enhancing glucose absorption in peripheral tissues. Chalcones are known to effectively inhibit α-glucosidase, dipeptidyl peptidase-4 (DPP4), peroxisome

proliferator-activated receptors (PPAR), and protein tyrosine phosphatase 1B (PTP1B), making them important agents in diabetes treatment. Compounds such as isoliquiritigenin, echinatin, “licochalcone A, lichochalcone C, and lichochalcone E, derived from *Glycyrrhiza inflata*, demonstrate antidiabetic effects by inhibiting PTP1B. Additionally, novel sulfonamide chalcones have been identified as potent α-glucosidase inhibitors. (Adelusi et al., 2021).

2.10.1.6 Neuroprotective Activity

Chalcones are known to have neuroprotective effects because they decrease oxidative stress, lessen neuroinflammation, and encourage the survival and regeneration of neurons. According to Barber et al., 2023), they have the potential to be used as therapeutic agents for neurodegenerative diseases including Parkinson's and Alzheimer's disease because they affect signaling pathways involved in neuronal growth and differentiation. As a strong transglutaminase inhibitor, thienylchalcone may be used to cure or prevent Alzheimer's disease. The catechol-O-methyltransferase enzyme inhibitor properties of nitro-substituted chalcones are helpful in the treatment of neurodegenerative conditions such as Parkinson's syndrome. Synthetic chalcones show inhibition against both cathepsin B and μ-calpain, making them useful in the treatment of Alzheimer's disease. Furthermore, a hybrid of coumarin and chalcone functions as an AChE inhibitor, which may be helpful in the treatment of neurological illnesses (Królicka et al., 2022).

2.11 Chalcone as efflux pump inhibitor

The compound chalcone and its derivatives exhibit a broad range of receptor-targeting capabilities, including but not limited to: (i) inhibition of the efflux pump (EPI); (ii) disruption of DNA replication; (iv) inhibition of filamentous temperature-sensitive mutant Z (FtsZ); (v) targeting virulence factors; and (vi) inhibition of protein tyrosine phosphatases (PTPs) (Le et al., 2022). Notwithstanding the complexity and numerous causes of antibiotic resistance, bacterial efflux pumps are essential for the quick excretion of antibacterial medications, which greatly contributes to the sustainability of pharmaceuticals.

Chalcones have demonstrated antibacterial effects in the past by blocking the efflux

pump. (Holler et al., 2012;Gaur et al., 2015). The efficiency of 117 chalcones in inhibiting the NorA was assessed by Holler et al., 2012.This experiment showed that in everted membrane vesicles, twenty chalcones inhibit the NorA efflux pump. Furthermore, two chalcones were shown to have potential efficacy similar to reserpine, a well-established efflux pump inhibitor. These findings suggest that chalcones, which are based on the microbial efflux transporters, could be developed into drugs to fight.

Plant-derived EPIs that target the upregulation of NorA efflux in *S. aureus* include chalcones, piperine-like compounds, citral amide derivatives, N-cinnamoylphenalkyl amides, dihydronaphthyl, indole, 2-chloro-5-bromophenyl, and piperidine (Handzlik *et al.*, 2013). Thai *et al.* investigated the novel NorA efflux pump inhibitors for *Staphylococcus aureus* by using molecular docking and virtual screening on a collection of 47 natural chemicals, including chalcones. Of them, seven were NorA inhibitors. Compounds that inhibit efflux pumps are linked to antibiotics in an attempt to restrict bacterial resistance via this mechanism. The chalcones ACLOPHENY and APCHAL were studied in this work, as well as the potentiating effects of antibiotics and antimicrobials on them. Furthermore, the medicines efflux pump inhibition was evaluated. The two strains of *S. aureus* that were employed were strain K2068, an MDR mutant strain that possesses the MepA efflux pump, and strain 1199B, which overexpresses the NorA gene (Ferraz et al., 2020).

2.12 Chalcone as biofilm inhibitor

In their 2019 study, Ušjak et al., identified and demonstrated the synergistic action of certain synthetic chalcones when used with commercial antimicrobials to inhibit biofilm formation and virulence factors in multidrug-resistant bacteria. It has been documented that cationic chalcones have medicinal promise; they exhibit strong antibacterial activity and little cytotoxicity. When Akemi et al., 2022 investigated the antibiofilm activity of hydroxylated and unhydroxylated chalcones, they discovered that the hydroxyl group's oxygen content is necessary for the compound's bioactivity. Production of glycocalyx was greatly reduced when MRSA strains were incubated with subinhibitory doses of chalcones. Compounds with minimum inhibitory

concentration values between 0.5% to 97% caused harm to the biofilm structures created by bacteria (Tutar et al., 2019). By inhibiting agrA in a concentration-dependent manner, licochalcones A and E decreased.

Bozic et al., 2014 demonstrated inhibition of adherence and biofilm formation of MRSA using novel chalcones. Their research highlights the critical impact of biofilm development as an element of virulence in MRSA infections and the increasing resistance of MRSA strains to antibiotics. The study evaluated the effects of three chalcones on glycocalyx production, biofilm formation, and adherence to human fibronectin of MRSA strains. The compounds were found to diminish glycocalyx and biofilm formation, resulting in reduced bacterial adherence to fibronectin. The most effective chalcone was 1,3-Bis-(2-hydroxy-phenyl)-propenone. This study suggested that chalcones could be potential antimicrobial agents for preventing staphylococcal infections or as adjuncts to conventional antibiotics.

Messery et al., 2018 researched the synthesis and characterization of amide chalcones conjugated with secondary amines. The study involved comprehensive spectroscopic techniques such as 1H NMR, 13C NMR, and ESI-MS for thorough characterization of the synthesized compounds. These compounds were then subjected to in vitro testing for antibacterial activity, revealing that compounds 36, 37, 38, 42, and 44 displayed notable efficacies against a range of bacterial strains. Notably, compound 36 exhibited comparable effectiveness to the standard drug Ampicillin specifically against *Staphylococcus aureus*. Furthermore, the study investigated the anti-biofilm properties of the synthesized compounds, with compounds 36, 37, and 38 showing promising results in inhibiting biofilm formation. Molecular modeling was employed to gain insights into the potential mechanisms underlying the anti-biofilm activity, emphasizing the significance of specific amino acid residues and the role of hydrophobicity in the compounds' activity. Overall, the study highlights the potential of these novel chalcone linked amine derivatives as effective antimicrobial agents with anti-biofilm properties, offering valuable contributions to the field of combating bacterial infections and biofilm-related challenges.

The research study titled "A chalcone with potent inhibiting activity against biofilm formation by nontypeable *Haemophilus influenzae*" conducted by Kunthalert and colleagues in 2014 investigated the efficacy of chalcone compounds in inhibiting biofilm formation by non-typeable *Haemophilus influenzae* (NTHi), a significant respiratory pathogen known for biofilm-related infections. The study evaluated a series of natural and synthetic chalcones for their biofilm inhibition potential against NTHi strains that form robust biofilms. Notably, 3-hydroxychalcone (chalcone 8) displayed superior biofilm inhibitory effects compared to natural chalcones and outperforming the reference drug azithromycin. Importantly, chalcone 8's inhibitory action on biofilm formation is non-antimicrobial, suggesting a unique mechanism of action. The investigation into the structure-activity relationship of chalcones provided important insights into the development of successful tactics to address the biofilm-related problems posed by NTHi infections. Furthermore, the findings suggest that chalcone 8 may be a promising novel therapeutic agent for controlling NTHi biofilm-associated infections.

CHAPTER 3: HYPOTHESIS AND OBJECTIVES

3. Hypothesis

Staphylococcus aureus is a notorious Gram-positive pathogen causing various infections. It has developed resistance to multiple antibiotics through mechanisms like target site mutation, enzyme synthesis, and efflux pumps. Moreover, *S. aureus* forms biofilms, encased in an extracellular polysaccharide matrix, rendering it up to 1000 times more resistant to antibiotics.

Researchers are exploring new approaches to combat antibiotic resistance. One strategy involves combining existing drugs with natural or synthetic molecules that inhibit resistance mechanisms, such as efflux pumps. This enhances drug efficacy and reduces resistance development. However, bacteria can form biofilms, limiting drug penetration and effectiveness.

The current study was conducted with a hypothesis to screen natural/synthetic molecules which show dual activity and inhibit two mechanisms, i.e., efflux pump and biofilm formation, at the same time. Chalcones show high potential as a dual inhibitor of efflux pump and biofilm formation. When these molecules combine with ciprofloxacin, it increases the efficacy of antibiotics by many folds. We tested series of chalcones in combination with ciprofloxacin and evaluated the combinations for other pre-clinical studies. Moreover, to establish the hypothesis, a NorA overexpressing strain was used in which chalcones were able to modulate the effect of ciprofloxacin by inhibiting NorA efflux pump and Agr gene expression (gene involved in biofilm formation). We further used *in silico* model also to validate our results.

Research Objectives

1. *In-vitro* evaluation of Chalcone in combination with Ciprofloxacin against planktonic cell of *S. aureus*.
2. *In-vitro* evaluation of Chalcone in combination with Ciprofloxacin against biofilm associated *S. aureus*.
3. Evaluate the effect of Chalcone on *Agr* expression and *Nor*A expression in sessile and biofilm forming *S. aureus* using qRT –PCR.

CHAPTER 4: MATERIALS AND METHODS

4: Materials and Methods

4.1 Bacterial cultures and chemicals used:

Staphylococcus aureus ATCC 29213 was obtained from the American Type Culture Collection located in Manassas, Virginia. Dr. Nitin Pal Kalia from NIPER Hyderabad, India kindly contributed two more strains of *S. aureus*: SA1199B, which overexpresses NorA; *S. aureus* 1199, which is its wild type. The Hi Media labs in India provided all of the chemicals, including antibiotics, needed in the research project. Dr. Gopal Kathik from Department of Medicinal Chemistry, National institute of Pharmaceutical Education and Research, NIPER, Raebareli, Uttar Pradesh synthesize and provide us the purely characterized chalcone derivatives. The primers and RT-PCR kit was procure from Invitrogen.

4.2 Growth conditions and media:

Mueller-Hinton Broth (MHB) from HiMedia Labs, India, was used for all screening assays, time-kill kinetics studies, and Minimum Inhibitory Concentration (MIC) determinations. For mutation studies, Mueller-Hinton Agar (MHA) from HiMedia Labs, India, was utilized. Trypticase Soya Agar (TSA) from same source was used as preferred medium for bacterial culture maintenance and colony-forming unit (CFU) counts. Time-kill investigations were conducted at 37°C with shaking at 175 rpm, while microdilution and agar diffusion assays were performed at 37°C without shaking.

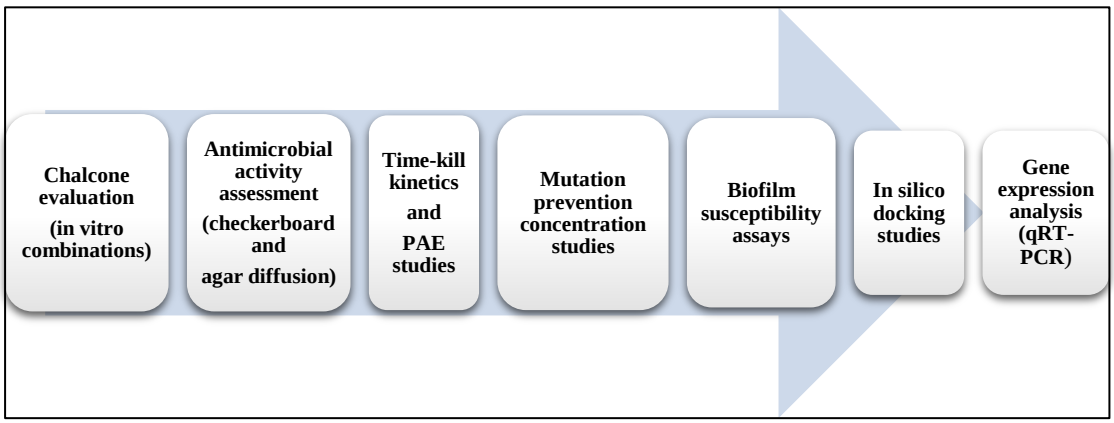
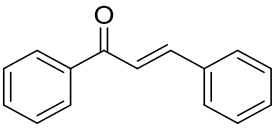
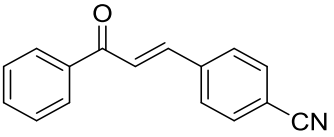
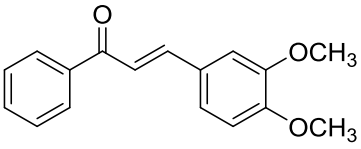
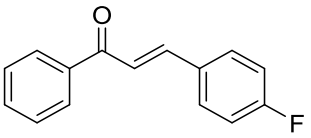
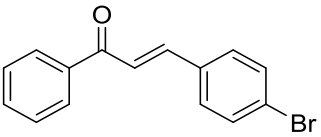
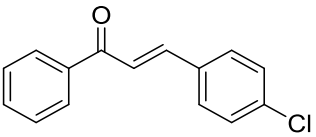
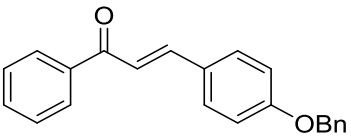
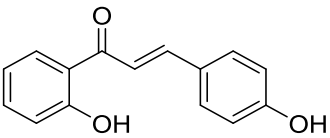
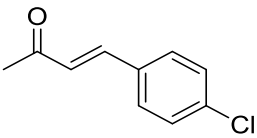
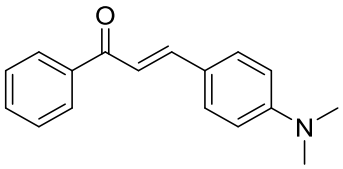
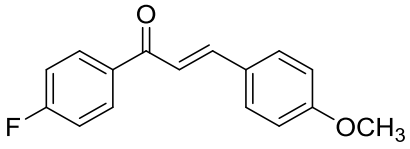
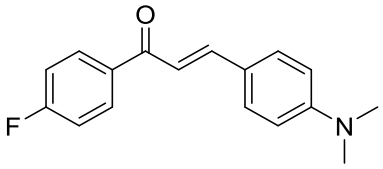
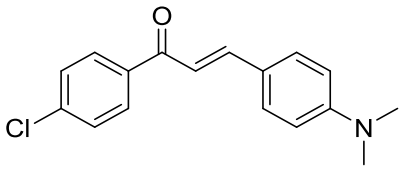


Figure 4.1: Workflow Diagram

4.3 *In-vitro* combination studies of Chalcones

Table 4.1: Structures of chalcones employed in the research study along with their molecular weights

Code	Structure	Mol. Wt.
C1		208.26
C3		233.27
C4		268.31
C5		226.25
C6		287.15
C7		242.70
C8		314.38
C9		240.25
C10		180.63

C11		251.32
C13		256.27
C15		269.31
C16		285.77

4.3.1 Evaluation of chalcones by checkerboard method: The broth checkerboard method was used to conduct combination trials (Eliopoulos, 1996). In U bottom 96-well microtiter plates (Tarson, India), a combination of two fold serial dilutions of ciprofloxacin in MHB and two fold dilutions of chalcones was evaluated. Ciprofloxacin and chalcone ranged at final concentrations from 0.03 mg/L to 64 mg/L and 0.8 mg/L to 50 mg/L, respectively. *S. aureus* 1199B and *S. aureus* 1199 inoculums were made by reducing the density of the cultures that were cultivated overnight to 0.5 McFarland in sterile normal saline. 100 µl of the inoculum was dispensed in each well after it had been diluted 1:100 in MHB. Each well received a final bacterial inoculum of 5×10^5 CFU/mL. The incubation of the plates was carried out at 37°C for a period of 24 hours. The minimal Effective Concentration (MEC) of the chalcone thus examined was determined by visually reading the plates and determining the minimal concentration of the chalcone molecule that, when combined with an antibiotic, decreased the MIC concentration.

4.3.2 Agar diffusion assay: The disk diffusion assay was performed according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI). The overnight culture of *S. aureus* ATCC 29213 was standardized to a turbidity equivalent to 0.5 McFarland and then final bacterial inoculum of 10^6 CFU/mL was mixed with molten MHA. Chalcone derivatives [C1, C3, C4, C5, C7-C11, C13, C15, and C16] were suspended in DMSO, at test concentrations of 12.5, 25, 50 mg/L, respectively. To check for individual antimicrobial activity, the 50µl volume of above-mentioned test concentrations of chalcone derivatives were loaded in wells punched using cork borer. For combination study, 25µl of each chalcone derivative at specified test concentrations and 25µl of ciprofloxacin at 5mg/L were employed. Following overnight incubation at 37°C, the plates were examined for zones of inhibition (ZOI), and the diameters of any observable zones were recorded in millimeters (Atef et al., 2019).

4.3.3 Time kill studies of chalcone with ciprofloxacin: Time-kill kinetics were assessed using the protocol previously described by Xedro et al. Ciprofloxacin combined with chalcone C9 in MHB was used to generate a time-kill curve against the *S. aureus* strain SA-1199B. A bacterial suspension was made at a concentration of $1x10^6$ CFU/mL. Separately, ciprofloxacin at a concentration of 2mg/L [1/4 XMIC] was examined separately and in combination with of chalcone C9 at 25mg/L [MEC of chalcone C9 determined earlier]. Moreover, MIC concentration of ciprofloxacin [8mg/L] was evaluated. The bacterial suspensions were incubated at 37°C. At set time periods, i.e., 0, 2, 4, 6, and 24 hours, 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. (Xedro et al., 2022)

4.3.4 Mutation Prevention Concentration and Rate of Mutation frequency: After an 18-hour culture on TSA, 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 4x, 8x, and 16x 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 as certained (Sharma et al., 2010).

4.3.5 Post Antibiotic Effect (PAE) of effective combinations: The PAEs of the ciprofloxacin alone and in combination with the chalcone were measured by methods described by Sharma et al (Sharma et al., 2010). 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 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 minutes, samples were collected for the CFU count until visible cloudiness was seen. The calculation of post-antibiotic effect (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.

4.4 Biofilm susceptibility of ciprofloxacin in combination with chalcone (C9):

4.4.1 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 publication (Nair et al., 2016). In a 96 well flat-bottom microtiter plate, 100µl of SA-1199B bacterial culture (10^6 CFU/mL) and 100µl of ciprofloxacin and chalcone C9 alone and in combination prepared as two fold serial dilutions in TSB supplemented with 2% sucrose, were added to each well and incubated for 24 hours at 37°C. After incubation, each well was rinsed with 200µl of 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 minutes under dark conditions at room temperature. Afterwards the plates were washed three times with sterile PBS to remove unbound CV, and finally resolubilized in 33% glacial acetic acid. The absorbance at 595nm 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,

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

4.4.2 Scanning electron microscopy (SEM) for biofilm inhibition: Scanning electron microscopy 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 25mg/L and ciprofloxacin at 1/4X 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. Afterwards, dehydration was done by employing ethanol at sequential concentrations of 10%, 50%, 70%, 80%, 90%, 95%, and 100% for 20 minutes each at a time. Final dehydration was carried out with isoamyl acetate and acetone (1:1) for 30 minutes 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 (Chino et al., 2017).

4.5 qRT-PCR study:

4.5.1 Expression studies to establish relation between *agrA* and *norA* genes in *S. aureus*

4.5.1.1 Primer designing for Quantitative Real-Time PCR analysis

The sequences of all primers used in this study together with their amplicon length are listed in table 4.2. The ORF sequences of NorA, AgrA gene and 16S gene were taken from 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 Muller Hinton Broth (MHB) (Sharma et al., 2010).

Table 4.2: List of the sequences of all primers with their amplicon length

Gene	Primer Sequences	Annealing Temp.
16s	F: TGAGTAACACGTGGATAACCTAC	62°C
	R: CGGATCCATCTATAAGTGACAG	
norA	F: CAGCTATTAAACCTGTCACACC R: AGCTATTAAACCTGTCACACCAG	62°C
agrA	F: TGTCTACAAAGTTGCAGCGATG R: TAAATGGGCAATGAGTCTGTGAG	62°C

4.5.1.2 RNA Extraction: Muller Hinton Broth (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 ten-milliliter 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 minutes at room temperature. Following that, the samples were centrifuged for 15 minutes at 4°C at 12,000 × 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 minutes of room temperature incubation, the tubes were centrifuged for 8 minutes 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 minutes 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 ten minutes.

4.5.1.3 Quantification of RNA: Quantification of RNA was done using nanodrop. The sterile MQ was used to set blank. Ratio of 2.1 of 260/280 wavelengths is indication of good quality of RNA. All the RNA quantification was done using the same procedure.

4.5.1.4 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, 1µg of pure sample of RNA was quickly treated in gDNA wipeout buffer at 42°C for 2 minutes. 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 minutes of incubation at 42°C, the reaction tubes were inactivated for three minutes at 95°C. The same procedure was followed for all cDNA synthesis process.

4.5.1.5 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. For the 16S rRNA gene, a standard curve was plotted as previously mentioned. In a 20 µl reaction volume, a two-step real-time PCR was conducted.

Ten pmol of each primer (2 µl), ten µl of SYBR Green I master mix, two µl of cDNA (1:10 dilution of cDNA from one 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 minutes. 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. Effect of proposed Chalcone (a known NorA EPI) on expression of norA and agrA genes were compared.

4.5.1.6 Quantification of RNA: Quantification of RNA was done using the same procedure as described in the section 4.5.1.3.

4.5.1.6.1 First Strand cDNA synthesis: The same procedure was followed as described in section 4.5.1.4.

4.5.1.6.2 Relative expression studies: The expression of both the genes was quantified in *S. aureus* 1199B treated with C-9 w.r.t untreated *S. aureus* 1199B as described in the section 4.5.1.5

4.6 *In-silico* studies

4.6.1 Preparation of the NorA Target via homology modeling: To better comprehend the binding relationship with a NorA MFS protein, a 3D homology model was constructed using Uniprot Q5HHX4 protein sequence with UniParc - UPI00000522D0 and compared to EmrD MFS from *E. coli* with pdb ID: 2G, following previous studies with slight modificationsas (Yin et al, 2006; Zarate et al, 2019). The model was further assessed using Swiss Model (Waterhouse et al., 2018), with the binding site selected based on previous research (Zarate et al., 2019; Kalia et al., 2012).

4.6.2 Molecular docking C9 compared with Capsaicin for NorA and Agr inhibition: AutoDock Vina, a molecular docking software, was used to investigate the interactions of the 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.

4.7 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 $P < 0.05$.

CHAPTER 5: RESULTS AND DISCUSSION

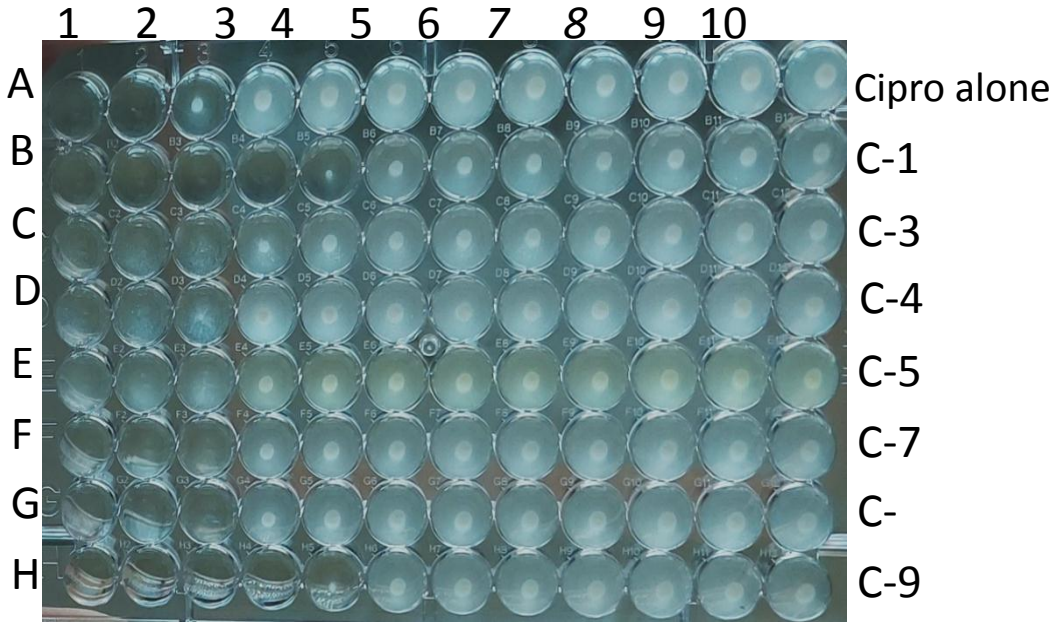
5. Results

5.1. *In-vitro* combination studies of Chalcones

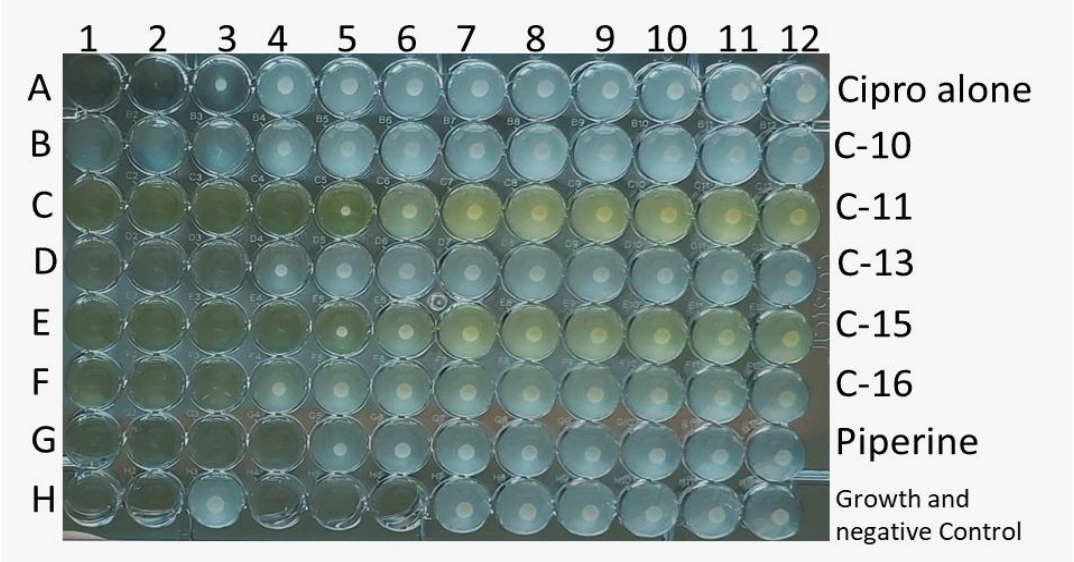
5.1.1 Evaluation of chalcones by checkerboard method: The MIC of ciprofloxacin, with and without chalcone C9, are listed in Table 5.1 and results depicted in Figure5.1. The presence of chalcone C9 led to a significant reduction in ciprofloxacin MIC, with values decreasing by 2- to 8-fold . The difference in MIC between *S. aureus* SA-1199 (wild-type) and SA-1199B (NorA-overproducing) was more distinct at 12.5 mg/L of chalcone C9 concentration (Table 5.2). Additionally, a decrease in MIC with chalcone C9 was not observed at concentrations above 25 mg/L Chalcone C9 exhibited potent antibacterial activity against *S. aureus* in broth microdilution assays.

Table 5.1: Screening of chalcones as EPI at single concentration (25mg/L) in combination with ciprofloxacin against *S. aureus* ATCC 1199B

Sr. No	Test Compound (25mg/L)	MIC of Ciprofloxacin (mg/L)
1	Ciprofloxacin	8
2	Ciprofloxacin + C-1	1
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



(A)



(B)

Figure 5.1: Screening of Chalcones as EPI at single concentration (25mg/L) in combination with ciprofloxacin against *S. aureus* ATCC 1199B (Plate pictures)

Table 5.2: In vitro Ciprofloxacin/Chalcone combination studies

Chalcone-C9 (mg/L)	MIC of Chalcone-C9 (mg/L)	**MIC (mg/L) of ciprofloxacin against strains of *S.aureus* with/without test molecule (Fold reduction)**	
		SA-1199 (WT)	SA-1199B (NorA overexpressing)
C9 (25)	>100	0.125/0.25(2)	1/8(8)
C9 (12.5)	>100	0.125/0.25(2)	2/8(4)
C9 (6.25)	>100	0.25/0.25(0)	4/8(2)
C9 Piperine (25)	>100	0.125/0.25(2)	2/8(4)

5.1.2 Agar diffusion assay: Agar well diffusion results brought to light the findings that when administered alone, the chalcone derivatives C1, C3, C4, C5, C7-11, C13, C15, and C16 showed no zone of inhibition (ZOI) against *S. aureus* ATCC 29213. Later, the chalcones were investigated for their antibiotic potentiating activities with ciprofloxacin which revealed that at 5mg/L concentration of ciprofloxacin, of all the chalcone derivatives, Chalcone C9 produced ZOI measuring 16.5mm, 18mm, and 20mm in diameter at doses 12.5, 25, and 50 mg/L, respectively. The results for the same have been indicated in Figure 5.2.

Figure 5.2: Agar diffusion assay: showing increase in zone of inhibition of ciprofloxacin in presence of chalcone (C9) at different concentration Agar well diffusion result as Cipro (5mg/L)- 15.5mm, C9(50mg/L)- No ZOI,C9(25mg/L)- No ZOI,C9 (12.5mg/L)- No ZOI,C912.5 +C- 16.5mm,C9 25 +C- 18mm, C9 50 +C- 20mm

5.1.3 Time kill studies of chalcone 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 2mg/L (1/4 XMIC), but at 8mg/L, it showed bactericidal efficacy (99.9% kill) within

6 hours. Moreover, bactericidal activity was reported when the combination of ciprofloxacin at 2mg/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 log10 CFU below the initial inoculum at the time of commencement of the experiment (0h) (Figure 5.3). These results imply that chalcone C9 can enhance ciprofloxacin action against mutant *S. aureus* strain 1199B, thus providing a novel strategy for combating the antibiotic resistance in this bacterium.

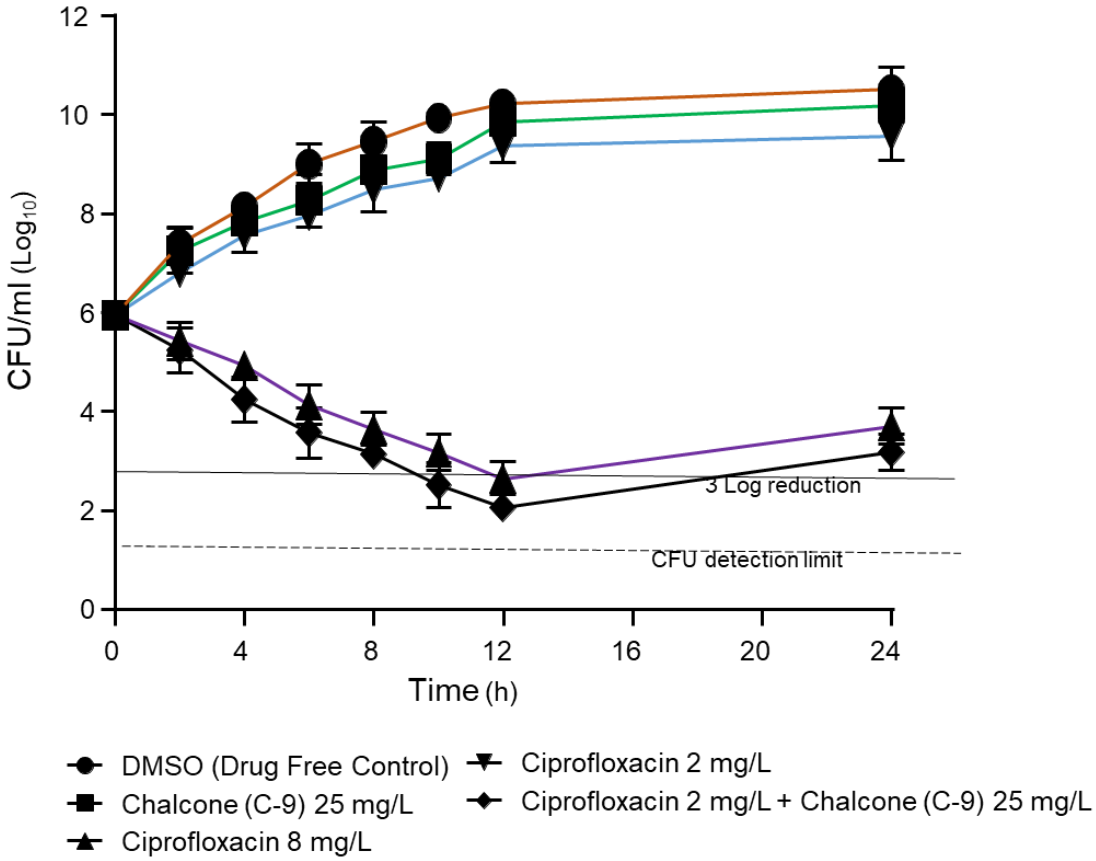


Figure 5.3: Time–kill curves of *S. aureus* SA-1199B demonstrating the bactericidal action of ciprofloxacin (1/4× MIC, 2 mg/L)combined with chalcone C9 (25 mg/L). The individual time pointwas the average log_{10} + SD of the three readings.

5.1.4 Mutation Prevention Concentration and Rate of Mutation frequency: The probability for 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 XMIC), 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 25mg/L, the MPC values dropped to 2 and 1mg/L, respectively. Moreover, the combination's MPC was lower than ciprofloxacin's maximum concentration, (i.e., $\mathbf{C_{max}}$ value of 4 mg/L), implying that these combinations could be clinically significant in preventing the evolution of resistant bacterial mutants (Table 5.3)

Table 5.3: Mutation frequency of *S. aureus* ATCC 29213

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

5.1.5 Post Antibiotic Effect (PAE) of effective combinations: Post antibiotic effect (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 5.4).

Table 5.4: Post antibiotic effect (PAE) with chalcone C-9 and Ciprofloxacin

Chalcone (EPI mg/L)	PAE's(h) ± SD at		
	¼ x MIC(2 mg/L)	½ x MIC(4 mg/L)	MIC(8 mg/L)
Without C-9	0.4 ± 0.1	1.25 ± 0.5	1.45 ± 0.5
C-9(25 mg/L)	1.0 ± 0.3	1.45 ± 1	2.5± 0.25

5.2 Biofilm susceptibility of ciprofloxacin in combination with chalcone (C9)

5.2.1 Biofilm inhibition (96 well plate): The study investigated the impact of chalcone C9 on the minimum biofilm inhibitory concentration ($MBIC_{50}$) of ciprofloxacin against *S. aureus* ATCC 29213. The results confirm a notable influence of C9 on the efficacy of ciprofloxacin in inhibiting the biofilm formation. Although the chalcone on its own had no significant effect in biofilm inhibition at concentrations of 50mg/L, 25mg/L, and 12.5mg/L respectively, but in conjunction with ciprofloxacin, it was observed that lower dose concentration of C9 of 12.5mg/L produced almost negligible potentiation of ciprofloxacin's efficacy. At 25mg/L of C9, $MBIC_{50}$ of ciprofloxacin enhanced by 13%, whereas with 50mg/L concentration of C9, most pronounced reduction in $MBIC_{50}$ was obtained potentiating the antibiotic's biofilm inhibition potential by almost 58% (Data represented in Table 5.5, Figure 5.4).

Table 5.5: $MBIC_{50}$ of Ciprofloxacin against *S. aureus* ATCC 29213 when used in combination with Chalcone C9

Chalcone C-9 (mg/L)	$MBIC_{50}$ of ciprofloxacin against *S. aureus* ATCC 29213 in combination with C9
0	**0.33**
12.5	**0.32**
25	**0.29**
50	**0.14**

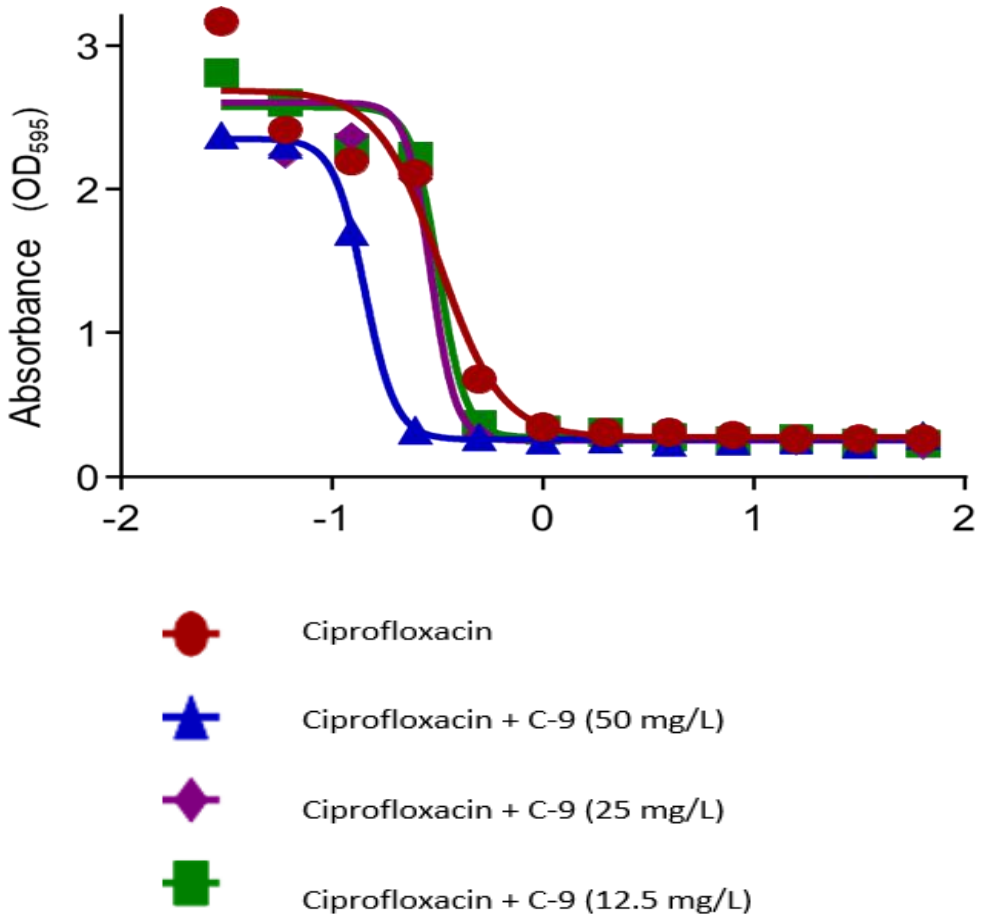
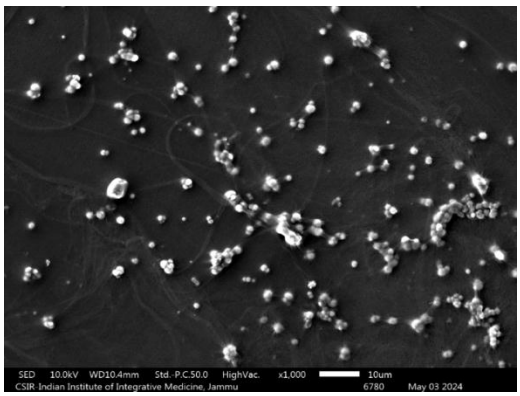
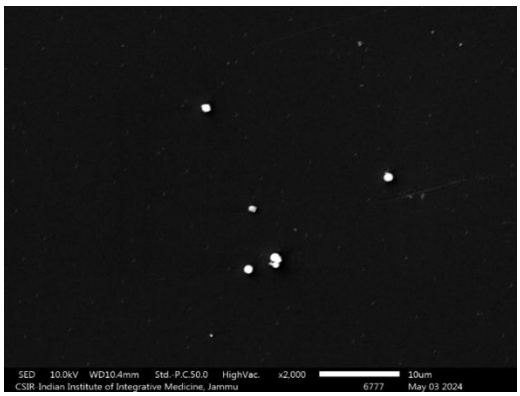


Figure 5.4: $MBIC_{50}$ of Ciprofloxacin against *S. aureus* ATCC 29213 when used in combination with Chalcone C9 (represented in terms of OD)

5.2.2 Scanning electron microscopy (SEM) for biofilm inhibition: The SEM micrographs so obtained demonstrated 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 (Figure 5.5.C), but treatment with 1/4 XMIC concentration of ciprofloxacin resulted in a moderate reduction in biofilm cell mass (Figure 5.5.A). However, when a combination of C9 (25mg/L) and ciprofloxacin (1/4 XMIC) was evaluated, the SEM pictures revealed a significant inhibitory effect of this synergistic combination (Figure 5.5.B) on *S. aureus* SA-1199B biofilm development compared to biofilms produced by untreated control (Figure 5.5.D).

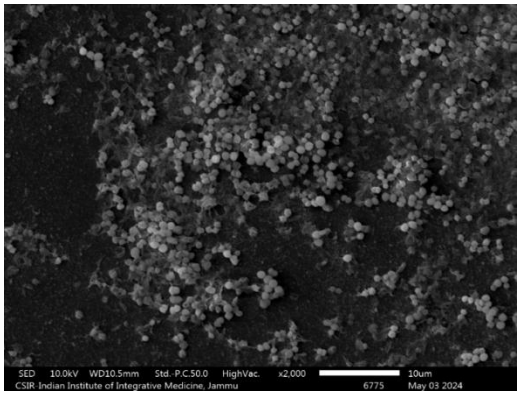
A

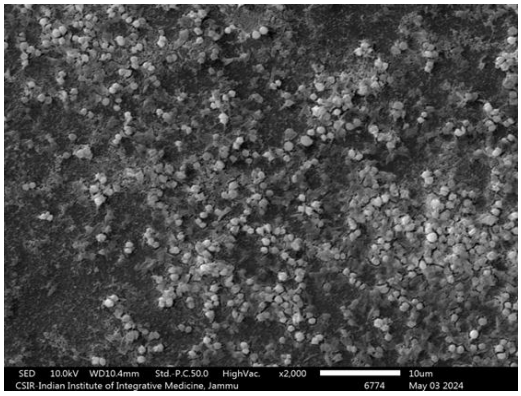




B

C





D

Figure 5.5: SEM images demonstrating the antibiofilm effect induced on the *S. aureus* ATCC 1199B treated with ciprofloxacin at 2mg/L, (A) *S.aureus* ATCC 1199B treated with ciprofloxacin at ¼ X MIC(2mg/L)(Reduced Biofilm Cell Mass),(B)Combination[Ciprofloxacin(2mg/L)+ChalconeC9(25mg/L)](Significant Biofilm Inhibtion),(C)Chalcone C9 at 25mg/L alone (No Noticeable effect),(D)Untreated control (Dense Biofilm Formation).

5.3 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, *agr*A 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 to significantly downreguralted to decimal points in biofilm associated cells in presence of C9 when compared with plantonic cells.

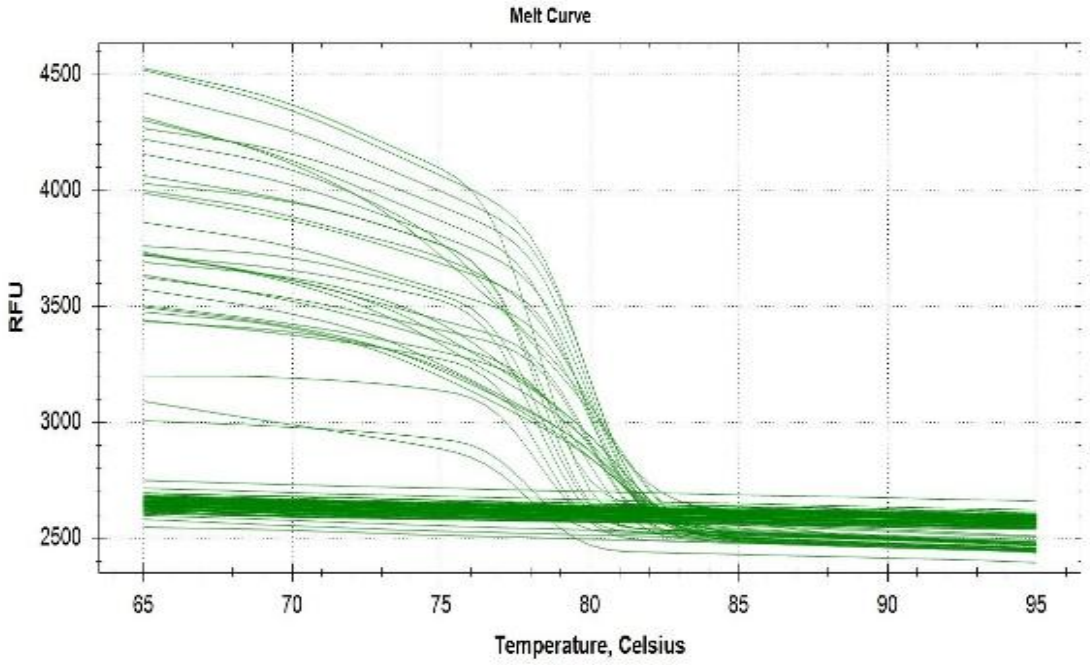


Figure 5.6.1: Melt curve Biofilm C9

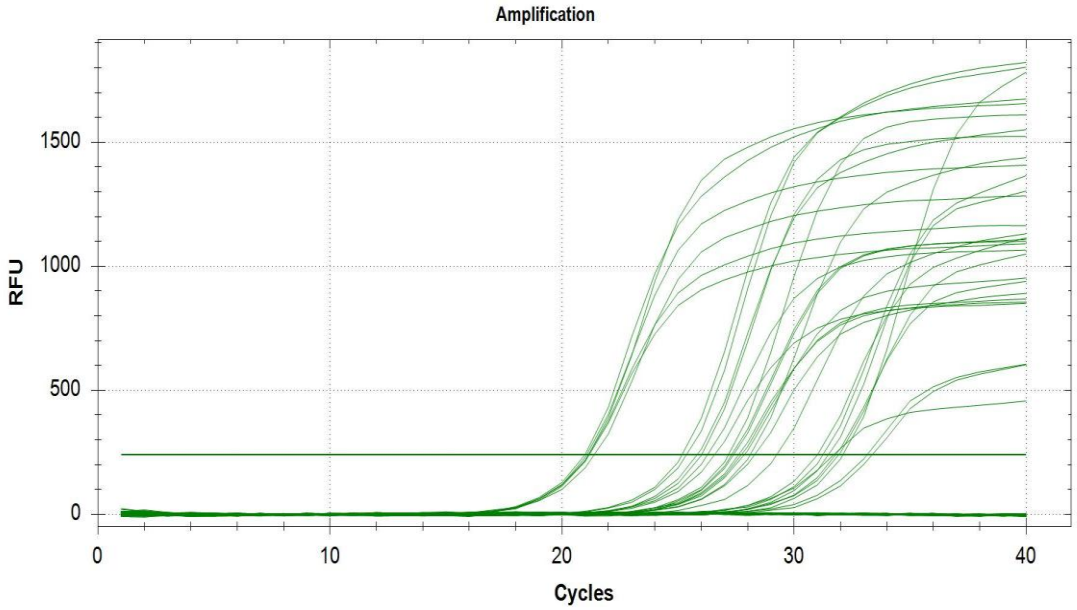


Figure 5.6.2: *S. aureus* C9 biofilm amplification

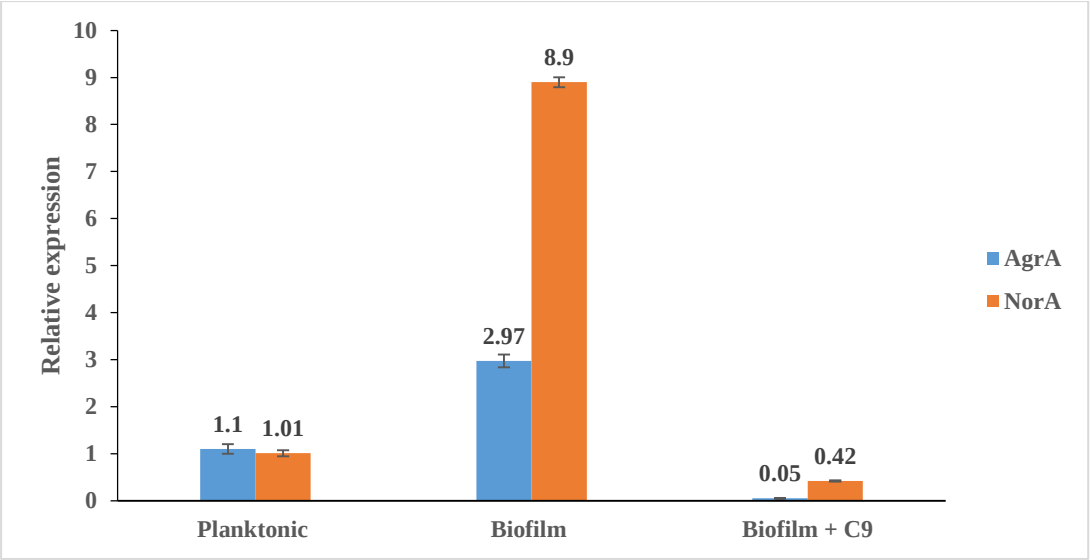


Figure 5.6.3: Biofilm induced co-overpexpression of agrA and norA and impact of chalcones on expression of both the genes

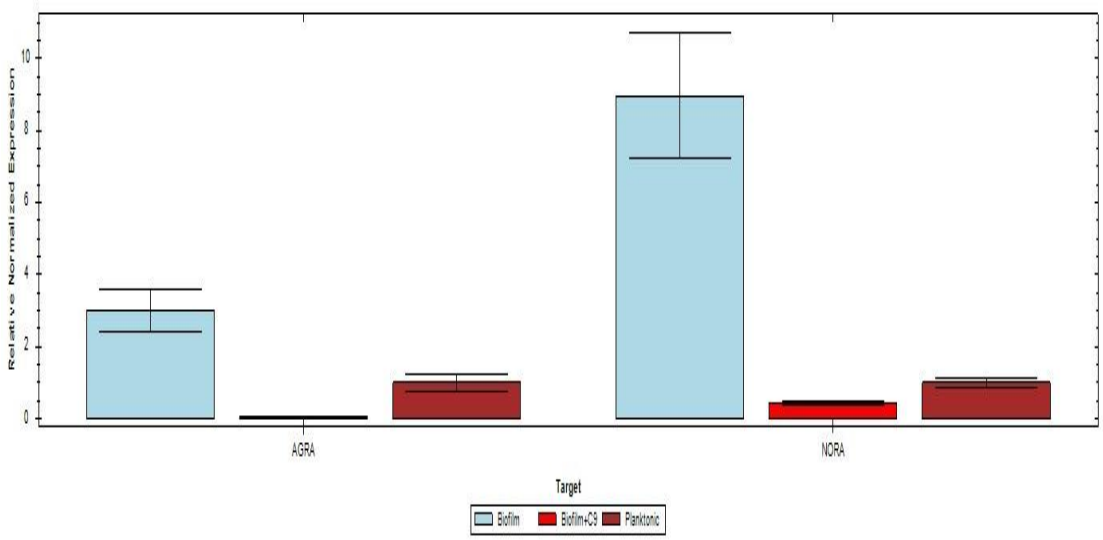


Figure 5.6.4: Relative normalized expression of AgrA and NorA

5.4 *In-silico* studies

5.4.1 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 5.6) C9 is involved in hydrophobic interactions at amino acid residues such as Phe140, Ile141, and Ile244 and shows π-stacking of the aryl ring with Phe140 (Figures 5.6 a and 5.6 b).

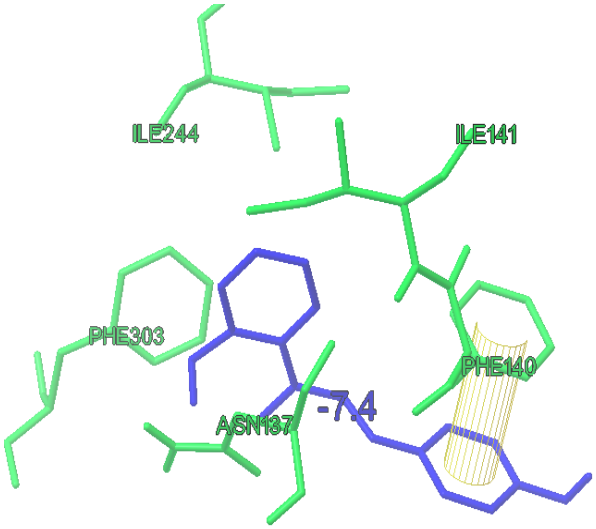
The binding site residues observed in the Agr gene are Ile285, Glu286, Leu288, Lys294, Ile297, Thr298, and Ile313. The binding affinities of capsaicin and C9 to the Agr gene were 6.3 and -6.3 kcal/mol, respectively. C9 molecules were observed to have hydrophobic interactions with Ile285, Leu288, Ile297, and Ile313. It was also found to form a hydrogen bond with Thr298 and its hydroxyl functional group. Furthermore, a strong cation- π interaction was observed between the aryl ring and Lys294 (Figures 5.6 c and 5.6 d).

Table 5.6: Docking results using Autodock vina

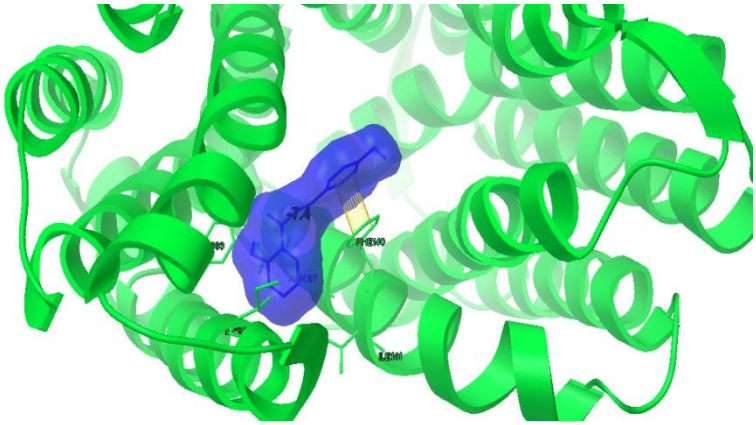
S.No.	Molecule	NorA		Agr	
		Binding residues	**Binding affinity (kcal/mol)**	**Binding residues**	**Binding affinity (kcal/mol)**
1	Capsaicin	Phe16, Ile19, Ile23, Gln61, Met109, Ile244, Phe303, and Asn340	-6.7	His379, Phe382, Phe386, Lys401	6.3
2	C9	Asn137, Phe140, Ile141, Ile244, and Phe303	-7.4	Ile285,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

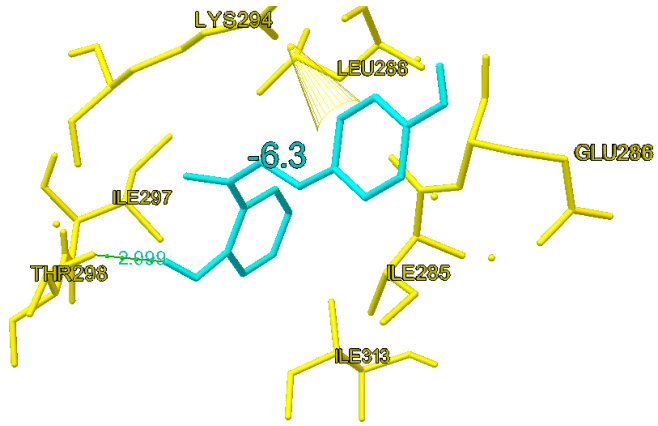
(a)



(b)



(c)



(d)

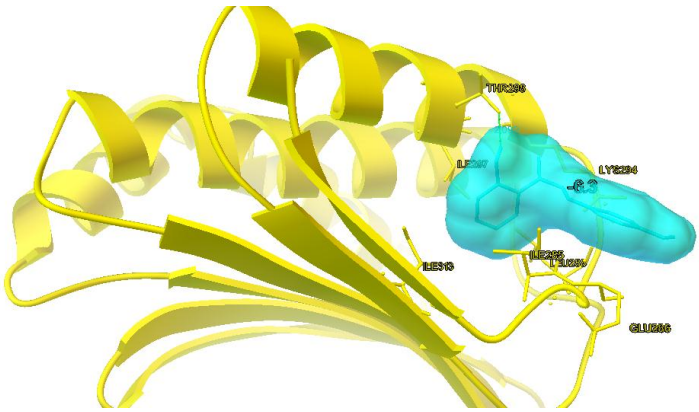


Figure 5.7: Binding interaction of C9 (a) ball and stick model NorA(green), C9 (blue), (b) ribbon structure NorA(green), C9 (blue), (c) ball and stick model Agr(yellow), C9 (blue), (d) ribbon structure Agr(yellow), C9 (blue).

5.5 Discussion

The bacterial pathogen, *Staphylococcus 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 (Costa et al., 2016).

Chalcones are natural molecules containing two aromatic rings linked by an α,β-

unsaturated carbonyl group, exhibiting numerous biologically essential properties, including antimalarial, antibacterial, anticancer, antifungal, antioxidant, neuroprotective, and anti-inflammatory effects (Rammohan et al., 2020). Notably, chalcones are considered relatively safe compounds with low toxicity, displaying a wide range of biological activities with minimal adverse effects (Nowakowska, 2007; Singh et al., 2016). Their safety profile and diverse pharmacological properties make them promising therapeutic candidates. Chalcones exert antibacterial effects by inhibiting efflux pumps, which remove detrimental chemicals and drugs from bacterial cells, contributing to multidrug resistance (Holler et al., 2012). Specifically, chalcones block NorA, an efflux pump in S. aureus, providing resistance to various antibiotic classes. By inhibiting NorA, chalcones may enhance antibiotic efficacy against Methicillin-Resistant Staphylococcus aureus (MRSA). Recent studies have focused on synthesizing and assessing chalcone derivatives as NorA inhibitors and modulators of ciprofloxacin activity, a widely used antibiotic for treating S. aureus infections (DaSilva et al., 2021; Siqueira et al., 2021; Rezende-Junior et al., 2020).

The investigation into the antibacterial properties of chalcone derivatives against *S. aureus* revealed significant findings regarding their potential as an efflux pump inhibitors and antibiotic adjuncts, highlighting their ability to enhance antibiotic efficacy and combat resistance 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 zones 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 so used (Rana et al, 2024; Da Silva et al, 2021).

However, further testing of chalcones in conjunction with ciprofloxacin demonstrated a significant increase in antibacterial activity, particularly for chalcone C9 suggesting its potential as a therapeutic adjunct. With ciprofloxacin at a dose concentration of 5mg/L, C9 showed potentiation forming ZOIs equal to 16.5mm, 18mm, and 20mm at employed concentrations of 12.5mg/L, 25mg/L, and 50mg/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 Siqueira et al (Siqueira et al, 2021).

Moreover, the in vitro checkerboard results in our research work demonstrated that combining chalcone C9 with ciprofloxacin reduced the minimum inhibitory concentration of ciprofloxacin by 2-8 folds in the two strains of *S. aureus*, i.e., *S. aureus* ATCC 1199, and *S. aureus* ATCC 1199B. This synergistic interaction likely results from chalcone C9's inhibition consistent with Nargotra et al. (2009), who reported enhanced antibacterial activity upon NorA inhibition. While these findings demonstrate promise, further studies are warranted to assess the consistency of this synergistic interaction across a broader range of *S. aureus* strains, including clinical isolates with diverse resistance profiles.

The time-kill curve assay confirmed 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 post-antibiotic effect (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 (Aboelnaga *et al.,* 2024). 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 behavior and adaptation. In the case of *S. aureus,* QS is mediated via the accessory gene regulator (agr) system, which regulates the production of bacteria's virulence factors such as alpha-hemolysin (hla)

and biofilm associated genes like icaA (Wang et al, 2016). Biofilm inhibition studies suggested that Chalcone C9 has the potential to enhance ciprofloxacin's ability to penetrate or disrupt biofilm formation, or it is likely that it may have a direct role in inhibiting the bacteria's ability to form biofilms. This prediction is based on the observation that increasing concentrations of Chalcone C9 strengthened ciprofloxacin's biofilm inhibition capacity against *S. aureus* ATCC 29213, with the most pronounced effect observed at a C9 concentration of 50 mg/L. 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 a proposed NorA inhibitor, inhibited the expression of both the genes. (as depicted in figure 5.6). Subsequently, the Scanning electron microscopy (SEM) imaging revealed that C9 when used at sub-inhibitory concentration of 25mg/L had no obvious impact in suppressing *S. aureus* SA-1199B bacterial biofilms, whereas treatment with 1/4xMIC concentration of ciprofloxacin induced mild reduction in biofilm cell mass. However, in case combination of C9 (25mg/L) with ciprofloxacin (1/4xMIC) was tested, SEM images visually confirmed substantial inhibitory effect of this synergistic combination on 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 mutant prevention concentration (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 the binding affinities of -6.7 kcal/mol, and -7.4 kcal/mol respectively on NorA protein Table (5.6). C9 is involved

in the hydrophobic interaction at the amino acid residues like Phe140, Ile141, Ile244 and also showed the π -stacking of aryl ring with Phe140 (Figure 5.6 a and 5.6 b).

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 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 hydrogen bond with Thr298 and its hydroxyl functional group. Further a strong cation – π interaction was noted between aryl ring and Lys294 (Figure 5.6 c and 5.6 d).

To summarize, our findings emphasize Chalcone C9 as a potential adjuvant to combat MRSA infection by enhancing ciprofloxacin potency and addressing bacterial resistance mechanisms. Specifically, C9 blocks the NorA efflux pump and inhibits biofilm growth, lowering ciprofloxacin's MIC and increasing bacterial eradication. While these results contribute to the growing body of research on chalcone derivatives as promising adjuvants against MDR *S. aureus* infections, further in vivo and clinical studies are warranted to validate their therapeutic efficacy and fully realize their potential in addressing antibiotic resistance.

CHAPTER 6: SUMMARY AND CONCLUSION

Staphylococcus aureus is an important human pathogen and quite often causes life threatening infections within the healthcare settings. The widespread use of antibiotics to combat S. aureus infections has been countered by the pathogen's capacity to develop resistance, resulting in the rise of antibiotic-resistant strains that pose a major challenge and contribute to hospital-acquired infections. The infection caused by *S. aureus* becomes life threatening to patients admitted for long term in health care facilities. These nosocomial pathogens develop resistance to antibiotics and emerge as MRSA, MSSA which raises serious global concerns.

This study investigated the potential chalcones derivatives as as efflux pump inhibitors (EPIs) in combination with ciprofloxacin against *S. aureus*. The checkerboard method revealed that chalcone C9 reduced the MIC of ciprofloxacin by 2-8 fold, with a notable 8-fold reduction among all the chalcones tested. The combination of ciprofloxacin and C9 exhibited enhanced bactericidal effects in kill kinetics studies and significantly reduced the emergence of resistance in mutation prevention concentration experiments. Furthermore, the combination extended the post-antibiotic effect (PAE) compared to ciprofloxacin alone. This PAE is critically important for pharmacological studies which help in deciding the dose of antibiotics during treatment. Moreover, the combination was tested for its potential to suppress the formation of biofilms. Notably, the combination of ciprofloxacin and C9 at minimum effective concentration (MEC) inhibited biofilm formation, as confirmed by SEM analysis.

NorA and Agr are involved in *S. aureus* resistance due to efflux pump and biofilm development respectively. qRT-PCR results showed that agrA and norA expression were upregulated 2.97 fold and 8.9 fold, respectively, in biofilm-associated cells compared to planktonic cells. However, in the presence of C9, the expression of both genes was significantly downregulated. Molecular docking analysis revealed that C9 had a higher binding affinity to NorA compared to capsaicin, a known EPI.

Significant findings include:

1. Chalcone derivative C9 significantly reduced the minimum inhibitory concentration (MIC) of ciprofloxacin by 2-8 fold, with an optimal reduction of 8-fold.
2. The combination of ciprofloxacin and C9 exhibited enhanced bactericidal effects, reduced emergence of resistance, and extended post-antibiotic effect (PAE).
3. C9 effectively reverses the upregulation of norA (8.9-fold) and agrA (2.97-fold) genes in biofilm-associated *S. aureus* cells, significantly downregulating their expression and suggesting its potential to combat antibiotic resistance by inhibiting efflux pumps and biofilm development.
4. Molecular docking analysis revealed that C9 had a higher binding affinity to NorA efflux pump protein compared to capsaicin, a known EPI.

In conclusion, to the best of our understanding, this study provides sufficient evidence for the potential use of chalcone derivatives as dual inhibitors targeting for NorA efflux pump and biofilm formation regulatory genes (agr). The chalcones derivatives (C9) significantly increases the in vitro efficacy of ciprofloxacin against MRSA. Our finding suggests that using an *in-silco* approach to screen larger libraries of molecules with dual activity, specifically targeting efflux pumps and biofilm formation could lead to the discovery of more effective compounds to combat the emergence of antibiotics resistance. However, further detailed studies and more effective studies in wet labs are warranted to validate the in-vivo and clinical efficacy of such combinations for therapeutic applications. These results contribute to the growing body of research on chalcone derivatives as promising adjuvants in the treatment of MDR *S. aureus* infections.

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LIST OF PUBLICATIONS

- **Bhawandeep Kaur,** Jeena Gupta, Sarika Sharma, Divakar Sharma, Sandeep Sharma **(2021)** Focused review on dual inhibition of quorum sensing and efflux pumps: A potential way to combat multi drug resistant *Staphylococcus aureus* infections. **International Journal of Biological Macromolecules.** 190: 33-43. **(Impact factor: 8.5)**

- **Bhawandeep Kaur,** Avleen Kour, Sarika Sharma, Gopal Kathik, Nitin Pal Kalia, Sandeep Sharma **(2024)** Chalcone derivatives as efflux pump inhibitor of *Staphylococcus aureus*. **African Journal of Biological Sciences.** 6(5), pp. 1831–1838. **(SJR- 0.15).**

- **Bhawandeep Kaur,** Shashikant Sau, Suman K. Jana, Nitin Pal Kalia, Gopal Lal Kathik, Ashish Suttee, Sarika Sharma, Sandeep Sharma **(2025)** Potentiation of Ciprofloxacin Activity By Chalcones By Modulating Efflux Pump And Biofilm Regulatory Gene In *Staphylococcus Aureus* **International Journal of Applied Pharmaceutics. ISSN: 09757058Volume: 17 Issue:** 4Pages: 290 - 298pp. 1831–1838. **(SJR- 0.219).**

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Review

Focused review on dual inhibition of quorum sensing and efflux pumps: A potential way to combat multi drug resistant *Staphylococcus aureus* infections

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ABSTRACT

Staphylococcus aureus is a common cause of skin infections, food poisoning and severe life-threatening infections. Methicillin-Resistant *Staphylococcus aureus* (MRSA) is known to cause chronic nosocomial infections by virtue of its multidrug resistance and biofilm formation mechanisms. The antimicrobial resistance owned by *S . aureus* is primarily due to efflux pumps and formation of microbial biofilms. These drug resistant, sessile and densely packed microbial communities possess various mechanisms including quorum sensing and drug efflux. Quorum sensing is a cooperative physiological process which is used by bacterial cells for social interaction and signal transduction in biofilm formation whereas efflux of drugs is derived by efflux pumps. Apart from their significant role in multidrug resistance, efflux pumps also contribute to transporting cell signalling molecules and due to their occurrence; we face the frightening possibility that we will enter the pre-antibiotic era soon. Compounds that modulate efflux pumps are also known as efflux pump inhibitors (EPI's) that act in a synergistic manner and potentiate the antibiotics efficacy which has been considered as a promising approach to encounter bacterial resistance. EPIs inhibit the mechanism of drug efflux s as well as transport of quorum sensing signalling molecules which are the supreme contributors of miscellaneous virulence factors. This review presents an accomplishments of the recent investigations allied to efflux pump inhibitors against *S. aureus* and also focus on related correspondence between quorum sensing system and efflux pump inhibitors in terms of *S. aureus* and MRSA biofilms that may open a new avenue for controlling MRSA infections.

1. Introduction

Staphylococcus aureus (*S. aureus*), a common human opportunistic pathogen is associated with minor to major skin infections with a high mortality rate. Although *S. aureus* is not always pathogenic, yet it is a common cause of skin infections (e.g. boils) and food-poisoning [1]. Disease-associated strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that not only bind antibodies but also and inactivate. Staphylococcal infections can turn deadly if the bacteria invade deeper into the human body, entering bloodstream, joints, bones, lungs or heart [2]. Also, people are developing life-threatening staphylococcal infections and majority of these infections are no longer respond to common antibiotics is use. The emergence of antibiotic-resistant forms of pathogenic *S. aureus* (e.g. MRSA) is a worldwide challenge in chemotherapy [3].

S. aureus in both hospital-acquired and community-acquired infections can cause a wide variety of diseases, ranging from minor skin infections to life-threatening endocarditis, pneumonia, blood infections, and toxic shock syndrome [4]. However, soon after β-lactam antibiotics were introduced in 1940's, bacteria became resistant to such antibiotics by producing β-lactamases. Methicillin, the first β-lactamases resistant antibiotic was released in 1960 and within the span of a decade; resistance to methicillin was evident and today, 60 to 80% of all clinical isolates of *Staphylococcus aureus* are resistant to methicillin (oxacillin). Methicillin resistant *Staphylococcus aureus* (MRSA) can cause chronic nosocomial infections which are due to its ability to adhere to medical devices such as catheters and form a biofilm [5]. Biofilm harbour sessile densely packed microbial communities, contains numerous protective

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features, including extracellular polymeric material and eDNA, that render biofilms impermeable to conventional antimicrobial agents [6]. Cell to cell signalling by quorum sensing systems play an important role in virulent pathogens associated with biofilm formation.

The biofilm forming MRSA are inherently cross-resistant to virtually all β-lactam antibiotics, the most effective and widely used class of antimicrobials [7]. Vancomycin is the drug of choice to treat MRSA, but the bacteria have smartly developed its own arsenal to evade antimicrobial treatment [8]. In 1996, for the first time, a vancomycin intermediate resistant strain was reported in Japan, whereas in 2002, the first fully resistant species of *S. aureus* was reported in Michigan, United States [9]. This naturally occurring antibiotic resistance mechanism is directed by resistant genes that commonly reported in bacterial species.

The four main mechanisms responsible for drug resistance are (1) drug inactivation, (2) alteration of the target site [10], (3) reduced intracellular drug accumulation by efflux pumps and (4) alteration of metabolic pathway [11] (Fig. 1). Out of these four mechanisms, efflux-related multi-drug resistance (MDR) is a significant means by which bacteria can evade the effects of selected antimicrobial agents [12]. Efflux pumps reduce the concentration of drug inside the cell and hence decreases its efficacy [13]. Increasing the antibiotic concentration inside the bacterial cell by inactivating or inhibiting the efflux pump can significantly is an effective measure against drug resistance.

Development of efflux pump inhibiting compounds; efflux pump inhibitors (EPIs) that can restore the antibiotic activity have been considered as a promising approach to overcome bacterial resistance [14]. These efflux pump inhibitors when used in combination with antibiotic can bind to antibiotic substrate (or can be antibiotic binding site) on efflux pump proteins. This effectual nexus reduces drug-efflux pump interactions and leads to drug accumulation [15]. Efflux pump inhibitors belonging to different categories have been evaluated against staphylococcal infections. Plant alkaloids such as reserpine, piperine, berberine and other synthetic or modified molecules have been effective against the efflux pump. Studies showed that the combined effect of phyto-therapeutics and EPI has been used to increase the drug efficacy against resistant microorganisms [16]. Cell to cell signalling by quorum sensing also plays an important role in pathogen's virulence associated with biofilm formation [17]. *Agr* locus is a global regulator of biofilm associated MRSA infection and controls the virulence expression [18]. Its specific biofilm dispersing characteristics is an upcoming field of investigation. Interaction of EPIs with cell signalling system is another upcoming approach against biofilm associated *S. aureus* infections [19].

Multidrug resistant pathogens are still a remedial problem; it requires additional research to analyze the current advancement in the search of new approaches to combat it. The extended knowledge of *S. aureus* resistance mechanisms and drug efflux mechanisms involved in the capability to form biofilm, may contribute to design the preventive strategies for *S. aureus* infections. This timeline review is focused on the recent studies evaluating new compounds for developing drug restoration by mean of MDR pump inhibition and quorum quenching. The enhancement of drugs efficacy with support of such compounds can be a helpful therapeutic antagonistic approach toward emergence of drug resistance.

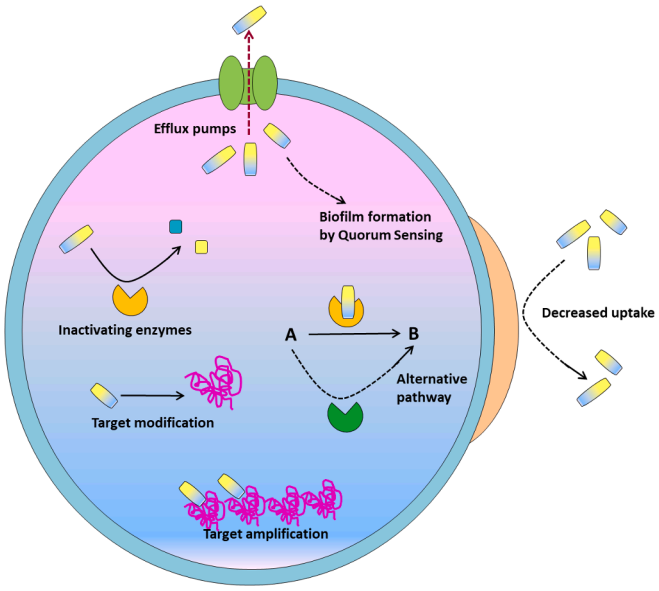

Fig. 1. Mechanisms adopted by *S. aureus* for its survival. A) Efflux pumps help bacteria to survive by removing drug from inside of cell. B) β-Lactamases played a crucial role in developing resistance against β-lactam drugs. C) Mutations in the target site helps bacteria to survive in even in the presence of drug due to absence/change alteration or in the target site. D) Overexpression or amplification in target is another mode of resistance developed by bacteria to survive in the presence of drug. E) Bacteria manage its survival by following alternative pathways to bypass the effect of drugs. F) Permeability is another challenge for drug efficacy and bacteria produces various lipids or proteins to strengthen the cell wall to escape from effect of drug by reducing its penetration into the cell. G) Various proteins produced by bacteria during different stress conditions are being excreted out by efflux pumps and these proteins helps bacteria to form biofilm by establishing a communication with surrounding cells.

2. Biofilms, efflux pumps and quorum sensing: triangular junctures of infections

2.1. Biofilms

Previous concepts successfully led to the development of antibiotics for treating both chronic and acute microbial infections [20]. However, in natural ecology, bacteria reside in dynamic and complex communities named as biofilms in spite of planktonic cells [21]. Biofilms are characterized by well-organized specialized configurations regulating intimate relationships by interactions [21]. Self-sufficient, social lifestyle of bacteria derived by their coordinated communication has led researchers to ascertain more details about these well-designed communities [22]. Microbiologists continuously keep on discovering the coordinated behaviour of microorganisms involved in biofilm formation [23]. The Staphylococcal biofilms show remarkable resistance to host immune system and other chemotherapies currently used in clinical settings [24]. These virulent microbial communities are significantly characterized by specific proteins, polysaccharides, and extracellular DNA which are supposed to be paramount components [25]. Pathogens flourish biofilm with the aid of four consecutive physiological stages (a) initial attachment, (b) accumulation of multi-layered slimy cell structures, (c) biofilm maturation and (d) dispersal (release of bacteria from the biofilm) [26]. These drug resistant microbial communities adhere to a suitable surface by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), adhesion proteins and other non -proteinaceous adhesions like polysaccharide intracellular adhesion (PIA). *Ica* operon (*icaA*, *icaD*, *icaB* and *icaC*) is responsible for synthesizing PIA whereas oleic acid, glucose, urea and glucosamine have been found to be influential factors for PIA expression [27]. Disparaging of *S. aureus* biofilm formation is also attributed by decreased level of PIA [28]. Accumulation includes cell multiplication, fusion of slime (PNAG: Poly-s (1,6)-*N*acetyl-D-glucosamine), surface proteins and extracellular DNA. Maturation leads to carbohydrate consumption and nutrition depletion that leads to emergence of persister cells (Fig. 2). These persister cells also involve into the reoccurrence of Staphylococcal infection. It was also reported that the process of biofilm dispersal is mediated by *agr* system in *S. aureus* [18].

Intracellular communication, often referred to as quorum sensing, influentially participates in biofilm organization by diverse bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus* mutants, *Aeromonas hydrophila* and many others [29]. Cell-cell communication via quorum-sensing systems affects the expression of

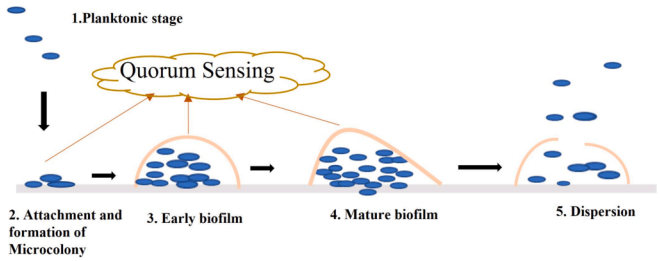

Fig. 2. Quorum sensing allows individual bacteria in colonies to coordinate and carry out colonial functions such as: sporulation, virulence, conjugation, and biofilm formation.

virulence factors in bacterial biofilms and primarily targeted to combat biofilm associated infections [30]. However, it's tough to treat the biofilm microbial territories which cause interruption in antimicrobial therapies because of their prohibitive resistance potential.

2.2. Quorum sensing

Bacteria synchronize diverse physiological activities to perpetuate communications through a unique mechanism known as quorum sensing [31,32]. Production, perception and response to tiny diffusible signalling molecules are versatile and unique features of quorum sensing [33]. Pertinently, it has long been known that quorum sensing-derived activities in any microbe need a maximum cell density in biofilm to invade the host immunity and communicate virulence [34].

S. aureus virulence factors include muster of adhesion molecules, toxins, and assorted molecules which present challenge to host immune system. Infections such as endocarditis and osteomyelitis are not caused by biofilm associated *S. aureus*. The investigation of social behaviour of *S. aureus* in biofilm represents a substantial force in inventing preventive strategies to treat such biofilm associated MRSA infections [35]. *S. aureus* quorum sensing includes a framework, unrelated to *Pseudomonas aeruginosa* AHL system and other pathogens, where auto-inducer proteins (AIP) play an important role in virulence expression [36]. Virulence is also regulated via accessory gene regulator (*agr*) system which is facilitated by virulence accelerating factors such as hemolysin, toxins, surface associated adhesions and autolysin.

The *agr* system of *S. aureus* comprises of 3 Kbp loci consisting of 4 genes (*agrA*, *agrC*, *agrD*, and *agrB*) and transcribes genes for two effector molecules of the *agr* system RNAII and RNAIII. These two divergent transcripts are driven by P2 & P3 promoters. Two-component system includes *agr*A (cytoplasmic regulator) and *agr*C (transmembrane receptor-histidine kinase) which is encoded by P2 operon. Two component systems respond to AIP encoded by *agr*D (propeptide) when processed through *agr*B (integral membrane protein). On reaching a threshold accumulation of AIP in extracellular environment, two-component system gets phosphorylated and P3 promoter initiates transcription [37]. P3 transcript RNAIII, mediate up-regulation of virulence factors secretion as well as encodes the gene for d-toxin (hld) that acts as strong surfactant and prevent microbial adhesion to the surface [38]. This indicates the dependence of biofilm dispersion on activated *agr* system (Fig. 3).

Studies about the interdependence among *agr* expression and *S. aureus* biofilm-uncovered the fact that *agr* gene is associated with extortionate morbidity and mortality among patients. Schweizer in 2011 had reported that *agr* QS system mediates biofilm dispersal in *S. aureus*. This evidence has brought QS signal transduction system forward as an inexpensive approach to combat biofilm associated MRSA infections [39]. MDR pumps, deriving transportation of quorum sensing signalling molecules also plays specific role in biofilm formation. However, "How these two junctures (QS system and Efflux pumps) interplay?" this question still needs extensive investigation.

2.3. Efflux pumps

Efflux pumps are membrane associated proteinaceous transporters of pathogens expelling out the drug causing reduction of drug

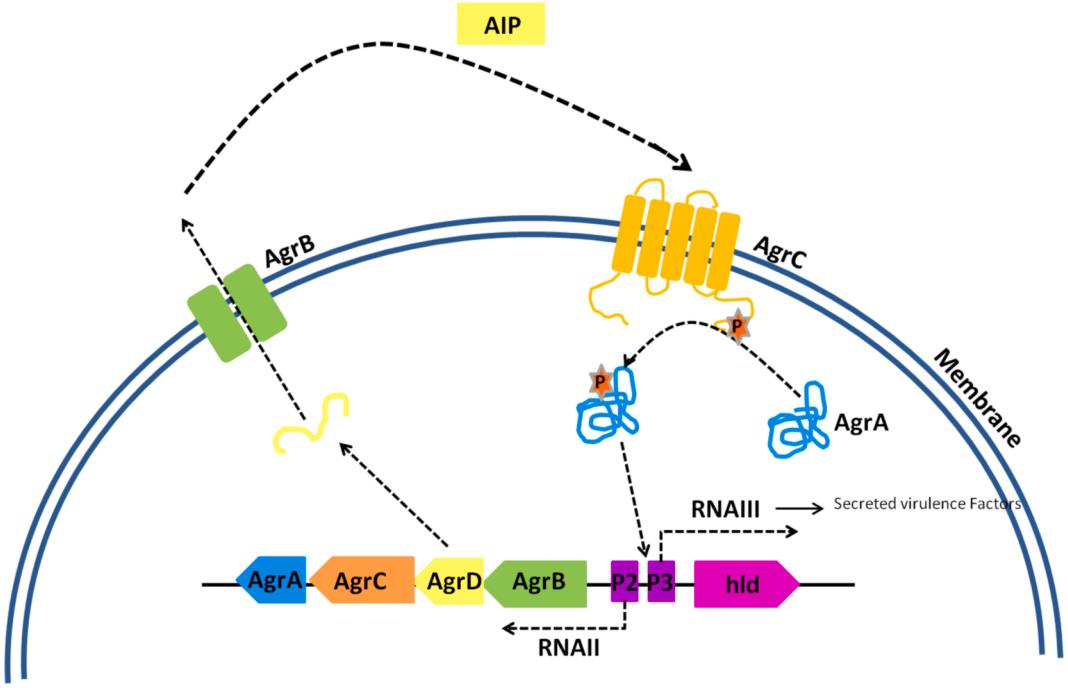

Fig. 3. Diagrammatic representation of agr system in *Staphylococcus aureus*. AgrB (integral membrane protein) involved in secretion of autoinducer proteins. AgrD (propeptide) processed by AgrB into the AIP. AgrC (transmembrane receptor-histidine kinase) is the sensor part of a two-component system agr locus. AgrA is the cytoplasmic regulator of AgrC, and acts on the P2 or P3 promoter to upregulate *agr* and RNAIII expression. RNA molecule acts on several gene transcripts and through post-transcriptional control modulate gene expression through.

accumulation and causing drug resistance (Fig. 4). Physiologically their role in bacteria is the extrusion of noxious metabolites and production of virulence determinants, providing a view that the drugs are sudden substrates of these exporters [40]. Microbial efflux system can either transport a single class of drug or antibiotic or more than one class of chemotherapeutic agents, so they named as multidrug resistance efflux pumps. Active efflux of antibiotics is one of the supreme mechanisms of drug resistance in *Staphylococcus aureus*. Though these pumps alone are not resistance conferring yet plays a major role in hampering efficient antimicrobial therapy [41]. To date, in *S. aureus* more than ten chromosomes encoded or plasmid encoded MDR efflux pumps have been reported (Table 1).

2.3.1. Classes of efflux pump

There are five families of multidrug resistance associated efflux-pumps which are: the major facilitator superfamily (MFS), the ATP binding cassette (ABC), the multidrug and toxic-compound extrusion (MATE), the resistance nodulation division (RND) and the small multi-drug resistance (SMR). These five families are further grouped in to two separate types: primary exporters i.e., ABC transporter using energy from ATP hydrolysis and secondary exporters i.e., MFS, RND, MATE, SMR families potentiated by proton gradient or sodium ion gradient [12,42]. Classification of these families is established on the basis of constituting components of these transporter pumps i.e., energy head spring that the pump use for extrusion of various components and substrate, which the pump expels. Microorganisms can exhibit efflux pumps that belong to more than one family, like *E. coli* having *Acr* efflux pump and *Pseudomonas aeruginosa* having more than one type of *Mex* efflux pump whereas both types belongs to RND family [43]. Exporters of MATE family use proton motive force as well as sodium membrane gradient for expelling out their substrate where as other families such as MFS, RND and SMR derive the energy for drug extrusion from proton motive force, like wise ABC family use ATP for their drug extrusion function [44]. In biofilm associated infections efflux pump disabling can be a helpful exercise for abolishing biofilms [45]. Heightened activity of such resistant efflux pumps can be a result of different environmental stimuli or chemical compounds and is suggested as a leading reason for potentiating the consistency of antibiotic resistant *S. aureus* [108].

Table 1
MDR efflux pumps and their identified substrates.

S. no	Efflux pump	Substrate	Family
1.	QacA	Benzalkonium chloride, dequalinium Diamidines, Biguanidines, tetraphenylphosphonium, ethidium bromide, rhodamine	MFS
2.	Smr	Cetrimide, benzalkonium chloride, ethidium bromide	SMR
3.	QacG	Benzalkonium chloride, cetyltrimethylammonium, ethidium bromide	SMR
4.	QacJ	Benzalkonium chloride, cetyltrimethyl ammonium, ethidium bromide	SMR
5.	NorA	Ciprofloxacin, norfloxacin, tetraphenylphosphonium, benzalkonium chloride, ethidium bromide, rhodamine	MFS
6.	NorB	Ciprofloxacin, norfloxacin, moxifloxacin, sparfloxacin, Tetracycline, tetraphenyl phosphonium, cetrimide ethidium bromide	MFS
7.	NorC	Ciprofloxacin and moxifloxacin rhodamine	MFS
8.	SdrM	Norfloxacin, ethidium bromide, acriflavine	MFS
9.	MdeA	Ciprofloxacin, mupirocin, norfloxacin, fusidic acid, Virginiamycin, novobiocin, tetraphenylphosphonium, benzalkonium chloride, dequalinium, ethidium bromide	MFS
10.	Tet38	Tetracyclines	MFS
11.	MepA	Fluoroquinolones (e.g. hydrophilic: ciprofloxacin, sparfloxacin, norfloxacin moxifloxacin, Tigecycline, tetraphenylphosphonium, benzalkonium chloride, cetrimide, ethidium bromide)	MATE
12.	LmrS	Erythromycin, linezolid, choramphenicol, fusidic acid kanamycin, florfenicol Trimethoprim, tetraphenylphosphonium, sodium docecyl sulphate, ethidium bromide	MFS

2.3.2. Efflux pump inhibitors (EPIs)

With the arrival of novel resistance paradigms, drug resistance in pathogens continues to heighten globally. Exploring new promising therapeutic agents or upgrading the molecular motif of conventional antibiotics that circumvent resistance mechanisms is a blueprint to withstand drug resistance. To combat this drug resistance, efflux pump inhibition appears to be a captivating approach for enhancing drug

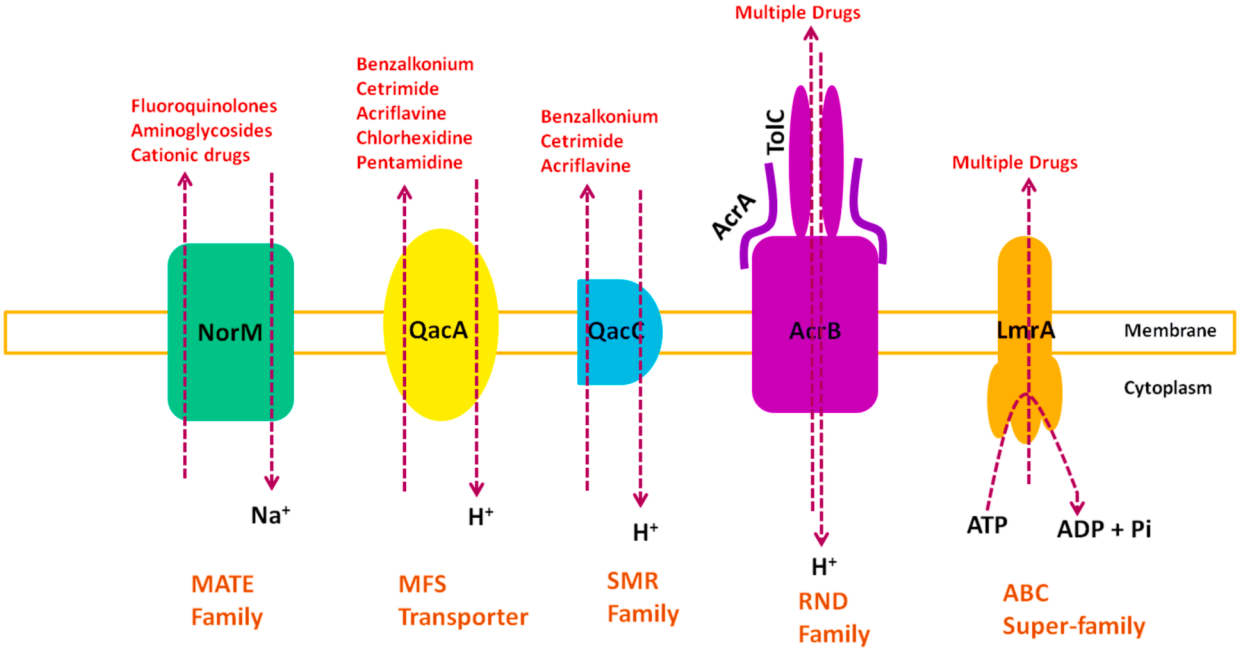

Fig. 4. Bacterial efflux pumps. The figure shows diagrammatic representation of the five structural classes of antibiotic transporters. Abbreviations: ABC: ATB-binding cassettes, MFS: major facilitator superfamily, RND: resistance-nodulation-division, SMR: small multi-drug resistance, MATE: multidrug and toxic compound extrusion.

efficacy by its accumulation inside the cell. A series of efflux pump inhibiting compounds are nominated for their vital role in interfering with efflux activity. These structurally diverse efflux interrupting derivatives act synergistically with most frequently used antibiotics, influencing their substantially increased activity. Several molecules either isolated from a natural source or synthesized/modified compounds have been used to potentiate the efficacy of antibiotics against pathogens in past few decades [46]. Huge number of EPIs has been isolated from natural sources but many of these do not find uses in the clinical settings due to menacing toxicity, as is the case with reserpine. Studies had reported number of structurally manifold inhibitors of NorA, a multi-drug transporter of *S. aureus*, that pursue an activity in a synergistic manner with the most widely used antibiotics and can increases their activity against both wild type and NorA- over-expressing *S. aureus* isolates [47]. In-vitro evaluation of numerous compounds of various groups has emphasized novel efflux pump inhibitors of NorA [47,48].

2.4. Plant alkaloid

Plant alkaloid reserpine, an antihypertensive mammalian multidrug efflux inhibitor, first isolated from the roots of *Rauwolfia vomitoria*, Afz, is active in potentiating antibiotic activity by inhibiting bacterial efflux pump. Significant reduction in the MIC of drug in combination with reserpine, a multidrug resistance efflux pump inhibitor, against MRSA was perceived [49]. Based on this precedents Mullin and his co-workers in 2004 evaluated, biricodar (VX-710) and timcodar (VX-853), two novel mammalian multiple drug efflux pump inhibitors for activity against *S. aureus* drug resistance, suggesting that VX-710 and VX-853 are evocative of a new stream of bacterial efflux inhibitors with the potential to improve the clinical efficacy of drug [50]. Stermitz et al. had identified berberine a plant alkaloid as a substrate for bacterial MDR pump [51]. In addition to these Flavonolignan and flavone inhibitors, methoxylated flavones [51] and isoflavones [52] flavones chrysosplenol-D and chrysoplenetin isolated from *Artemisia annua* (Asteraceae) [53], chalcone, catechin gallates and spinosan A and isoflavone extracted from 'smoke tree' [54], are the plant molecules which act significantly either alone or synergistically with other molecules, against NorA over-expressing *S. aureus* strains. Kaempferol rhamnoside extracted from *Persea lingue* Nees [55] and Caffeoylquinic acids isolated from *Artemisia absinthium* [56] act effectively as efflux pump inhibitor against pathogenic gram-positive bacteria. A study with *Lawsoniainermis* extract had demonstrated its bactericidal activity for *S. aureus* and other pathogenic Gram-positive and Gram-negative microbes [57] and in recent studies it has been assessed as potent efflux pump inhibitor of fluoroquinolones [58] and has profound probability for assessing further as efflux pump inhibiting potential.

Verapamil a calcium channel blocker, another P-glycoprotein inhibitor, was found to be an efficient intracellular activity modulator of ciprofloxacin and azithromycin against *Staphylococcus aureus* by increasing its cellular concentration but has reduced potency as compared to reserpine [59]. Piperine, another alkaloid extracted from *Piper longum* (black pepper) of great significance, assessed as an efficient putative multidrug inhibitor of P-glycoprotein i.e., ATP dependent drug efflux pump [60]. The nano liposomal formulations of piperine were also found effective against MRSA and act via efflux inhibitory activity [61]. This resistance obstructing molecule reduced inflammation and improved digestion as well as an array of disorders. A study with piperine expands the existing comprehensive concept of efflux inhibition in *S. aureus* that revert the drug resistance [62]. A clinical trial elucidates the potential of piperine to enhance the pharmacokinetics of Omeprazole, a proton pump inhibitor, designed as gastro-retentive microsphere by emulsification- solvent evaporation method and therefore, improving the drug potential by demolition of drug extrusion maintaining the required plasma level of drug [63]. Milk thistle seed contains a flavonolignan component, Silybin, which is effective in inhibiting the efflux system of MRSA by targeting NorA and disrupts its resistance to antibiotics [64].

2.5. Synthetic and modified inhibitors

Synthetic or modified inhibitors are also effective against drug resistant pathogenic bacteria. Evaluation of new synthetic efflux pump inhibitors that could be used in conjugation with novel antibiotics can restore the drug efficacy by suppressing the resistant phenotype that might emerge during chemotherapeutic or antibiotic treatment. Various documentations had signified progress of medicinal chemistry gadgets competent in search for efflux pump inhibitors of pathogenic gram-positive bacteria [65].

A study with ofloxacin based efflux pump inhibitor validated that chemical alteration by inserting a functional motif of an efflux pump inhibitor is a worthwhile approach in a direction to revert drug resistance. GG918 mammalian efflux hampering agents, have been assessed against efflux derived resistant *S. aureus* and found to be comparable to reserpine in intensifying the affectivity of ciprofloxacin and norfloxacin, therefore, confer enhanced drug sensitivity [66]. Butyrophenones and fractionalized 2-aryl-5-nitro-1H-indoles were also assessed as potent inhibitor of *S aureus* NorA efflux pump [67].

By modifying the flavones nucleus of known efflux pump inhibitors, a series of 2-phenyl-4(1H)-quinolone and 2-phenyl-4-hydroxyquinoline derivatives have been synthesized with dominant EPI activity [68]. Fabrication of (E)-3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid [69] and new aryl benzo[*b*]thiophene and diarylthiophene derivatives and their synergistic evaluation with ciprofloxacin against *S. aureus* results in 2-16 fold reduction in MIC of antibiotic in an ethidium bromide efflux inhibition assay. These modified EPIs in combination with ciprofloxacin display a potent inhibitory action against NorA over-expressing *Staphylococcus aureus* strain [68]. Citral amide-derivatives were evaluated as most potent efflux pumps inhibitors, that have been found to be highly effective to potentiate minimum inhibitory concentration of ciprofloxacin for *S. aureus* over expressing NorA pump as compared to well-known EPIs like verapamil, reserpine, piperine etc. [31,59]. Phenylpiperidine selective serotonin reuptake inhibitors (PSSRIs) are capable of obstructing the performance of some MDR efflux pumps. Paroxetine was one of the first identified PSSRI that inhibits both NorA and MepA efflux pumps. On this basis, additional chemical modifications were performed in order to find out more potent EPIs [66]. Piperine analogs were synthesized and their quantitative structure activity relationship (QSAR) was studied and further evaluated for its efflux pump inhibition activity. Ethidium bromide assay confirm their potency to inhibit efflux pump activity and surprisingly they were found to be more potent than other EPIs such as reserpine and verapamil [70]. Ferulic acid derivatives 4-((*E*)-2-(diethylcarbamoyl)vinyl)-2-methoxyphenyl acetate and (E)-methyl 3-(4-((p-tolylcarbamoyl)methoxy)-3-methoxyphenyl) acrylate have been screened for their putative efflux pump inhibitory activity and were found to be effective by inhibiting Nor type efflux pumps by both *in-silico* docking studies and in-vitro in clinical isolates of MRSA. The latter one also showed synergistic activity with Ciprofloxacin [71].

Cinnamaldehyde derivatives were also found to be effective against NorA overexpressing *S. aureus* [72]. These synthetic and modified EPIs provide broader spectrum and are highly potent inhibitors of *S. aureus* MDR efflux pumps.

Previous studies have analyzed a synergistic effect of a proton pump inhibitor omeprazole and an antibiotic in human clinical trials for the growth of human tumor cells [73]. Further three β-lactam antibiotics carbapenem, meropenem, and imipenem have been combined with clavulanic acid in clinical trial and explored for its efficacy to restore the drug activity against *Mycobacterium tuberculosis*. This recent study had demonstrated that the clavulanic acid in synergy with meropenem exhibits bactericidal bustle [74].

3. Relation between biofilm, efflux pump and quorum sensing

'Biofilms' as exceptionally drug resistant microbial territories have highly energetic efflux pumps which extrude antibiotics as well as transport cell signalling molecules. So, efflux pump prohibition by EPI can also simultaneously interrupt biofilm formation and cell signalling (QS) pathway [75]. Resistance to quorum quenching molecules is simultaneous to unregulated efflux mechanism. On the other hand, efflux pump inhibiting agents also interact with quorum sensing system. Many studies demonstrated that NorA is core part of *S. aureus* genome and its expression were induced in many *S. aureus* strains [76]. Involvement of NorA efflux pump in the biofilm formation was tested in SA 199B strain (*Nor*A overexpressing strain) and it was found that using NorA (EPI) inhibitors, biofilm formation was also inhibited [77]. Moreover, [70], explained that over-expression of NorA increase the intracellular invasion of *S. aureus* in macrophages.

These evidences conclude that efflux pumps and quorum sensing system simultaneously derive each other and hindrance with one will upregulate or downregulate another one. In previous studies, it had been shown that multidrug efflux pumps, biofilm formation and quorum sensing are significantly related to each other [78]. Mechanisms that play an important role in the antimicrobial resistance acquired by certain micro-organisms within biofilms include the presence/over expression of several gene-encoding efflux pumps. Studies showed that plasmid exchanges at a very higher rate in biofilms compare to planktonic microbes, increasing the chances of developing resistance naturally as well as antibiotic-induced [79].

It is also been demonstrated that inactivation of bacterial efflux pumps led to deficiencies in secretion of quorum sensing signals and biofilm production. The signals of quorum sensing produced by bacterium to inhibit competing bacterial growth can also be considered as foreign molecules and expelled out promoting biofilm formation. Efflux pump functions are required for responding to quorum sensing signals as the secretion of these signals are dependent on bacterial main efflux pump system and any noxious agent produced internally are expelled out through them [80]. Furthermore, in mixed cultures efflux pumps acts as a defense system when quorum sensing inhibitors are utilized and do not allow the transfer of inapt message from other species. Therefore, efflux pumps and quorum sensing affect the functions of each other in addition to their own activity in response to extracellular environment.

3.1. Quorum sensing inhibitors

Inhibitors of quorum sensing are also known as quorum quenchers. Quorum sensing inhibition by enzymatic degradation of AIPs is emerging as an important strategy to address antibiotic resistance. AIPs can be different molecules like oligosaccharides ranging from low to high molecular weight, of which most common are N-acyl homoserine lactones (AHLs). Several organisms produce enzymes like laconases which can degrade AHL and inhibit numerous phases of quorum sensing controlled pathogenicity. A lactonase isolated from *B. thuringiensis* diminishes *Erwinia carotova* pathogenesis by quorum sensing inhibition [81]. Porcine acylase-I had been demonstrated to degrade AHLs of *Microbacterium* and *Pseudomonas* spp. and also reduces their biofilm formation [82]. However, the therapeutic application of these enzymes is limited by the fact that they can also hydrolyze some non-AHL molecules and can be toxic to the host, therefore careful examinations are required.

The *S. aureus* virulence can also be constrained by inhibiting quorum sensing (Table 2). Various toxins like delta-toxin, *Staphylococcal* enterotoxin C and Panton-Valentine leukocidin whose production itself is regulated by quorum sensing are potent enough to kill this pathogen however less selective pressure must be experienced by the bacteria to drive through this route for development of resistance against conventional antibiotics [83]. Wealth of research data had been devoted to the discovery of quorum quenchers as an alternative strategy for anti-

Table 2
Molecules found to interfere with *Staphylococcus aureus* quorum sensing.

Sr. no	Molecule	In vitro effects	Mechanism of action
1	(5Z)-4-bromo-5-(bromo-nethylene)-3-butyl-2(5H)-furanone	Inhibits RNAIII expression, enhances biofilm formation	Leads to increased PIA production enhancing biofilm formation
2	(*S*)-3-Decanoyl- 5-(2-hydroxyethyl)tetramic acid (5-HE-C10-TMA 5)	Inhibits P3 activity	Prevents AgrC receptor dimerization or interference with the interactions between AgrC and AgrA (Not Confirmed)
3	2-(4-Methyl-phenyl)-1,3-thiazole-4-carboxylic acid	Unknown	Interferes with AgrA DNA binding activity
4	3-Dodecanoyltetronic acid (C12-TOA, 16)	Inhibits P3 activity	Prevents AgrC receptor dimerization or interference with the interactions between AgrC and AgrA (Not Confirmed)
5	3-Tetradecanoyltetronic acid (C14-TOA 17)	Inhibits P3 activity	Prevents AgrC receptor dimerization or interference with the interactions between AgrC and AgrA (Not Confirmed)
6	9H-Xanthene-9-carboxylic acid	Unknown	Interferes with AgrA DNA binding activity
7	Alpha-cyperone	Inhibits hla and agrA expression	Unknown
8	Ambuic acid	Inhibit hemolytic activity, RNAIII transcription, AIP-I production	Prevents AIP biosynthesis (Not Confirmed)
9	Avellanin	Unknown	From sponges; functions via competitive inhibition of AgrC
10	Chromatography eluates	Inhibits P3 activity and hemolytic activity	Unknown
11	Cochinmicin	Unknown	From actinomycetes, functions via competitive inhibition of AgrC
12	Cyclic dipeptides: cyclo (L-Phe-L-Pro) and cyclo (L-Tyr-L-Pro)	Inhibits TSST-1, downregulates RNAII and RNAIII, SarA and SaeRS	Competitive inhibitors of AIP-mediated P3 promoter activation, affecting sarA and saeRS
13	Furanone 202	Inhibits biofilm formation by *S. epidermidis*	Unknown
14	Licochalcone A	Inhibits sea, seb and agrA expression	Unknown
15	Luteolin	Inhibits hla and agrA expression	Unknown
16	N-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL)	Inhibits production of exotoxins and fibronectin-binding proteins but enhances	May affect SarA functionality; potentially antagonizes membrane-associated

(*continued on next page*)

Table 2 (*continued*)

Sr. no	Molecule	In vitro effects	Mechanism of action
		protein A expression	regulators (e.g., sensor components of ArlRS, SaeRS and SsAB)
17	Naphtalene and biaryl compounds	Inhibits hla, psmo, RNAIII expression	Targets AgrA (N) phosphoryl binding pocket; may interfere with AgrA DNA binding activity
18	Naringenin	Unknown	Reduces agrA and hla transcript levels
19	Oxacillin	Pleiotropic effect on toxin expression	Unknown
20	PLNA34	Unknown	Specifically and significantly reduces agrA mRNA levels
21	Polyhydroxyanthraquinones	Inhibits hld expression and P3 activity	Unknown
22	RIP	Unknown	Inhibits synthesis of agr transcripts RNAII and RNAIII
23	RIP derivatives (16P-AC)	Unknown	Inhibits the expression of biofilm-related genes in *S. aureus*
24	RIP-V, RIP-L	Unknown	Down-regulates RNAIII expression and -hemolysin production
25	Savarin	Inhibits RNAIII expression (only in *S. aureus*, not *S. epidermidis*), psma promoter	Interferes with AgrA DNA binding activity
26	Solonamide A/B, Ngercheumicins	Reduces hla, rnalll and increases spa transcripts, reduces *S. aureus* mediated killing of human neutrophils	Competitive inhibitor of AgrC
27	*Staphylococcus schleiferi*	Unknown	Functions by cross-inhibition of the pathogenic agr system
28	Thymol	Inhibits sea, seb, hla and agrA expression	Unknown
29	Truncated AIP-I, II, III,	Unknown	Inhibits autoinduction of all four *S. aureus* subgroups
30	Vaccination with hapten-linked AIP IV	Unknown	Provides passive immunity and reduces the pathology of agr IV strains
31	w-Hydroxyemodin (OHM)	Unknown	Prevents agr activity by all four *S. aureus* agr group strains

virulence of MRSA. These studies have been targeted to identify synthetic, natural as well as peptide quorum quenchers.

Synthetic quorum quenchers can be small molecules or antisense nucleic acids. Biaryl hydroxyketones were demonstrated to disrupt AgrA-P3 interaction with less production of virulence factor [84]. A combinatorial library of biaryl hydroxyketone had been investigated to find out the most efficacious compound. Of these some showed much effectiveness with 98% inhibition of hemolysis in rabbit erythrocyte by MRSA at 1 μg/ml concentration or against MRSA infection model of Galleria mellonella larva [85]. Savirin another synthetic molecule was also found to inhibit the activation of *agr*-P3 in *S. aureus* with reduced production of RNAIII [36]. It also inhibited the transcription of agr-specific major virulence factors for skin and soft tissue infections (SSTIs) (George [86]). A study showed oxacillin (synthetic antibiotic) employed at sub-MIC, was effectively down regulate the *agr* system and attenuate MRSA virulence but increases MRSA methicillin resistance [87]. Two antisense oligonucleotides have been synthesized targeting most conserved *agr*A. These oligonucleotides were modified ribonucleotides (RNA) where an extra bridge was present between 4′ carbon and 2′ oxygen of ribose sugar and were thus called as locked nucleic acid (LNA). They were conjugated with (KFF) 3K, a cell-penetrating peptide and were found to reduce transcription of *agr*A and RNAIII [88]. RNAIII-inhibiting peptide (RIP) is an endogenously produced peptide by *S. aureus* which can also inhibit quorum sensing. RIP derivatives like a tetrapeptide FS10 have been screened to break quorum sensing in *S. aureus* but were however found to be less effective [89].

Natural products isolated from both plants and fungi have been screened for their inhibitory effect on quorum sensing in *S. aureus* ([90,91]) (Fig. 5). Xenobiotics derived from fungus were found to potentiate the activities of antibiotics. Polyhydroxy anthraquinones isolated from fungus like *Penicillium restrictum* were found to be very effective [92]. ω-hydroxyemodin (OHM) has been shown to potentially inhibit *agr* signalling in MRSA. Molecular docking studies demonstrated that OHM inhibits binding of *agr* AC to *agr* promoter and reduced expression of all four types of *agr* [93]. In-vivo studies revealed that OHM treatment in mouse, whose skin was infected with MRSA, reduced the bacterial burden in abscess, reduced the size of ulcer and also increased the in-vitro clearance of bacteria by both human polymorphonuclear leukocytes (PMNs) and mouse macrophages. Treatment of OHM in *agr* null *S. aureus*, showed no effect highlighting that *agr* system is the target of OHM [94]. Another metabolite of fungal origin: ambuic acid was found to inhibit AIP synthesis by targeting *agr*B. Furthermore, it inhibited production of RNAIII and alpha-toxin by MRSA in dose-dependent manner. A single intradermal 25-μg injection of ambuic acid completely abolished the formation of skin ulcers in mouse model with MRSA infected skin [95]. Leaf extract of *Castanea sativa* and berry extract of *Schinus terebinthifolia*, both were found to inhibit *agr* expression (IC_{50} ranges 1.52-32 μg/ml) and production of delta-toxin by *S. aureus*. They also reduced morbidity and abolished skin ulcer formation on MRSA infected mouse models [96].

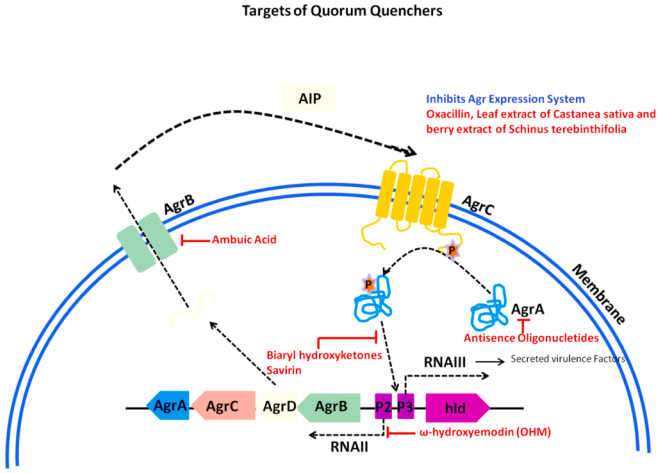

Fig. 5. Targets of Quorum quenching during bacterial biofilm formation.

3.2. Efflux pump inhibitors and quorum sensing inhibition

As multidrug resistant biofilm associated MRSA bacteria have been and still is a current therapeutic problem, it is of great importance to analyze the recent progress in the search for new tools to combat such resistant strains [25]. Drug extrusion, impermeability of drug inside the biofilm matrix, quorum sensing and virulence factors such as bacterial adhesion factors & toxin are prime resistance contributors in biofilms. Efflux pumps are involved in antibiotic transport as well as cell signalling in quorum sensing. They have been found to be AIP secretor and therefore appear to be an important target for antimicrobial therapies [97]. Molecules that target this track way could be applicable as new therapeutic agent. The existence of anti- resistance weapons such as an efflux pump inhibitor [55] and quorum sensing inhibitor [98] with their impact on biofilm inhibition, has now taken fully into account for the selection of novel antimicrobial therapies against biofilm derived infections. For this reason, correlation of efflux pump inhibitors and quorum sensing system can possibly be a significant approach to abolish biofilms [108]. These groups of molecules represent the most significant antibacterial arsenals in order to restore the drug efficacy (efflux pump inhibition) and biofilm destruction (Table 3).

A cooperative endeavour is underway to find out a productive molecule that may revert the mechanism of biofilm formation. Previously interaction of quorum sensing signals and efflux pump inhibitors in biofilm associated infections has been reported [99]. Some compounds which are having inhibitory activity against efflux pump has also been found interactive to quorum sensing signalling pathway which results in suppression of virulence gene expression [99,100]. Most recently, seleno compounds and benzene derivative selenoester moieties showed strong inhibition of bacterial *Acr*-AB and *Tol*C system in *E. coli* AG 100 strain [101]. These results can be exploited for rational drug designing to inhibit the formation of biofilm in MRSA derived infections and to reduce emergence of antibiotic resistance.

Though therapeutic potential of such vital and efficient compounds is very much attractive and should inspire a huge research but unfortunately to date, no EPI has reached the clinical settings for anti-infective purpose antagonistic toward bacterial infections in human beings or veterinary settings. Likewise, *agr* dysfunction with the aid of EPI is still under experimentation. Moreover, we are lacking with the evidences that how virulence factors are regulated during *agr* dysfunction.

4. Conclusions and future prospect

Over the last few decades resistance against conventional antibiotics have been becoming a major threat for antibacterial chemotherapies employed for treatment of numerous recalcitrant life-threatening infections. Drug efflux, a leading contributor in resistance mechanism has been gaining a great attraction by researchers for combating drug resistance. In this concern inhibitors of such membrane associated diligent transporters have been observed to possess a promising role to restore the drug accumulation leading to the increased efficacy of the drug. Efflux pump inhibitors suppress resistance phenotype of pathogens, rehabilitate drug efficacy and hence proved to be an innovative approach for defeating pathogens. Efflux pump inhibitor approaches can be applied to pathogenic *S. aureus* and MRSA with a goal of improving the clinical performance of antibacterial therapy.

The hallmark of MRSA infection is its ability to form slime matrix encased “smart microbial communities” with modified genetic program that has the ability to cope up with adverse conditions. Bacteria in biofilms with manipulated and more protected genetic expressions are less susceptible to antimicrobial agents as compared to planktonic cells because of drug penetration limitation, drug extrusion, decreased oxygen and nutrient, decreased growth rates and metabolism, quorum sensing and formation of persister cells. We are hindered by the fact that how these microbial communities can be made susceptible to antibiotic therapies. It is very important to find out therapies that can eradicate antibiotic resistant biofilms and make these pathogenic cell clusters susceptible to treatments. Anti-biofilm therapies are an integral approach for the infections which faces difficulties to treat and can enhance the spectrum of drug activity as well as reduce the emergence of resistance.

Table 3
List and structure of molecules having efflux pump and biofilm inhibition activity.

Name	Reference
Chlorpromazine	Alav et al. [75], Chan et al. [102,103]
1-(1-Napthylmethyl) piperazine (NMP)	Alav et al. [75], Kvist et al. [104]
CCCP	Mahamoud et al. [105]
Phenylalaninearginine b-naphthylamide (PABN)	Chan et al. [103], Liu et al. [106]
4′,5′-*O*-dicaffeoylquinic acid	Fiamegos et al. [56]
Thioridazine	Kvist et al. [104], Nzakizwanayo et al. [107]
Reserpine	Alav et al. [75]
Piperine	Mirza et al. [62]

Efflux pump inhibitors enlightened a new way for their coming clinical application for life threatening bacterial infections. These drug adjuvants have shown their potential to reduce antibiotic resistance and abolish biofilm formation. Interactions of efflux pump inhibitor and quorum sensing cell signalling route have surely been shown as a new way to suppress virulence by interrupting biofilm formation. The expected fruitful outcomes for biofilm associated MDR infections significantly can provide emerging therapeutic trends in preventive therapy against recalcitrant MRSA infections. Efflux pumps and quorum sensing in bacteria holds a well-established association. In spite of all the above information, the interaction of EPI and quorum sensing inhibitors still prone to vast investigation for expected fruitful results. Specifically, an additional experimental analysis should be focused on quorum sensing evaluation for developing efficacious biofilm abolishing strategies to potentiate drug bustle.

Ethics approval and consent to participate

Not applicable.

Consent to publish

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CRediT authorship contribution statement

SS (Sandeep Sharma) designed and reformed the concept, SS and BK wrote the manuscript draft, JG created the figures and editing. Sar S (Sarika Sharma), DS and SS (Sandeep Sharma) edited the draft and finalized the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

There is no competing or conflict of interest among the authors.

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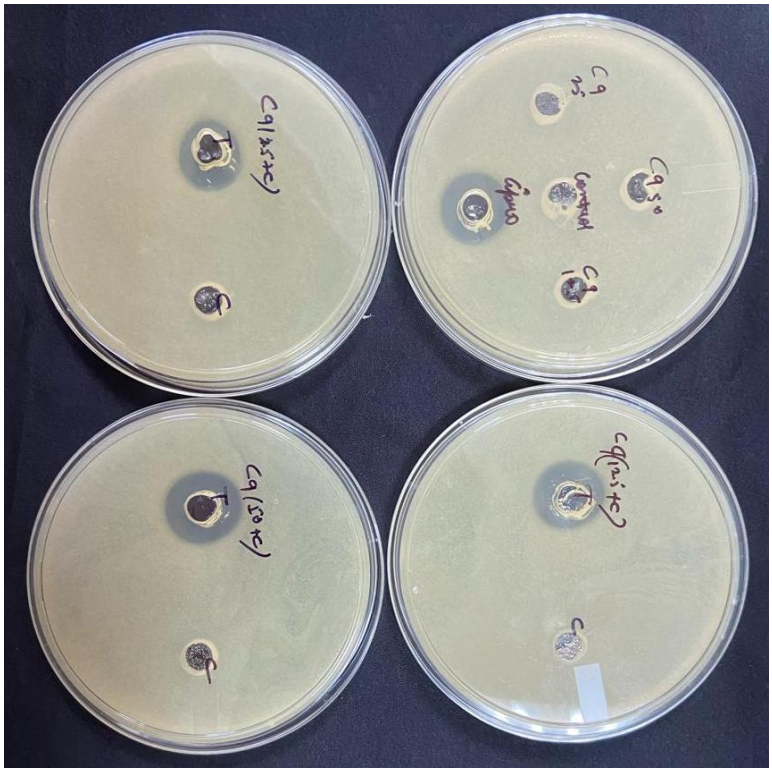
Table 1: In vitro checkerboard study to screen Chalcones as Antibiotic Modulators at single concentration (25µg/ml) in combination with ciprofloxacin against *S. aureus* SA 1199B.

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



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Original Article

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

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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 (HZT) 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 1×10^6 CFU/ml. Separately, ciprofloxacin at a concentration of 2 mg/l [1/4×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×, 8×, and 16× 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,

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

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 × 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. aurues:* 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: CAGCTATTAAACCTGTCACACC	62 °C
	R: AGCTATTAAACCTGTCACACCAG	
agrA	F: TGTCTACAAAGTTGCAGCGATG	62 °C
	R: TAAATGGGCAATGAGTCTGTGAG	

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 × 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, 1µg 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 × 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.

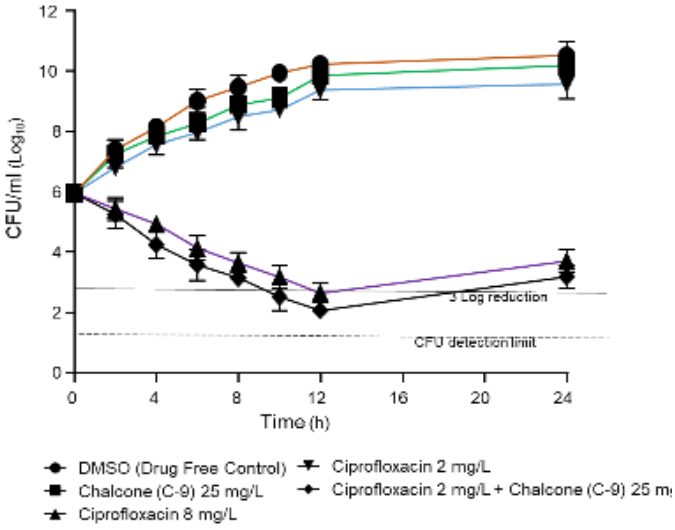

Fig. 1: Time–kill curves of *S. aureus* SA-1199B demonstrating the bactericidal action of ciprofloxacin (1/4× MIC, 2 mg/l) combined with chalcone C9 (25 mg/l). The individual time point was the average $\log_{10}$+SD of the three readings

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 × 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_{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 × MIC (0.5 mg/l)	4 × MIC (1.0 mg/l)	8 × MIC (2.0 mg/l)	16 × MIC (4.0 mg/l)
0	4×10^{-8}	1×10^{-8}	$2 \times <10^{-9}$	$<10^{-9}$
12.5	8×10^{-8}	4.5×10^{-9}	$<10^{-9}$	$<10^{-9}$
25	2.0×10^{-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 ($MBIC_{50}$) 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, $MBIC_{50}$ of ciprofloxacin enhanced by 13%, whereas with 50 mg/l concentration of C9, most pronounced reduction in $MBIC_{50}$ 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)		
	¼ ×MIC(2 mg/l)	½ ×MIC(4 mg/l)	MIC(8 mg/l)
Without C-9	0.4±0.1	1.25±0.5	1.45±0.5
C-9(25 mg/l)	1.0±0.3	1.45±1	2.5±0.25

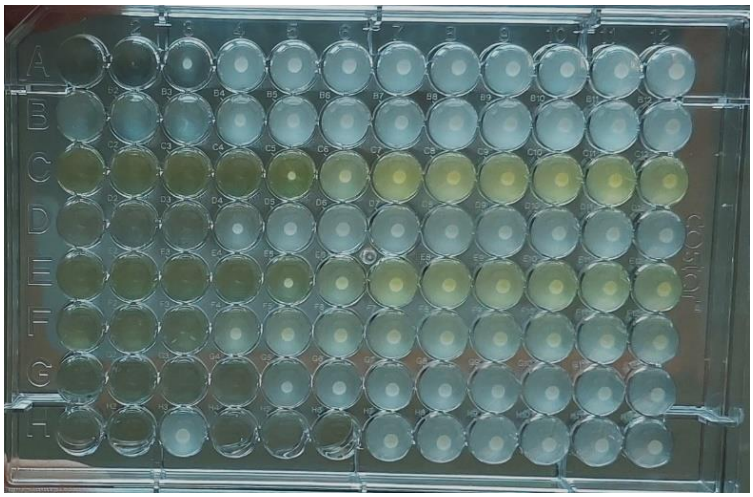

Fig. 2: 96 well plate showing a synergistic effect

Table 4: $MBIC_{50}$ of ciprofloxacin against *S. aureus* ATCC 29213 when used in combination with Chalcone C9

Chalcone C-9 (mg/l)	$MBIC_{50}$ of ciprofloxacin against *S. aureus* ATCC 29213 in combination with C9
0	0.33
12.5	0.32
25	0.29
50	0.14

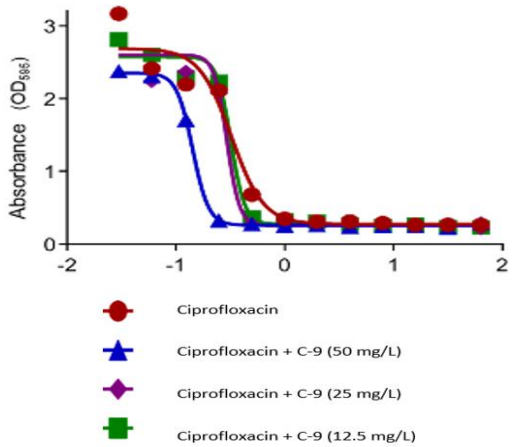

Fig. 3: MBIC$_{50}$ of ciprofloxacin against *S. aureus* ATCC 29213 when used in combination with chalcone C9 (represented in terms of OD)

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 × 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 × 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).

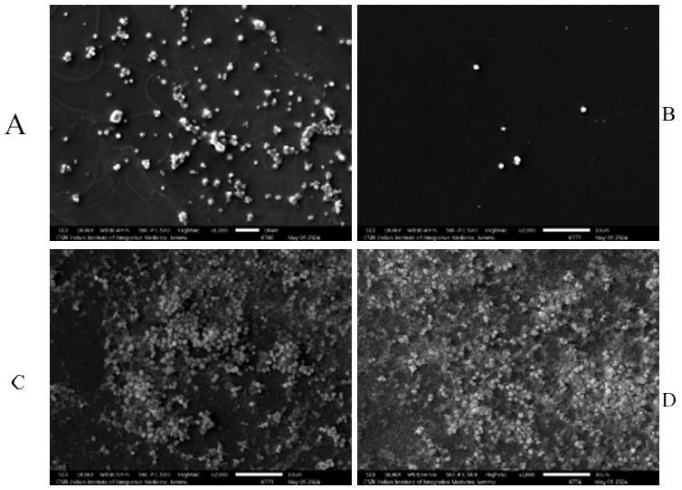

Fig. 4: SEM images demonstrating the antibiofilm effect induced on the *S. aureus* ATCC 1199B treated with ciprofloxacin at 2 mg/l, (A)*S. aureus* ATCC 1199B treated with ciprofloxacin at 1/4×MIC(2 mg/l)(Reduced Biofilm Cell mass), (B) Combination[Ciprofloxacin(2 mg/l)+ChalconeC9(25 mg/l)](Significant Biofilm Inhibition), (C) Chalcone C9 at 25 mg/l alone(No Noticeable effect), (D) Untreated controlled (Dense biofilm formation)

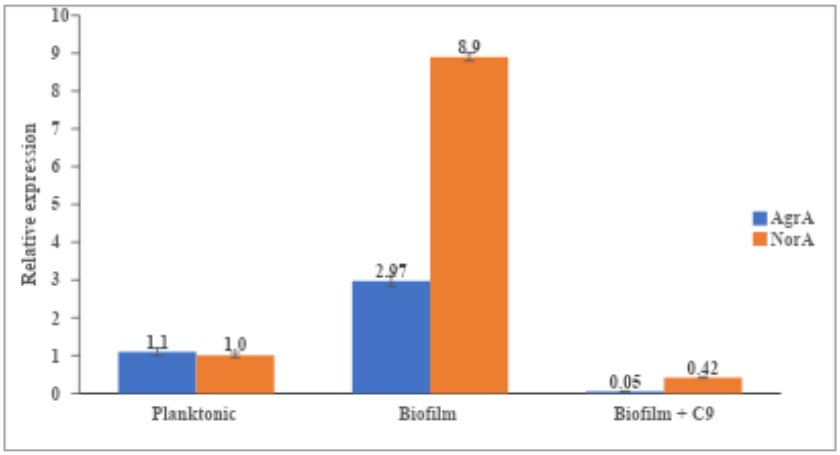

Fig. 5: Biofilm induced co-overexpression of AgrA and NorA and impact of chalcones on expression of both the genes

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 *Agr*A 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 NorAwere 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 π-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-π interaction was observed between the aryl ring and Lys294 (fig. 6d and 6c).

Table 5: Docking results using Autodock vina

S. No.	Molecule	NorA		Agr	
		Binding residues	**Binding affinity (kcal/mol)**	**Binding residues**	**Binding affinity (kcal/mol)**
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.

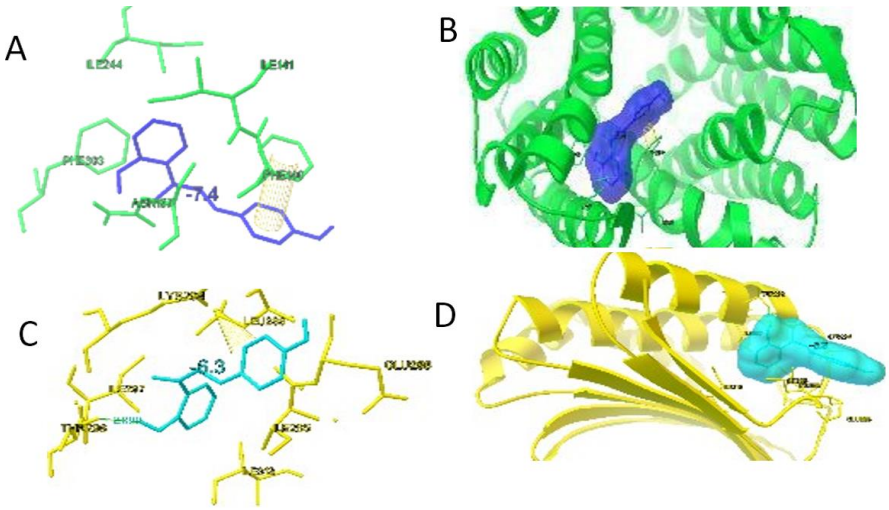

Fig. 6: Binding interaction of C9 (a) ball and stick model NorA (green), C9 (blue), (b) ribbon structure NorA(green), C9 (blue), (c) ball and stick model Agr(yellow), C9 (blue), (d) ribbon structure Agr(yellow), C9 (blue)

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 α, β-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 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 ¼ × MIC concentration of ciprofloxacin induced mild reduction in biofilm cell mass. However, in case combination of C9 (25 mg/l) with ciprofloxacin (1/4 × 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 π-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 – π 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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AUTHORS CONTRIBUTIONS

The inspiration for the topic is given by Dr. Sandeep Sharma, Dr. Nitin Pal Kalia, Dr. Sarika Sharma, Dr. Gopal Lal Kathik and Dr. Ashish Suttee. The framework was designed by Bhawandeep Kaur, Dr. Sandeep Sharma and Dr. Nitin Pal Kalia. A literature survey was done by Bhawandeep Kaur, Suman K Jana and Dr. Sandeep Sharma.

CONFLICT OF INTERESTS

Declared none

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LIST OF CONFERENCES

- Presented a poster presentation entitled **Inhibition of Efflux Pump and Biofilm Formation in *S. aureus*** at **International Conference on Emerging Trends in Biosciences and Chemical Technology -2022, (ETBCT-2022, SMVDU, December 3-5,2022)** at Shri Mata Vaishno Devi University, Katra, J&K which was held in collaboration with CSIR-IIM Jammu and BRSI, India.

- Presented a poster presentation entitled **Chalcones as efflux pump and biofilm inhibitors against *S. aureus* in 2nd International conference on Biotechnology & Bioinformatics (ICBAB-2023) From 11-13 July, 2023** at Jaypee University of Information and Technology, Waknaghat, H.P.

- Presented a poster presentation entitled **Screening of Chalcones as Efflux and Biofilm Inhibitors Against Bacterial Pathogen** in **International Conference on Recent Trends in Biomedical Sciences (RTBS-2023)** organized by Department of Medical Laboratory Sciences **from 6th -7th October 2023** at Lovely Professional University, Punjab, India.

CSIR-IIIM

International Conference on Emerging Trends in Biosciences and Chemical Technology-2022

December 3-5, 2022

Organised by

School of Biotechnology, Shri Mata Vaishno Devi University, Katra

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. BHAWANDEEP KAUR

has presented ✓ Poster / Oral Presentation entitled Inhibition of Efflux Pump and Biofilm formation in S. aureus at

International Conference on Emerging Trends in Biosciences and Chemical Technology (ETBCT-2022) held at Shri Mata Vaishno Devi University, Katra, J&K in collaboration with CSIR-IIM Jammu and BRSI, India.

Dr. Indu Bhushan	Dr. Asha Chaubey	Prof. Ashok Pandey	Dr. Ratna Chandra
Convener	Convener	Conference Chair	Organizing Secretary
ETBCT-2022	ETBCT-2022	ETBCT-2022	ETBCT-2022

Department of Biotechnology & Bioinformatics

Certificate of Participation

Awarded to

Bhawandeep Kaur

for presentation (Oral/Poster) in 2nd International Conference on "Biotechnology & Bioinformatics (ICBAB-2023)" from 11-13 July, 2023 at Jaypee University of Information and Technology, Waknaghat, H.P.

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Jaypee University of Information Technology, Solan, Himachal Pradesh

International Conference on Recent Trends in Biomedical Sciences (RTBS-2023)

Certificate of Achievement

This is to certify that Prof./Dr./Mr./Ms. Bhawandeep Kaur of Lovely Professional University has presented Oral/Poster entitled Screening of chalcones as efflux pump and biofilm inhibitors against Bacterial pathogen. In International Conference on Recent Trends in Biomedical Sciences (RTBS-2023) organized by Department of Medical Laboratory Sciences from 6th-7th October 2023 at Lovely Professional University, Punjab, India.

Prof (Dr.) Sandeep Sharma
Covenor/Organizing Secretary
RTBS2023

Prof (Dr.) Monica Gulati
Chairperson
RTBS-2023