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Synergistic Activity of Curcumin Against the Biofilm forming Staphylococcus aureus Isolated from Poultry Chicken

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Abstract: ***The synergistic effects of curcumin on strain of biofilm-producing Staphylococcus aureus isolates from poultry chickens are examined in this study. The study employs a combination of microbiological techniques to assess the ability of curcumin to inhibit biofilm formation and degrade established biofilm. Biofilm are a major problem in both veterinary and clinical contexts because they cause recurring infections and are resistant to antibiotic therapy. The study utilizes a range of microbiological assays to determine the anti-biofilm activity and the disruption of mature biofilms by curcumin. Findings show that curcumin has a significant inhibitory action on the growth and biofilm formation of Staphylococcus aureus, especially when combined with conventional antibiotics. These results suggest that curcumin could be a useful complement to conventional antibiotic therapy for managing biofilm infections in poultry, potentially resulting in improved animal health and safer meat. Certainly, there is a clear need for further study on how curcumin functions and how to employ it in the clinic.More research is needed to understand how curcumin does this and how to use it in real-world situations.***
Keywords: Biofilm, Curcumin, Inhibition, Antibiotic, Anti-biofilm activity.

I. INTRODUCTION

Antimicrobials are substances that inhibit the growth of bacteria and vital cellular functions thereby causing rapid growth bacterial cell death. Antibiotics have been over-used by humans, not only by direct consumption but also through their extensive use in animal feed. Consequently, antibiotic resistance in several pathogenic organisms has occurred, causing a global health issue. The American centre for disease control for disease control and prevention has reported that more than two million diseases have been caused by microorganisms that are resistant to one or more class of antibiotics. The high mortality rate is due to the fact that these relate infections have become extremely difficult to treat, where example include extensively drug-resistant tuberculosis (XDR-TB) and community associated methicillin-resistant *Staphylococcus aureus* (MRSA).

Staphylococcus aureus, particularly methicillin-resistant strains (MRSA), poses a significant public health risk, especially through its reservoirs in poultry. These bacteria can contaminate meat products, leading to foodborne infections among consumers. Studies have detected *S.aureus* in various poultry samples, indicating a widespread presence that can contribute to antibiotic-resistant infections in humans. The multi-drug resistance often exhibited by these strains reflects a growing challenge for public health, with many commercial antibiotics losing efficacy against them. As highlighted in recent research, the presence of MRSA in food animals, including poultry, raises concerns regarding transmission pathways to humans, potentially increasing the incidence of resistant infections *(Bhargava et al.1975).*

A biofilm is a complex network of closely packed, membrane-like structures created by bacteria that attach to a surface and release a matrix of polysaccharides, fibrin, lipid proteins, and other substances *(Xu et al., 2022).*Bacterial biofilms enable survival in hostile conditions and frequently exhibit resistance to drugs and human defenses, therefore playing a role in developing persistent illnesses *(Kim Y. et al., 2022).*

In addition to being resistant to β-lactam antibiotics, methicillin-resistant *S. aure*us (MRSA) strains frequently exhibit resistance to other widely used antibiotic groups, including aminoglycosides, fluoroquinolones, macrolides, tetracycline, and chloramphenicol (Kot et al., 2020). Recent studies have shown that certain natural chemicals, including curcumin, cinnamaldehyde, eugenol, carvacrol, and thymol, not only prevent the production of biofilms but also remove fully developed biofilm formations *(Doke et al., 2014*; *Rangel et al., 2018).* Moreover, the concurrent administration of antibacterial medications and various natural compounds can serve as a highly efficient approach to addressing prevalent bacterial infections owing to its heightened potency and efficacy, diminished drug toxicity, optimized dosages, and decreased probability of acquiring resistance strains *(Ushimaru et al., 2012).*

Therefore, this study focuses on the interactions between curcumin and biofilm of *S. aureus*, as well as different pharmacological platforms utilized to enhance the effectiveness of natural compounds against this bacterial biofilm community.

A. CURCUMIN

Figure 1: Curcumin

Curcumin is an orange-yellow pigment found in the rhizome of *Curcuma longa (Borra et al., 2014).* Curcumin exhibits a wide range of therapeutic effects, including antimicrobial, and antiseptic activities *(Prakash et al., 2011; Kunnumakkara et al., 2017; Wang H. et al., 2019).* Curcumin has been shown in recent research to effectively suppress the development of biofilms, particularly in Gram-positive bacteria *(Moshe et al., 2011; Batista de Andrade Neto et al., 2021; Alqahtani et al., 2024).* An *in vitro* study demonstrated that a 100 µg/mL concentration of curcumin successfully inhibits the development of *S. aureus* biofilm *(Moshe et al., 2011).* Noteworthy, curcumin has the potential to disrupt the structural integrity of the bacterial cell membrane before the initial stages of biofilm development, which include the attachment of cells to a surface, the assembly of cells to form micro colonies, and the maturation of the biofilm into a cohesive structure *(Tan et al., 2019; Pamukçu et al., 2022).* Additionally, curcumin can interfere with the planktonic cells and further inhibit biofilm reformation *(Tan et al., 2019).*

The previously published research findings indicated that the curcumin concentration needed to suppress biofilm formation was far lower than the dosage needed to suppress *S. aureus* growth. Accordingly, the authors proposed that the inhibitory effect of curcumin on biofilm formation is attributed to its ability to impede the process of biofilm formation itself rather than its bactericidal properties *(Moshe et al., 2011).* Therefore, curcumin has shown good potential by targeting bacterial adhesion and preventing biofilm formation.

Additionally, curcumin can reduce the expression of some genes associated with QS and enhance the proliferation of *biofilms (Khaleghian et al., 2023;*

Curcumin has several ways to disrupt *S. aureus* biofilms, including inhibiting sortase A activity, interfering with attachment, changing bacterial surface properties, interacting with biofilm matrix, and inducing oxidative stress. Together, these factors diminish the ability of *S.aureus* to form or protect its biofilm communities, making them more sensitive to host defences and traditional antimicrobial treatments.

To combat this threat, exploring natural products such as curcumin is essential, as they may enhance the effectiveness of existing antibiotics and help mitigate the growing problem of resistant strains. Understanding these dynamics is crucial for developing better infection control strategies*(Sharma et al., 2023).*

B. BIOFILM

Biofilm are intricate bacterial communities found attached to living or abiotic surfaces and surrounded by a bacterially-produced extracellular matrix composed of exopolysaccharide, DNA and proteins. Biofilms develop in a complex and well co-ordinated manner that involves sensing and responding cues, such as bacterial cell density, nutrient availability and energy sources present in the environment.

The switch towards the biofilm mode of growth is often considered to be a survival strategy for bacteria.
Biofilms are microbial communites that adhere to biotic or abiotic surfaces and cells within a biofilm are encased in self-produced matrix biofilms are medically important because they have been implicated in the pathogenesis of numerous bacterial infection that are difficult to successfully eradicate with antibiotics.

II. AIM & OBJECTIVES

A. Aim

The aim of the present study to evaluate the combined effects of Curcumin (CCM) and antibiotics against biofilm formation among *Staphylococcus aureus.*
The objectives are:

1) Isolation of bacterial colonies from various samples
2) Identification of biofilm forming *Staphylococcus aureus* from the poultry chicken.
3) Qualitative detection of biofilm production in bacterial culture.
4) Determination of Minimum Inhibitory Concentration of CCM and antibiotics in *S.aureus.*
5) To perform checkboard assay to test interaction between CCM and antibiotics.
6) Evaluating the antibiofilm activity of Gentamicin, Curcumin, and Gentamicin combined with curcumin using microtiter plate assay (MTP).
7) Confirmation of biofilm inhibition by SEM analysis.

III. REVIEW OF LITERATURE

The genus *Staphylococcus* is the most important genus present in the family Micrococcaceae having in its ambit thirty-two species. The members of this group are Grams smooth, opaque, raised, with white to pigments of different colors. *Staphylococci* are known to be facultative anaerobes, usually oxidase negative and catalase-positive. Coagulase production by staphylococci organism cause hemolysis of blood, but the pattern of hemolysis depends on both the source of the blood and the staphylococcal strain *(Moraveji et al., 2014).* The biochemical characters of different species of staphylococci have been well documented. *Staphylococci* are known to be ubiquitous in nature and are usually isolated from the outer body surfaces of mammals and birds besides also from blood, genitourinary tract, intestines, upper respiratory tract and other organs of the body. Staphylococci are the most common bacteria found in the environment where poultry are hatched, reared, and processed. They are also isolated from the skin and nares, feet and beak of healthy chickens. *Staphylococcus aureus* is one of the major foodborne pathogens in fresh and ready-to-eat products and recognized for causing various infections around the world. There are many foodborne diseases associated with *Staphylococcus spp.* where food handlers who have staphylococcal lesions of the skin, especially of the nasopharyngeal region and the hands, or who are carriers. Most of the contamination of chicken meat due to*S.aureus*was found due to cross-contamination, inadequate heat treatment of the foodstuff and improper storage resulted into outbreaks of food poisoning.
Bacterial infections are one of the major causes of chronic infections and mortality. Antibiotics used in the treatment of these infections are preferred due to their potent effects. However, it is also known that the widespread use of antibiotics leads to the emergence of multidrug resistant (MDR) bacterial strains. In recent years, the increase in infections caused by resistant strains has attracted attention. MDR bacteria show resistance to three or more classes of antibiotics. High morbidity and mortality rates are observed in diseases caused by these bacteria.
In the following sections, we will provide a detailed description of the process of *S.aureus* biofilm formation to establish a theoretical foundation for the development of therapeutic strategies for treating *S. aureus* infections in the livestock and poultry industry. The development of *S. aureus* biofilms is tightly controlled by a complex global regulatory system that involves the regulation of numerous related proteins and can be divided into three primary stages: (1) initial attachment, (2) extracellular matrix generation and cell proliferation, and (3) biofilm deconstruction and bacterial dispersal.

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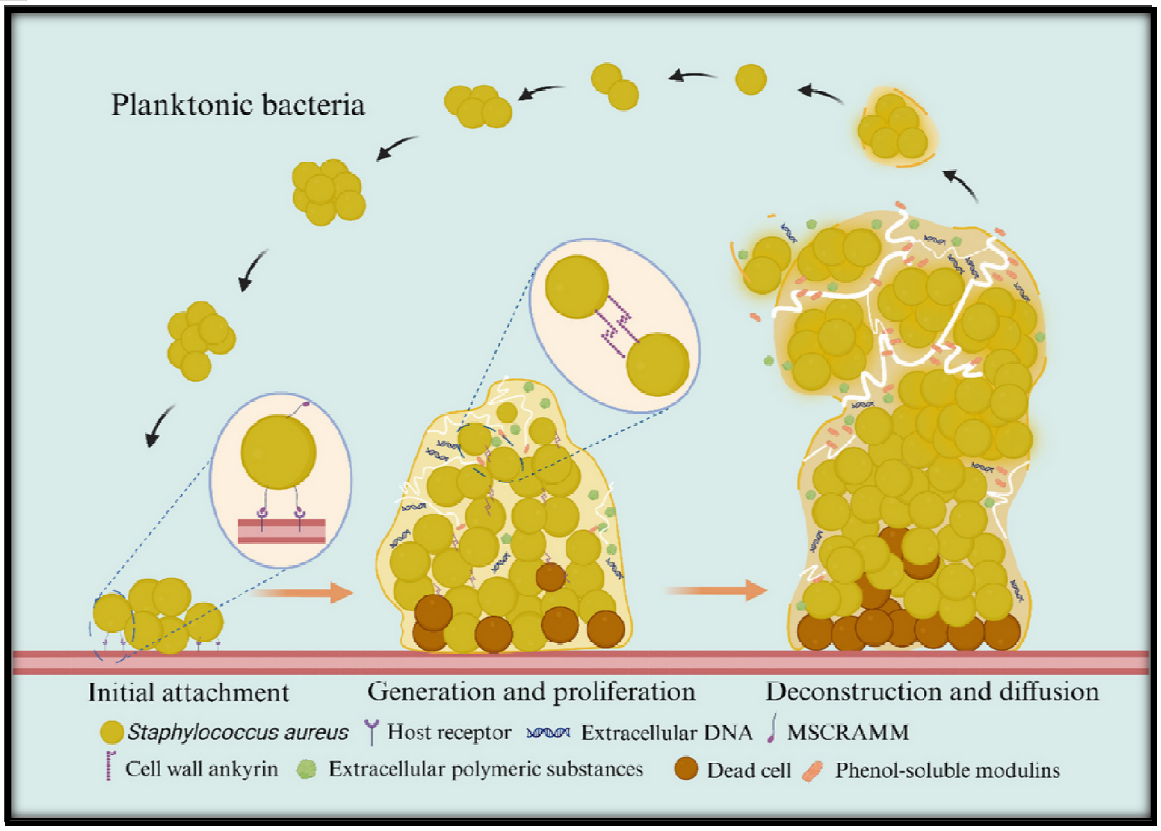

Figure 2: Formation of biofilm

The process of *S.aureus* biofilm formation. Previous reports have classified the biofilm formation process into several stages including initial attachment, proliferative growth, and deconstruction and diffusion. The attachment phase is subdivided into reversible and irreversible attachment, with free *S.aureus* first reversibly attaching to the surface of inert or active entities and then forming irreversible attachments by secreting extracellular substances such as proteins, polysaccharides, lipids, DNA, and other substances. Then,*S.aureus* further expands the scale of the biofilm through polysaccharide-dependent and polysaccharide-independent pathways. In addition, the binding of associated surface proteins results in tighter binding of adjacent cells. Finally, the mature tower biofilm diffuses through various extracellular polymeric substance cracking mechanisms

Biofilms are defined as an aggregate of microorganisms in which the microbial cells attach irreversibly to each other or to living or non-living surfaces to form self-organized communities. The cells in these complex communities are embedded in an adhesive matrix made up of the extracellular polymeric substances (EPS) that are produced by the cells in the community.The EPS matrix is an insoluble and oily secretion composed of polysaccharides, proteins and nucleic acids. This matrix encapsulates the cells residing in a biofilm, alters their phenotype by changing growth rates, gene regulation and therefore enables them to withstand harsh environmental conditions by seizing and captivating nutrients from the environment. EPS also prevents the incorporation of antimicrobial drugs and increases microbial tolerance against drugs. Microbes living in a biofilm have adopted several modifications with which they have become more resistant. They have altered their phenotype and have modified their enzymatic activity and have acquired a mutated genotype that includes more resistant genes which makes them resistant to antimicrobials. The cells in a biofilm communicate with each other with the help of different biochemical signals, this cell to cell interaction is known as quorum sensing. During biofilm formation the attachment of cells to the substratum is facilitated by adhesin proteins; formation of macro colony and finally dissemination of bacterial cells.

Staphylococcus aureus are Gram-positive cocci that inhabit the skin and mucous membranes of humans and animals *(Hanselman et al. 2009; Gharsa et al. 2012; Gundogan et al. 2012; Gutiérrez et al. 2012).* This species causes diseases through two different mechanisms: multiplying and spreading widely in tissues or production of extracellular enzymes and toxins. Staphylococcal food poisoning, a form of gastroenteritis characterized by rapid onset of symptoms, typically occurs after ingestion of food—usually meat, processed meat, milk, or dairy products—contaminated with a toxin produced by *S.aureus (Pereira et al. 2009; Pu et al.*

2011; Moura et al. 2012; Martins et al. 2013; Njage et al. 2013). The presence of *S.aureus* in food is often attributed to improper handling during processing and packaging of food, or to infection or colonization of animals that can contaminate carcasses during the slaughter process *(Devita et al. 2007; Greig and Ravel 2009; Pu et al. 2011; Weese et al. 2010).* Members of the *Staphylococcus* genus express a wide range of virulence factors, including cell wall components, biofilm formation proteins, proteases, lipases, coagulase, hemolysins, nuclease, fibrinolysin, enterotoxins, and toxic shock syndrome toxin *(Novick et al. 2001; Peacock et al. 2002; Gundogan et al. 2012; Moura et al. 2012; Tang et al. 2013).* Biofilms are a serious problem in many sectors of the food industry and are known to play a role in chronic infections *(Jessen and Lammert 2003; Otto 2013; Giaouris et al. 2014).* In food processing, biofilms are a potential source of product contamination that may lead to food spoilage and can cause issues through serious fouling of equipment. Biofilm formation in *S.aureus* is a complex process involving multiple phases. The first phase requires surface adhesin and autolysin proteins in the bacterial membrane, one of which—the autolysin A (AtlA)—is encoded by the atlA gene. The maturation phase is characterized by the production of extracellular factors that mediate contact with the bacterial cell. Staphylococcus spp. can mediate cell–cell adhesion using the polysaccharide intercellular adhesin (PIA) encoded by the operon icaADBC (PIA-dependent mechanism) or other proteins (PIA-independent mechanisms), such as the S. aureus surface protein G (SasG) (Otto 2013).

A. BIOFILM FORMATION AND INCREASING BACTERIAL VIRULENCE

In the presence of harsh environmental circumstances, such as low oxygen, UV damage, low nutrients conditions, pH, metal toxicity, hydrogen peroxide and human immune response, many bacterial species adhere to any surface and switch to the biofilm mode of growth (Witty, College and Myers, 2015).These bacteria show an increased tolerance to antibiotics, disinfectant chemicals, phagocytosis, and other components of the body's defense system. When an antibiotic enters the body, the bacteria contact their neighboring population by releasing chemical signals to promote gene expression for cell aggregation and induce them to switch to biofilm mode of growth in a process called quorum sensing. In addition, the transfer of antibiotic resistance genes between the bacteria cells increases in the biofilm population, hence rendering the antibiotic ineffective *(Adolphi et al., 2014).*

In order to prevent the increase in antibiotic resistance, there is a need for antimicrobial compounds that can be used as an alternative to conventional antibiotic therapy. This has led to the discovery of new natural or synthetic antimicrobial compounds. The side effects of synthetic drugs have led to a growing interest in natural plant-derived antimicrobial agents and a growing interest in treating infections naturally. Natural products are also being investigated in combination therapies to manage antibiotic resistance. Synergistic studies are expected to be important in the future to overcome antimicrobial resistance.

B. CURCUMIN AS AN ANTIMICROBIAL COMPONENT

Curcumin is a food spice that is a natural component of Curcuma longa (turmeric, turmeric) rhizomes. It has been widely used as a medicine in the treatment of various diseases in Asian and Middle Eastern countries for years. Curcumin, also known as turmeric, has been shown to have antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory and anticancer activities. In the society, it is known to be used for therapeutic purposes against various malignant diseases, diabetes, arthritis, gastritis, urinary tract infections, skin diseases and other chronic diseases. Studies have shown that combinations of curcumin with different agents, including various antibiotics, have synergistic effects against bacteria.

Curcumin is a polyphenolic compound naturally derived from the Curcuma longa plant, which is a member of the ginger family (Zingiberaceae). It is used as a spice and as a yellow colouring agent in foods. It is relatively insoluble in water, but shows greater solubility in some organic solvents such as acetone, ethyl acetate, acetonitrile, ethanol and Dimethyl sulfoxide (DMSO) *(Amalraj et al., 2016).*

Curcumin has a lot of pharmacological properties, such as: anti-inflammatory properties by cyclooxygenase inhibition; anti-oxidant activity by blocking the reactive oxygen species formation; anti-tumor properties by inhibiting cancer cell proliferation, suppressing the growth of tumors and inducing apoptosis of tumor cells in animal models *(Biology, Aggarwal and Harikumar, 2009);* laxative effects, anti-helminthic agent, carminative and many benefits in the cosmetic field*(Gunes et al., 2016).*

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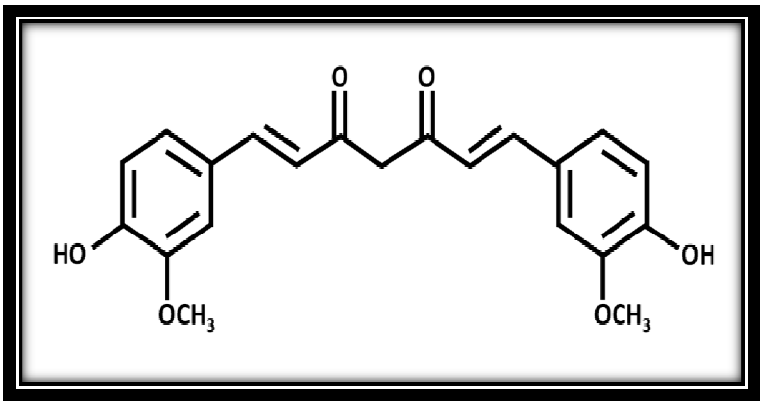


Figure 3: Structure of Curcumin

The antibacterial and antimicrobial effects of curcumin have been extensively studied. Curcumin has an antibacterial effect against: Staphylococcus aureus, Enterococcus faecalis, and P. aeruginosa through the interaction of their hydroxyl groups of phenolic compounds with the cell membrane that leads to leakage, alteration of fatty acids and phospholipid profiles in the cells and damage of the energy metabolism and synthesis of genetic materials. The mechanism of antibacterial activity of curcumin is determined depending on the strain type. For example, the effect against Bacillus subtilis is inhibition of the bacterial proliferation by blocking the assembly dynamics of FtsZ in the Z ring, whereas against P. aeruginosa, it functions by interrupting QS mechanism, biofilm initiation and virulence *(Negi et al., 1999; Loo et al., 2016).* Another mechanism of action of curcumin as an antibacterial agent is thinning of the membrane of S.aureus and Escherichia coli, as a result disrupting the plasma membrane *(Mukhopadhyay et al., 2015).* The studies for synergistic effects of antibiotics in combination with plant derivatives aim to develop antimicrobial activity with a wider spectrum and to reduce adverse side effects of anti-microbial agents. Studies on curcumin have shown it to have a synergistic effect with a lot of antibiotics, thus enhancing bacterial susceptibility towards antibiotics like tetracycline, vancomycin and cefixime against Staphylococcus aureus. The synergistic activity of curcumin and ampicillin demonstrated pronounced reduction in the minimum inhibitory concentration (MIC) of ampicillin against a clinical strain of *S.aureus* (ATCC 25923) *(Mukhopadhyay et al., 2015). (Pattiyathanee et, al.2009)* reported that the biofilm formation of Helicobacter pylori can be inhibited by curcumin in a dose-dependent manner. However, H. pylori could restore biofilm- 10 forming ability during a prolonged incubation period *(Pattiyathanee, Vilaichone and Chaichanawongsaroj, 2009).*

C. THE ANTI-BIOFILM EFFECT OF CCM

The antibiofilm effect of curcumin in a previous study about the activity of curcumin against biofilm formation, a visible reduction in the numbers of microcolonies, deterioration of the architecture of the biofilm, slight reduction in the size of the bacterial cells, and the cell wall becoming amorphous with unspecific filaments appearing around the cells were all shown to occur. These observational changes serve to prevent biofilm formation *(Loo et al., 2016).* In addition, the curcumin inhibited the swimming motility and swarming which is associated with biofilm formation by instigating the cell-to-cell surface attachment which plays an important role in the virulence of bacteria *(Moshe, Lellouche and Banin, 2012; Packiavathy et al., 2014)*

IV. MATERIALS AND METHODS

A. Collection and processing of samples

A total of 3 samples of poultry meat (Brain, Intestine & Heart) samples were collected from farm. Here, the samples were collected in dry, clean and sterile saline and transported to the laboratory for microbiological analysis within one hour or refrigerated at 4°C till further analysis was carried out. These samples were then processed no later than 48 hours after purchase. These samples were directly inoculated onto the Tryptic Soy broth (TSB) and then incubated overnight at 37°C. On the next day, the swabs were streaked onto the different media plates like Brain Heart Infusion Agar (BHI), Mannitol Salt Agar (MSA) for isolation of Staphylococcus spp.

B. Identification of bacterial isolates

The bacterial colonies were isolated after incubation. These colonies were subjected to Gram's staining for identification and requisite biochemical tests were carried out to further confirm the presence of the pathogen. *S.aureus* suspected colonies was subjected to biochemical tests like the Catalase test , coagulase test and oxidase test to confirm the *Staphylococcus aureus*.

C. Biofilm Formation and Quantification

1) Congo Red Agar method

Freeman *et al.*, 1989 described a simple qualitative method to detect the biofilm formation by Congo Red Agar (CRA) method.This method involves use of special media that is Brain Heart Infusion (BHI) agar with Sucrose and Congo red in the following composition: BHI agar-52 g/L; sucrose-36 g/L; agar-10 g/L; congored-0.8 g/L. Congo red was prepared as concentrated solution and autoclaved. It is added to the medium when agar is cooled to 55°C and poured into petriplates. Plates were inoculated and incubated for 24–48 h at 37°C. Black colonies with dry crystalline morphology was considered positive for biofilm producing organisms while weak or non-biofilm producing organisms appeared to be pink in colour. The experiment was performed in triplicates.

2) TCP method

This quantitative, gold standard method for biofilm detection was carried out as described by Christensen et al. In brief, a colony of S. aureus was isolated from a fresh agar plate and inoculated in 2 mL of trypticase soy broth. The broth was incubated overnight at 37 °C. The culture was then diluted to 1:100 with fresh medium. A sterile individual plate with 96 flat-bottom polystyrene wells was filled with 200 µL of the diluted culture. The control organisms were also processed in a similar manner. The plate was incubated at 37 °C for 24 hours. After incubation, the contents of each well were removed by gentle tapping. The wells were washed with 200 µL of phosphate buffer saline (pH 7.3) to remove free-floating bacteria. Biofilms formed by bacteria adherent to the wells were fixed by 99% methanol and stained with 0.1% crystal violet (CV). Excess stain was washed gently, and the plate was kept for drying. The optical density of the stained adherent biofilm was measured using a micro-ELISA auto-reader (HUMAN) at a wavelength of 570 nm. The experiment was performed in triplicate. Interpretation of biofilm production was performed as per the criteria described by Stepanovic et al. and the bacteria were categorized into biofilm nonproducers, or weak, moderate or strong biofilm producers.

3) Tube adherence method

The isolated organisms were inoculated in 5 mL trypticase soy broth in test tubes and incubated overnight at 37 °C along with the control organism.15 After incubation, the tubes were decanted, dried and stained with 0.1% CV. Subsequently, the tubes were washed gently and placed upside down for drying. Visible lining of the wall and bottom of the tube by a film was considered as positive. The results were scored visually as nonproducers, or weak, moderate or strong biofilm producer.

D. Minimum Inhibitory Concentration

Minimuminhibitory concentration of four different classes of antibiotics, penicillium (PencillinG),lincomycin (Clindamycin),Macrolides (Erythromycin), aminoglycosides (Gentamicin) and Curcumin were determined using the broth dilution method. MIC was carried in 96-well microtitre plates with a Muller Hinton Broth (MHB) overnight culture of Staphylococcus aureus adjusted to OD_{600} of 0.1. Plates were incubated at 37˚C for 18-20hrs the absorbance values were measured at 595nm.

Checkboard assay to test interaction between CCM and antibiotics

Interactions of each plates antibiotic with CCM was assessed using a checkboard assay in 96-well microtitre plate. After preparing each well with appropriate dilutions of CCM and antibiotic cultures were inoculated and incubated at 37˚C for 18-20hrs. The synergistic interactions were expressed as the fractional inhibitory concentration (FIC). The FIC index (FICI) was calculated using the following formula:

$$\Sigma FICI = FIC\ antibiotic + FIC\ CCM,$$

Where,

FIC antibiotic = MIC of antibiotic in combination/MIC of antibiotic alone,

FIC CCM = MIC of CCM in combination/MIC of CCM alone

A synergistic effect was defined at an FICI of ≤0.5; an indifferent effect at an FICI between 0.5 and ≤4 and an antagonistic effect at an FICI >4. Similar checkerboard assays were followed for all antibiotics.

Detection Of Biofilm Formation Both Treated And Untreated On Glass Slides Using SEM

Tryptic Soy Broth was prepared and inoculated with B01 strain and different concentrations of gentamicin, curcumin and curcumin combined with gentamicin. It was incubated for 24hrs at 37˚C after incubation glass slides are taken and allowed to fix in 3% glutaraldehyde for 5hrs after glutaraldehyde fixation glass slides are washed with PBS for 5 minutes.

V. RESULT AND DISCUSSION

A. Isolation of bacterial colonies from various samples

The samples were separately inoculated on the Tryptic Soy Broth and incubated for 37˚C for 24hrs. Then, the serial dilution was carried out up to the dilution of 10^{-7}. Then the nutrient agar plate was made to isolates the typical colonies.From, that five different strains were obtained. Those strains were named as B01, B02, B03, I02, I03.

B. Identification of biofilm forming Staphylococcus aureus from the poultry chicken.

Identification of bacteria using Mannitol Salt Agar (MSA) media showed the growth of Staphylococcus aureus bacteria colonies in all types of samples but with different amounts. Identification of bacteria was marked by the shape and colour of the specific colony of *Staphylococcus aureus* in MSA which was yellow and confirmed as a Gram-positivebacteria(blue and round) through Gram staining. The results of this study indicated that *Staphylococcus aureus* colonies were relatively high and were found in samples from the environment.

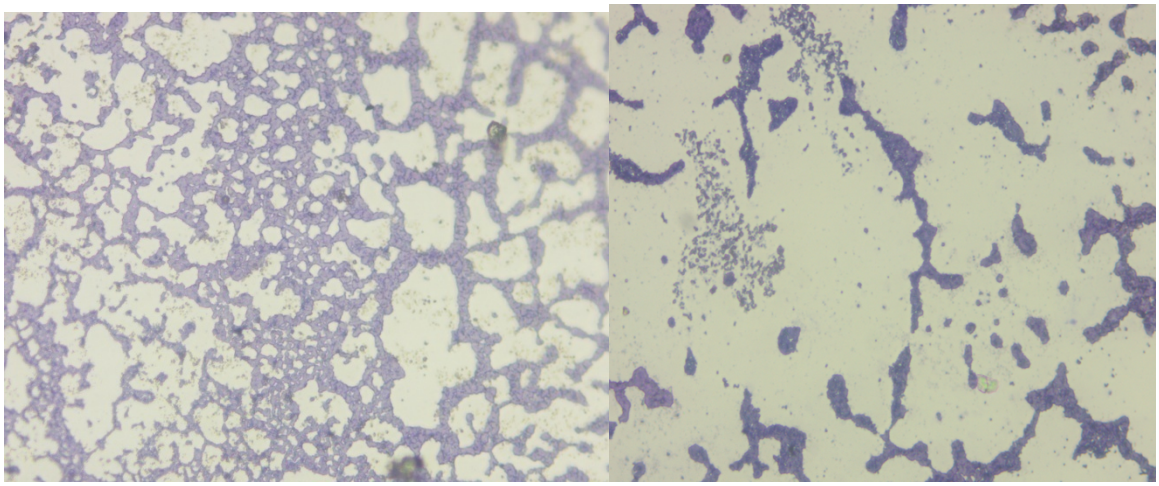


Figure 4 : GRAM STAINING – Staphylococcus aureus

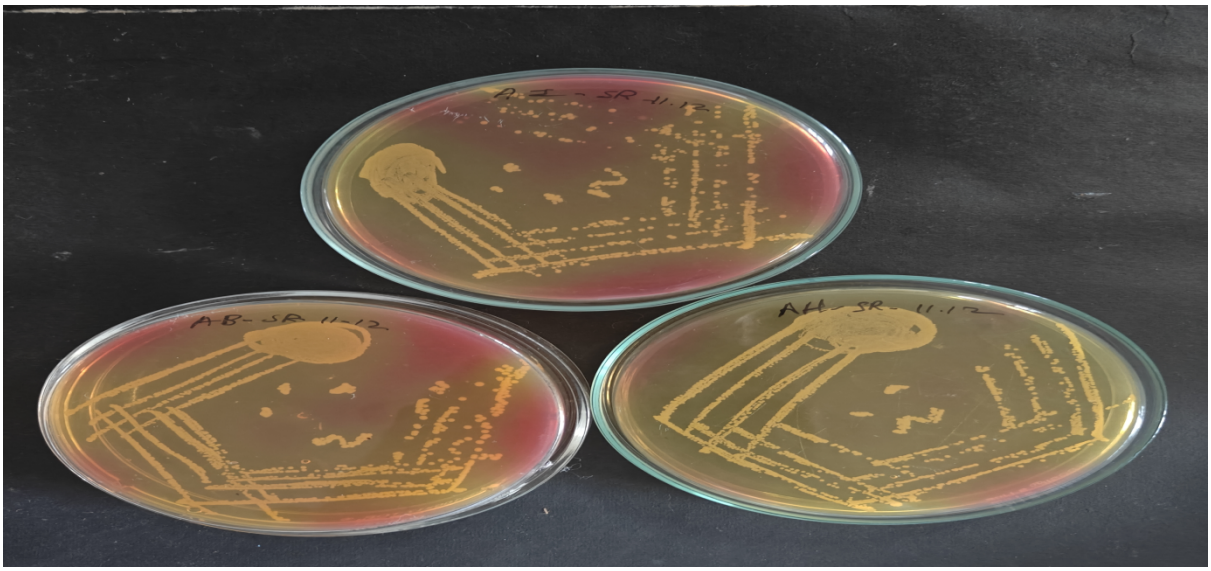


Figure 5 : Isolates on Mannitol Salt Agar Plate

C. Primary test for biofilm

Mostly bacterial biofilms are found to possess a commonly known amyloid protein. The amyloid fibers assemble into a cross-β quaternary structure that imparts resistance to proteases and harsh denaturing conditions. CR-supplemented brain heart infusion agar has been extensively used as a differential medium for identifying amyloid-producing bacteria.

Congo Red Agar (CRA) method is widely used method for detection of biofilm production clinically isolated organism such as *Staphylococcus aureus*, produced pink colonies on a CRA plates.

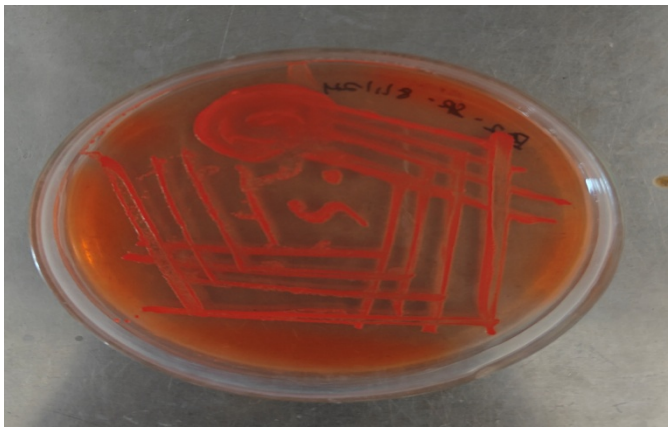


FIGURE 6: CRA PLATE OF B01STRAIN

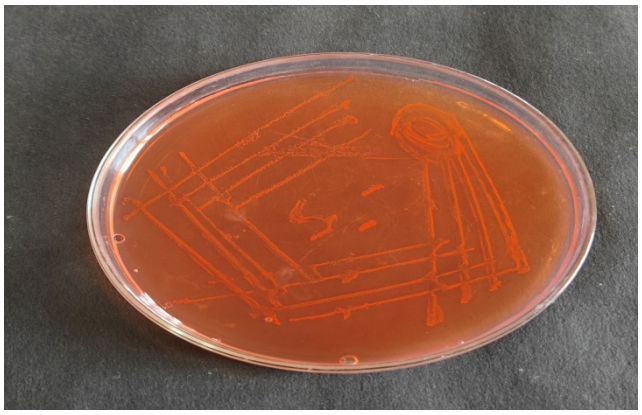


FIGURE 7: CRA PLATE OF B02STRAIN

FIGURE 8: CRA PLATE OF I02STRAIN

D. Secondary screening for Biofilm

Tube method and TCP Method are used to a quantitative assay for detection of biofilm formation, as a result of the occurrence of visible film in TSB Medium. The isolate B01 produced the visible films on the walls of polystyrene test tube whereas the strains of B02, B03, I02, I03 were less detectable in the test tubes. In also gives result

OD≤ ODc – no biofilm producer

ODc<OD ≤ 2×ODc – weak biofilm producer

2×ODc < OD≤ 4×ODc – moderate biofilm producer

4×ODc < OD – strong biofilm producer

From the obtained OD value result it is interfered that B01 is the moderate biofilm producer where as B02, B03, I02, I03 are the weak biofilm producers.

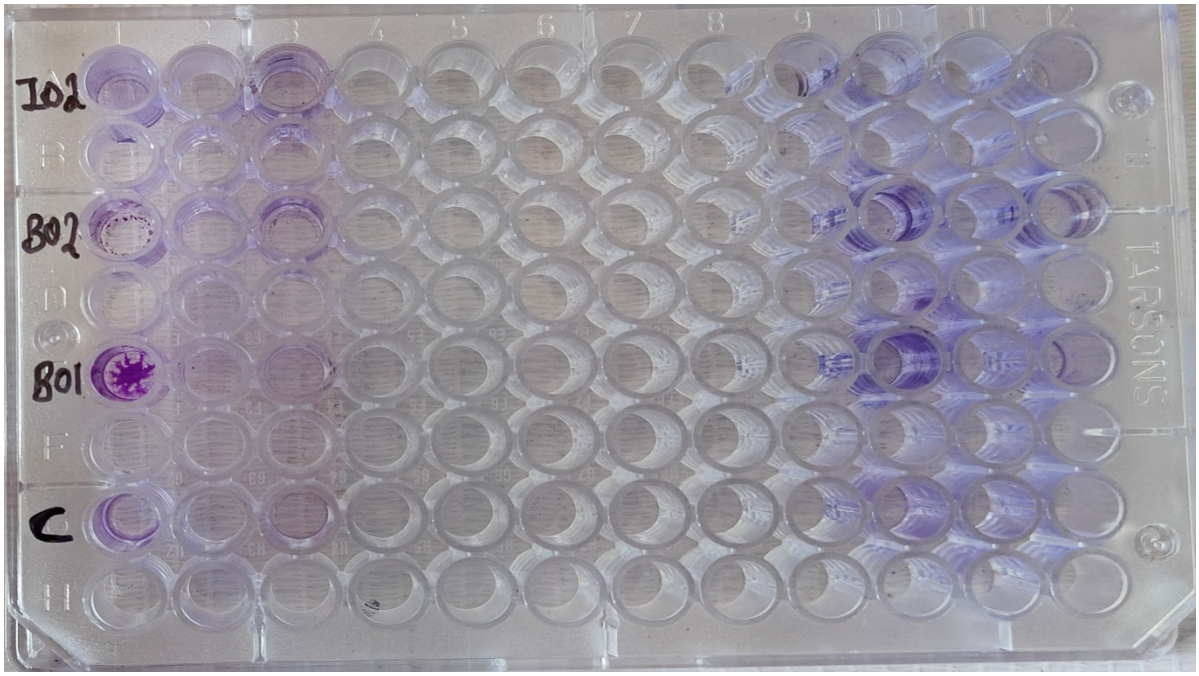


Figure 9: 96-Microtitre Plate of TCP Method

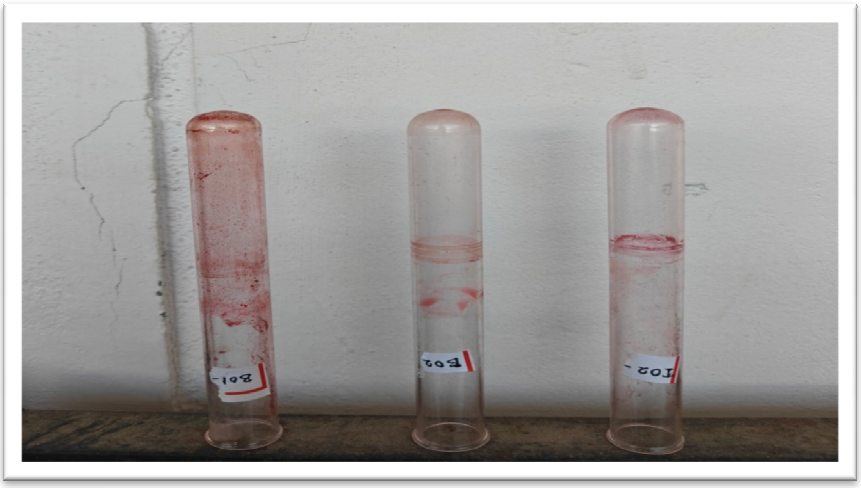


Figure 10: Tube Method

Minimum inhibitory concentration (MIC) of antibiotics

MIC'sis thelowest concentration of which resulted in inhibition of growth of the test organism. MICs of three different classes of antibiotics, penicillium (Pencillin G), lincomycin (Clindamycin), Macrolides (Erythromycin), aminoglycosides (Gentamicin) were determined with the planktonic cells of *Staphylococcus aureus* using the broth dilution method. MICs were then established the checkboard assay to study interaction between CCR and antibiotics with *Staphylococcus aureus*.

Table 1: MIC's of Antibiotic alone and in combination with CCM

ANTIBIOTICS	MIC ALONE	MIC COMBINED	FIC OF ANTIBIOTIC	FIC OF CURCUMIN	FICI
PENICILLIN	0.260	0.249	0.329	0.420	1.420
CLINDAMYCIN	0.404	0.339	0.750	0.892	0.704
GENTAMICIN	0.391	0.375	0.730	1.810	0.440
ERYTHROMYCIN	0.363	0.359	0.747	0.987	0.583

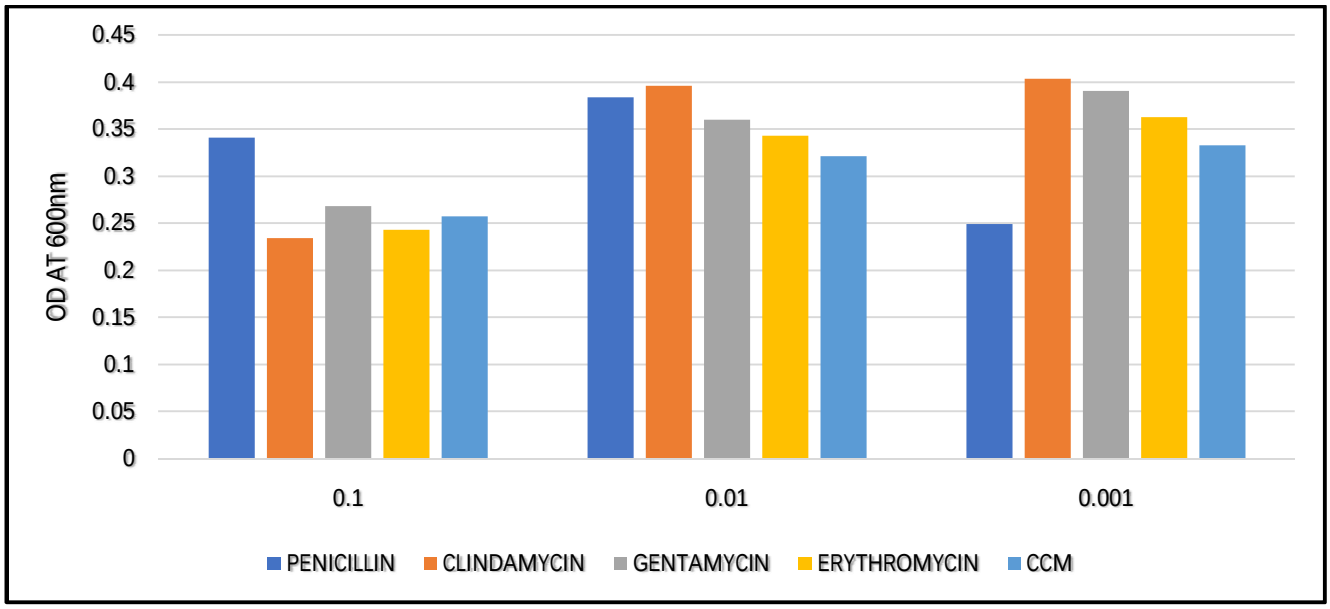


Figure 11: Determination of MIC and CCM against *Staphylococcus aureus*

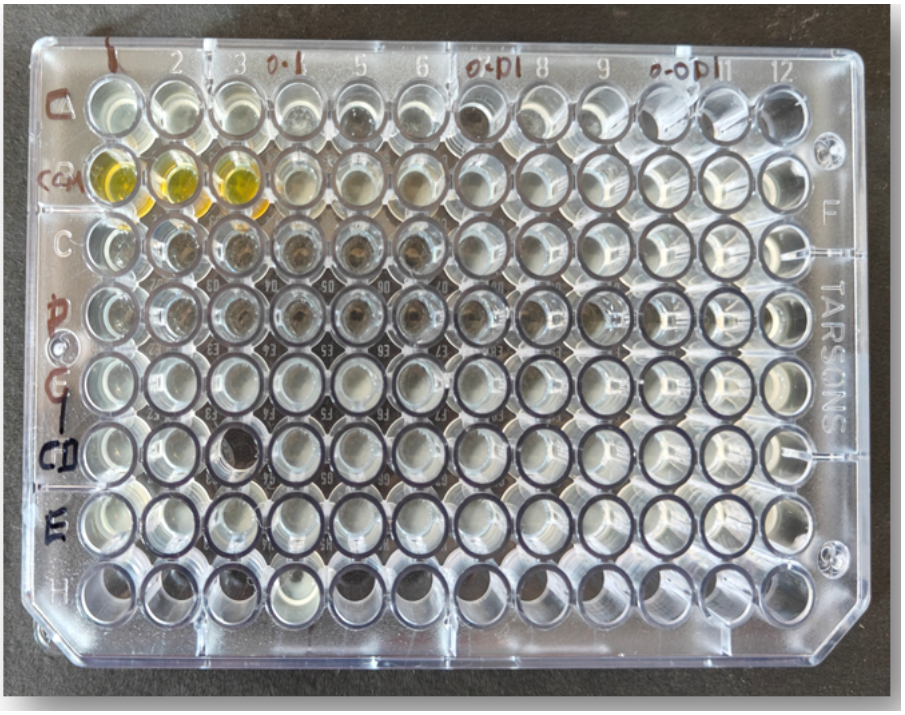


Figure 12: 96 Micro-titer plate contains Antibiotic alone and in combination with CCM

Checkboard assay to test interaction between CCR and Antibiotics

Checkerboard assay was performed to evaluate the ability of antibiotics combined with CCR to inhibit the growth of biofilm forming *Staphylococcus aureus.*This effect was defined by an fractional inhibitory concentration index FICI)

- A synergistic effect FICI of ≤0.5
- An indifferent effect at an FICI between 0.5 and ≤4
- An antagonistic effect at an FICI > 4.

ANTIBIOTIC	MIC		FICI	ACTIVITY
	ANTIBIOTIC	CURCUMIN		
PENICILLIN	0.1	0.1	1.420	Indifferent
CLINDAMYCIN	0.01	0.01	0.740	Indifferent
GENTAMICIN	0.1	0.1	0.440	Synergistic
ERYTHROMYCIN	0.01	0.01	0.583	Indifferent

Table 2: FICI and mg/ml concentration of Antibiotics and Curcumin

FICI of gentamicin is less than 0.5 when compared to other antibiotics, therefore it's is inferred that the Gentamicin shows synergistic activity. FICI of of Clindamycin, Penicillin, Erythromycin is greater than 4 it shows indifferent activity.

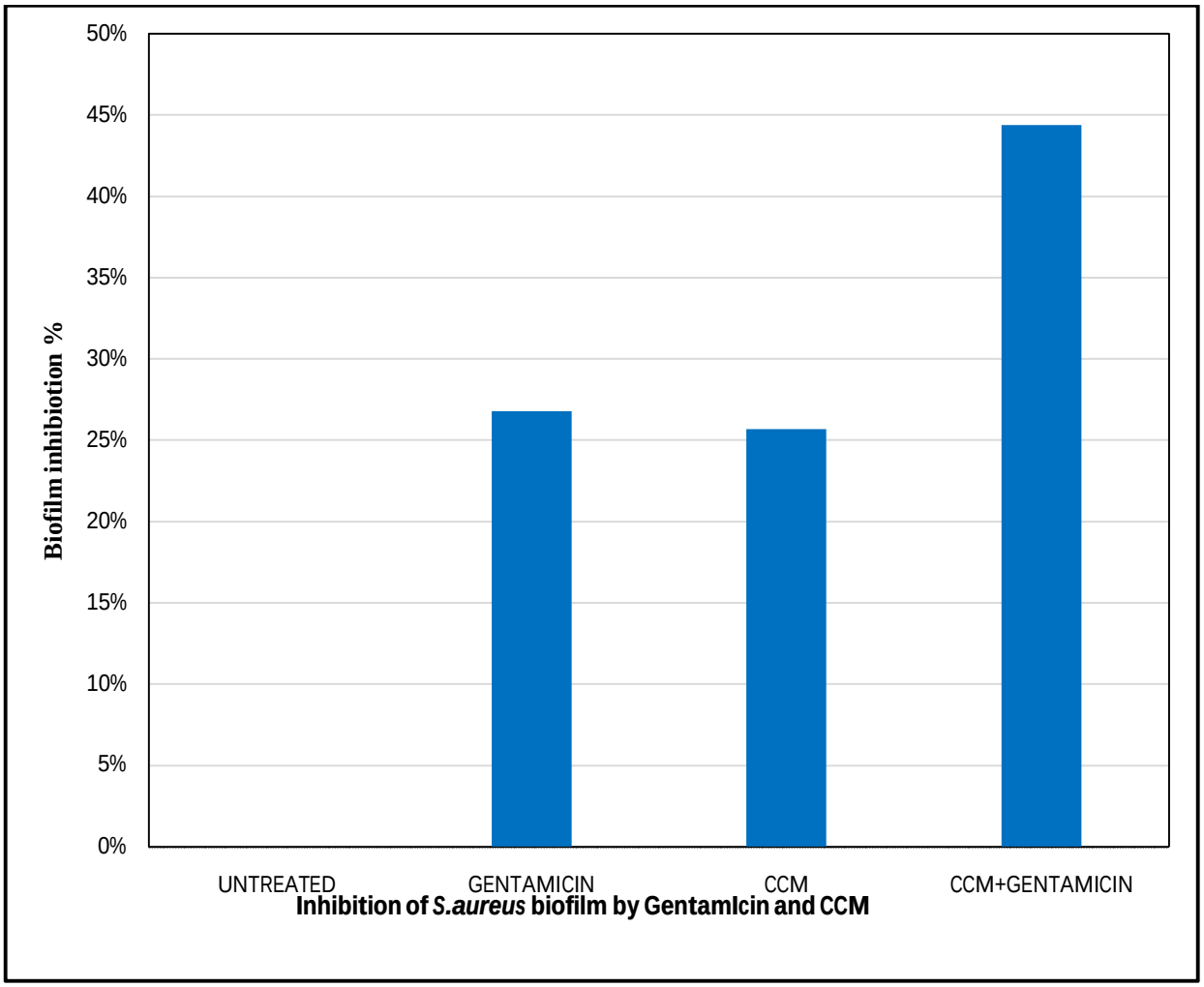
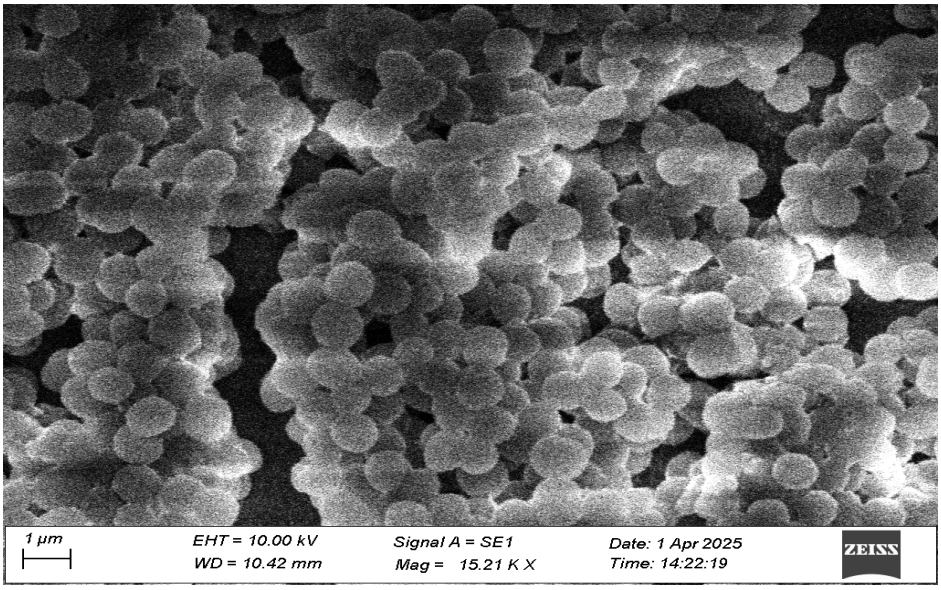


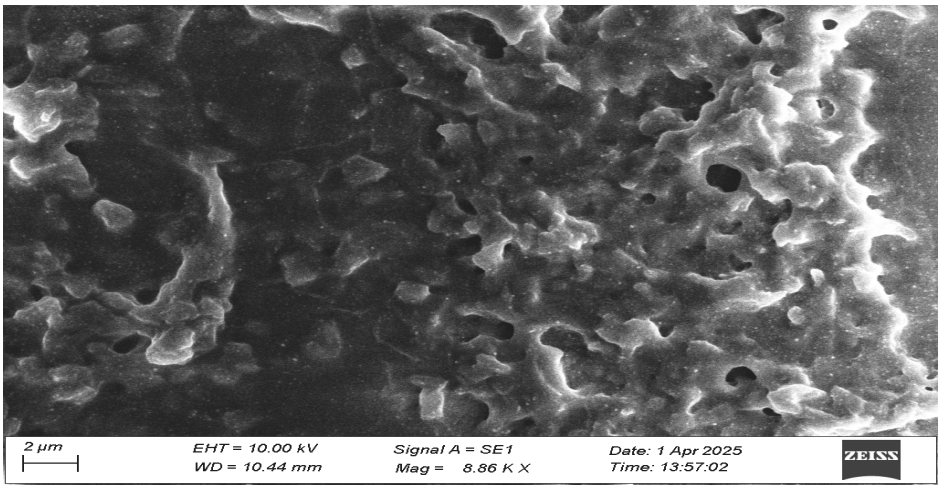
Figure 13: CCM (0.1mg/ml) combined with the Gentamicin (0.1mg/ml)- mediated inhibition of biofilm in *Staphylococcus aureus.*

Confirmation of biofilm inhibition by SEM analysis

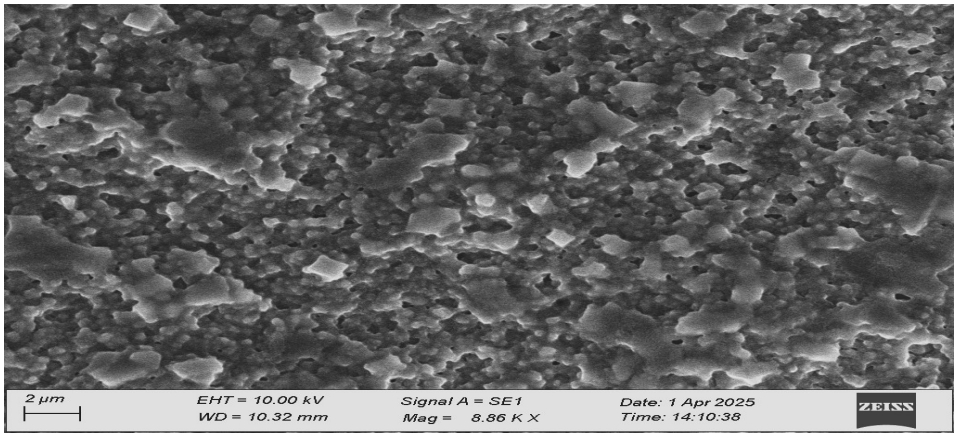
- Untreated B01 Strain



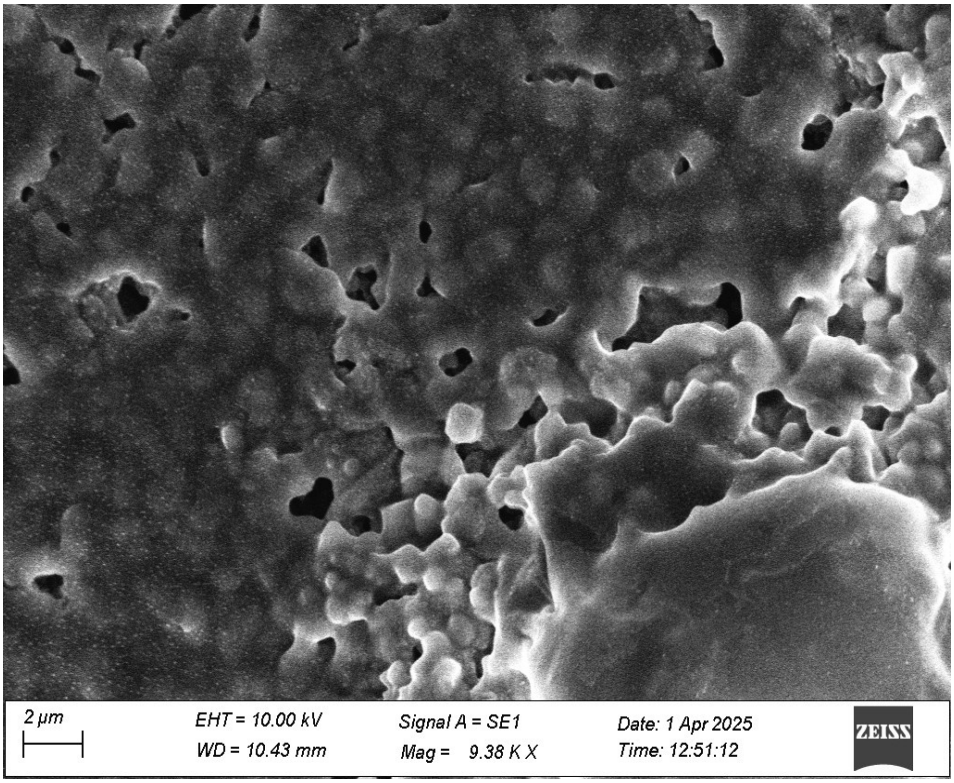
- B01 Treated with Gentamicin



- B01 Treated with CCM



- B01 Treated with Gentamicin + CCM



VI. SUMMARY AND CONCLUSION

In this study shows that the combined use of CCM and Gentamicin showed synergistically inhibiting bio-film formation among *Staphylococcus aureus.* At sub-MICs, CCM inhibits QS and the combined action of gentamicin and curcumin was also noticeable in QS inhibition. It was observed that CCM, a QS inhibitor enhances the efficacy of antibiotic therapy in combination therapy, more effectively disrupting QS and preventing biofilm formation. Future research is needed to optimize the molecular mechanisms in the inhibition of bio-film formation exhibited by the synergistic action of Curcumin(CCM) and Gentamicin.

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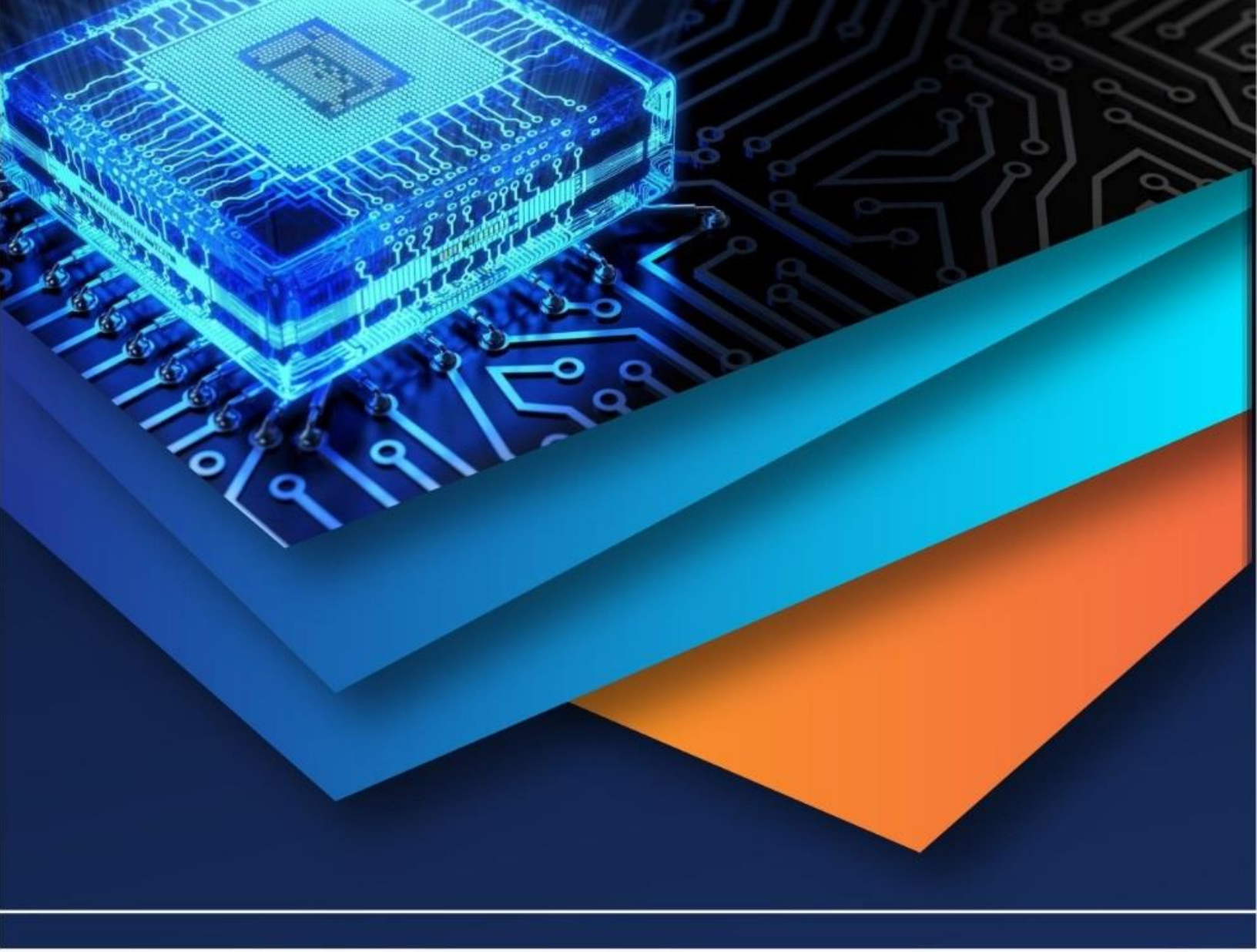
APPENDIX

Congo Red Agar:
Brain heart infusion - 37.3g
Sucrose - 36g
Congo red - 0.8g
Distilled water - 1000ml
Tryptic Soy Broth:
Tryptone - 17g
Soy peptone - 3g
Sodium chloride - 5g
Dipotassium hydrogen phosphate - 2.5g
Distilled water - 1000ml
pH - 7.3±0.2
Muller Hinton Broth:
(Equivalent to beef heart infusion) - 3000
Acicase (Equivalent to casein acid hydrolysate) - 17.500
Starch - 1.500
Distilled water - 1000ml
pH - 7.4±0.1
Peptone broth media:
Peptone - 20g
Magnesium chloride - 1.4g
Potassium sulphate - 10g
Distilled water - 1000ml
Nutrient broth:
Peptone - 20g
Sodium chloride - 5g
HM peptone B - 1.50g
Yeast extract - 1.50g
Final pH - 7.4±0.1
Luria Bertani Broth, Miller (Miller Luria Bertani Broth):
Tryptone - 10g
Yeast extract - 5g
Sodium chloride - 10g
Distilled water - 1000ml
Final pH (at 25˚C) - 7.5±0.2
Mannitol salt agar
Enzymatic Digest Of Casein - 5.0 g
Enzymatic Digest Of Animal Tissue - 5.0g
Beef Extract - 1.0g
D-Mannitol - 10.0g

Sodium Chloride	- 75.0g
Phenol Red	- 0.025g
Agar	- 15.0g
Distilled water	- 1000ml
pH	- 7.4 ± 0.2 at 25 °C



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