



INVITRO ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY OF CLOVE (*SYZYGIUM AROMATICUM*) EXTRACT AGAINST METHICILLIN -RESISTANT (MRSA) AND METHICILLIN-SUSCEPTIBLE (MSSA) *STAPHYLOCOCCUS AUREUS* STRAINS

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ABSTRACT

The increasing prevalence of antibiotic-resistant pathogens, particularly Methicillin-resistant *Staphylococcus aureus* (MRSA), has necessitated the exploration of alternative antimicrobial agents. This study aimed to evaluate the *in vitro* antimicrobial and antibiofilm activity of clove (*Syzygium aromaticum*) extract against MRSA, Methicillin-susceptible *Staphylococcus aureus* (MSSA), and *Staphylococcus aureus* (control strain). Clove extract was prepared using methanolic extraction. Preliminary identification of isolates was performed using culture on MacConkey agar, Mannitol Salt Agar, Blood agar, and Nutrient agar. Confirmation was done using biochemical tests such as catalase, oxidase, and coagulase. Antibiotic susceptibility testing (AST) was conducted to determine resistance patterns and to differentiate MRSA and MSSA strains. The biofilm-forming ability of the isolates was assessed prior to treatment. Antimicrobial activity of the clove extract was evaluated using the agar well diffusion method, and the Minimum Inhibitory Concentration (MIC) was determined

at different concentrations. Antibiofilm activity was analysed to evaluate the inhibitory effect of the extract on biofilm formation. The results demonstrated that methanolic clove extract exhibited significant antimicrobial activity against all tested strains. MRSA isolates showed stronger biofilm-forming ability compared to MSSA. The extract effectively inhibited both bacterial growth and biofilm formation in a concentration-dependent manner. These findings suggest that clove extract has potential as a natural antimicrobial and antibiofilm agent against resistant *Staphylococcus aureus* infections.

KEYWORDS: *Staphylococcus aureus*, MRSA, MSSA, clove extract, antimicrobial activity, antibiofilm activity.

INTRODUCTION

Antimicrobial resistance (AMR) is increasingly recognized as a major global health concern, threatening the successful treatment and prevention of infectious diseases. It arises when microorganisms develop the ability to survive exposure to antimicrobial drugs that were once effective against them. As a result, standard treatments become less effective, leading to prolonged illnesses, greater risk of spread, and increased rates of death. The escalation of AMR is largely attributed to the excessive and inappropriate use of antimicrobials in healthcare, agriculture, and livestock production, as well as poor infection control and limited development of new drugs. This growing challenge endangers the safety of routine medical procedures such as surgeries and therapies that weaken the immune system. Based on origin, resistance may be intrinsic, where microorganisms naturally possess resistance due to inherent structural or functional characteristics, or acquired, where previously susceptible organisms develop resistance through genetic mutations or horizontal gene transfer. Mechanistically, AMR involves several strategies such as enzymatic inactivation of drugs, alteration of antimicrobial target sites, active efflux of drugs from the cell, reduced membrane permeability, and the development of alternative metabolic pathways. Furthermore, based on the spectrum of resistance, AMR is categorized into Multidrug resistance (MDR), where organisms are resistant to multiple antimicrobial classes (Magiorakos *et al.*, 2012); Extensively drug-resistant (XDR), indicating resistance to most available agents except a few; and Pan drug resistance (PDR), where organisms are resistant to all known antimicrobial agents (N. Sharmila Devi *et al.*, 2024).

Between 2000 - 2024, multidrug-resistant organisms (MDROs) have rapidly emerged and proliferated, escalating into a severe public health emergency. Multidrug-resistant (MDR)

bacteria, many of which are highly pathogenic, are increasing rapidly and pose a severe risk to human health. While such resistant strains were once uncommon and largely confined to hospital-acquired infections, they have now become widespread. Initially, MDR microorganisms were connected with hospital-acquired illnesses. Consequently, this increase in the transmission of antibiotic resistance poses a major threat to society and the medications that are currently available. It also has a detrimental effect on cancer treatment, transplantation, and surgical procedures. It has been believed that the use of broad-spectrum antibiotics, which include both empirical and definitive therapy, is closely linked to the emergence of specific MDR bacterial strains. The formation of biofilm is a distinctive feature of the life cycle of the bacterial community. At the community level, MDR proliferate and have the capacity to form biofilm in the absence of foreign substances. The most prevalent instance of MDR bacteria is MRSA (methicillin-resistant *Staphylococcus aureus*), which is effectively disseminated from hospital-acquired infections to community-associated dissemination. The distinction between Methicillin-Sensitive *Staphylococcus aureus* (MSSA) and Methicillin-Resistant *S. aureus* (MRSA) is primarily determined by their genetic determinants of antimicrobial susceptibility. While both represent variants of the same bacterial species, MSSA retains sensitivity to methicillin and related β -lactam antibiotics. MRSA exhibits resistance not only to methicillin but also to nearly all penicillin and cephalosporins, agents that remain highly efficacious against MSSA. Biofilm-associated *Staphylococcus aureus* strains, particularly MRSA, are capable of producing a wide spectrum of infections, ranging from superficial skin and soft tissue infections (SSTIs) to severe invasive conditions such as bloodstream infections (BSIs), osteomyelitis, and infective endocarditis (IE). Within intensive care units, MRSA poses a major clinical challenge, as many isolates exhibit resistance to multiple classes of antibiotics. The burden of these infections is amplified by their frequent occurrence in hospital environments and their contribution to considerable morbidity and mortality. A further complication arises when MRSA transitions into a biofilm mode of growth, wherein the bacteria are embedded within a self-generated extracellular matrix that facilitates adherence to diverse surfaces. Such biofilms commonly colonize catheters, contact lenses, prosthetic heart valves, and other implanted prostheses, thereby complicating treatment and eradication efforts (Gustavo Andrés Urriago-Osorio *et al.*, 2025).

To combat MRSA infections and inhibit biofilm formation through natural methods, researchers have focused on plant-derived compounds, essential oils, and naturally occurring

peptides that interfere with bacterial communication and weaken the biofilm structure. Essential oils such as clove, oregano, tea tree, and cinnamon have demonstrated strong antibiofilm activity by disrupting bacterial membranes and interfering with quorum sensing signals. Among these, eugenol from clove is particularly effective in suppressing MRSA biofilm development and reducing virulence. (Abdallah *et al.*, 2011).

Syzygium aromaticum, commonly known as clove, is a widely used culinary spice with diverse medicinal applications. Owing to its rich composition of bioactive constituents including gallic acid, flavonoids, eugenol acetate, and eugenol clove finds utility across cosmetics, medicine, gastronomy, and agriculture. Clove essential oil has been extensively reported to possess antibacterial, antinociceptive, antifungal, and anticancer properties. Its anti-inflammatory agents, particularly eugenol and flavonoids, contribute to reducing inflammation and alleviating pain. These anti-inflammatory and analgesic attributes have established clove oil as a traditional remedy for dental ailments such as toothache and gum irritation (Vinay Kumar Pandey *et al.*, 2023). Eugenol, the predominant constituent of clove oil, is widely recognized for its diverse biological activities, including antimicrobial, antioxidant, anti-inflammatory, and antispasmodic effects. Beyond its pharmacological relevance, it holds considerable industrial importance, serving as a flavouring agent in food products and as a fragrance component in cosmetics. Recent investigations have expanded its therapeutic profile by demonstrating its ability to inhibit and eradicate biofilms formed by *Staphylococcus aureus*, including both methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) clinical strains. This dual action against planktonic bacteria and biofilm-associated communities highlights eugenol's potential as a natural antimicrobial agent with applications in combating persistent infections and supporting the development of novel therapeutic strategies (Nasser Abdulatif Al-Shabib *et al.*, 2017). Previous studies have shown that clove oil possesses the anti-quorum sensing activity against MRSA and it concentrated the strains of MRSA. Therefore, the present study was aimed at the potential of methanolic extraction of clove had the ability to inhibit the biofilm formation in MRSA, MSSA and *S. aureus*. The comparative analysis was studied between these strains.

MATERIALS AND METHODS

1. Isolation and Identification of Bacterial strains

1.1. Cultural Characteristics of isolates in different culture media

The bacterial isolates were cultured on different media to assess their growth characteristics and aid in identification. MacConkey agar without crystal violet and bile salts was used for isolation, while Mannitol Salt Agar (MSA), a selective and differential medium containing 7.5% sodium chloride, was employed to Favor the growth of *Staphylococcus* species and observe mannitol fermentation through colour change. Nutrient agar was used as a general-purpose medium to support overall bacterial growth. Blood agar supplemented with 5% blood was utilized to evaluate colony morphology and haemolytic activity, particularly noting β -haemolysis associated with *Staphylococcus aureus*.

1.2. Preliminary Identification Test

Gram Staining: Gram staining was performed to identify the cellular morphology and staining characteristics of the isolates, where *Staphylococcus aureus* appeared as Gram-positive cocci arranged in irregular clusters under oil immersion.

Biochemical Tests

Catalase and Oxidase Test: The catalase test is important for identifying bacteria that can break down hydrogen peroxide, helping to differentiate *Staphylococcus* (catalase positive) from *Streptococcus* (catalase negative) by forming bubble in H_2O_2 . The oxidase test is used to detect cytochrome c oxidase enzyme that forms purple colour in the oxidase disc, which helps distinguish oxidase-positive bacteria like *Pseudomonas* from oxidase-negative groups such as *Enterobacteriaceae*. Together, these tests provide quick preliminary identification of bacteria.

Coagulase Test: The coagulase test was performed using both slide and tube methods to identify *Staphylococcus aureus*. The slide coagulase test detects bound coagulase (clumping factor), which reacts with fibrinogen in plasma to produce visible agglutination, helping to differentiate *S. aureus* from other *Staphylococcus* species. The tube coagulase test is used to detect free coagulase, indicated by clot formation in plasma after incubation, providing further confirmation of *S. aureus* identification.

1.3. Antibiotic Susceptibility Test (AST)

A reliable, commonly used method where the bacterium is exposed to ceftazidime to test for resistance. MRSA carries the *mecA* gene, which encodes an altered penicillin-binding protein (PBP2a) that confers resistance to β -lactam antibiotics. Ceftazidime is a potent inducer of the *mecA* gene and is therefore used as a surrogate marker for methicillin resistance whereas the MSSA and *Staphylococcus aureus* are susceptible to those antibiotics. The antibiotics used are Ceftazidime, Oxacillin, Penicillin, Vancomycin and Erythromycin.

The Muller Hinton Broth were prepared and inoculate the overnight culture of the isolates into MHB and incubate for 30 minutes, then adjust with 0.5 McFarland (1.5×10^8 CFU/ml). McFarland standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland standard. The standard most commonly used in the clinical microbiology laboratory is the 0.5 McFarland standard which is prescribed for Anti-microbial susceptibility testing. 11.4 gm of Muller Hinton Agar dissolved with 300 ml of distilled water 0.9 gm of agar were mixed and sterilized. The media were poured into sterile petri plates and allowed to get solidify. The MHB culture are inoculated on MHA plates by using sterile cotton swab by lawn culture method. The antibiotics are placed using sterile forceps and incubate at 37°C for 24 hours in inverted position. After incubation, observe the results and measure the zone of inhibition.

1.4. Biofilm Activity of the isolates

The biofilm adhesion activity of all 4 strains of MRSA was carried out in a 96-well plate by Crystal violet method. The biofilm formation assay was carried out over three days using bacterial isolates cultured in LB broth supplemented with sucrose. On Day 1, a fresh LB broth containing sucrose was prepared, and the bacterial isolates were inoculated into the medium. The inoculated broth was then incubated at 37°C for 24 hours. On Day 2, the turbidity of the culture media was assessed to confirm bacterial growth. The cultures were then diluted in a 1:10 ratio by mixing 10 μ l of the sample with 90 μ l of LB broth containing sucrose. A sterile 96-well U-bottom microtiter plate was taken and designated for biofilm activity analysis. The wells were organized such that wells 1–3 contained MRSA strain 1, wells 4–6 contained MRSA strain 2, wells 7–9 contained MSSA, and wells 10–12 contained *Staphylococcus aureus* ATCC 25923. Each well was filled with 15 μ l of LB broth with sucrose, followed by the addition of 50 μ l of the diluted bacterial culture. The plate was incubated at 37°C for 1 hour to allow initial adherence. On Day 3, after incubation, the broth

was carefully removed using a micropipette, as the biofilm had adhered to the surface of the wells. The wells were washed four times with 1% phosphate-buffered saline (PBS) at pH 7.2 to remove non-adherent cells. Subsequently, 100µl of 2% sodium acetate was added to each well and incubated for 20 minutes. After incubation, the plates were stained with 125µl of 0.1% crystal violet and allowed to stand for 1 minute. Excess stain was removed by washing the wells with sterile distilled water. Finally, the optical density (OD) of the stained biofilm was measured at 595 nm to quantify biofilm formation.

2. Preparation of Methanolic extraction of Clove

10g of clove powder was soaked in 50ml of methanol in a conical flask and placed in a dark for 48 hours at room temperature. The solvent was filtered through Whatman no.1 filter paper. Then the filtered extract was centrifuged at 3000 rpm for 10 mins. Collect the supernatant and stored in 4°C.

3. Agar Well Diffusion Method

100ml of Muller Hinton Agar was prepared and sterilized. The sterilized MHA was poured into the sterile petri plates and allowed to solidify. The MHB was prepared and the standardized bacterial suspension in MHB was prepared by inoculating the organism and incubated for 30 minutes, then adjust with 0.5 McFarland. By using the sterile cotton swab, the MHB culture was inoculated on the MHA agar plates by using lawn culture method. The wells are made by using sterilized 1ml tips. The methanol extraction of clove was poured into the wells of different concentration (25µl, 50µl, 75µl, 100µl) and the Erythromycin disc was taken as positive control. Then incubate the plate at 37°C for 24 hrs. Observe the results and measure the zone of inhibition.

4. Determination of Minimum Inhibitory Concentration

The MIC was carried out in a 96- well plate method. The Muller Hinton Broth was prepared and the standardized bacterial suspension was prepared by inoculating the organism in the MHB and incubated for 30 minutes, then adjust with 0.5 McFarland. The bacterial suspension was subjected to made as 1:10 dilution. 100µl of MHB was added to each well (A1-A10). 100µl of clove extract was added to the first well (A1), then 100µl is taken from the first well and is further transferred to next well by twofold serial dilution up to last well(A10). 75µl of diluted culture is added to each well (A1-A11). 100µl of MHB and 75µl of culture is taken as positive control (A11) and 175µl of MHB is taken as negative control. The plate was incubated for 24 hours at 37°C.

5. Determination of Antibiofilm activity

The Antibiofilm adhesion activity of all 4 strains of MRSA was carried out in a 96-well plate by Crystal violet method. The antibiofilm activity of clove extract against bacterial isolates was evaluated using a microtiter plate assay over three days. On Day 1, fresh LB broth supplemented with sucrose was prepared, and the bacterial isolates were inoculated into the medium. The cultures were then incubated at 37°C for 24 hours. On Day 2, the turbidity of the culture media was checked to confirm bacterial growth. The cultures were diluted in a 1:10 ratio by mixing 10µl of the sample with 90µl of LB broth containing sucrose. A sterile 96-well U-bottom microtiter plate was taken, and rows were designated based on clove extract concentrations: the first row for 5%, the second row for 2.5%, and the third row for 1.25%. Within each row, wells 1–3 were assigned to MRSA strain 1, wells 4–6 to MRSA strain 2, wells 7–9 to MSSA, and wells 10–12 to *Staphylococcus aureus* ATCC 25923. Different concentrations of clove extract (5%, 2.5%, and 1.25%) were prepared. To each well, 75µl of LB broth was added, followed by 75µl of the respective concentration of clove extract in the designated rows. Subsequently, 50µl of the diluted bacterial culture was added to each well, and the plate was incubated at 37°C for 24 hours.

On Day 3, after incubation, the broth was carefully removed using a micropipette, as the formed biofilm adhered to the well surface. The wells were washed four times with 1% phosphate-buffered saline (PBS) at pH 7.2 to remove non-adherent cells. Then, 100µl of 2% sodium acetate was added to each well and incubated for 20 minutes. Following this, the plates were stained with 125µl of 0.1% crystal violet and allowed to stand for 1 minute. Excess stain was removed by washing the wells with sterile distilled water. Finally, the optical density (OD) was measured at 595 nm to quantify the extent of biofilm formation and evaluate the antibiofilm activity of the clove extract.

RESULT AND DISCUSSION

1. Isolation and Identification of Bacterial strains

1.1. Cultural characteristics of isolates in different culture media

The isolates showed characteristic growth on different culture media, aiding in their identification. On MacConkey agar without crystal violet, pink to red colonies indicated lactose fermentation. On Mannitol Salt Agar, the formation of golden-yellow colonies with a colour change of the medium from red to yellow confirmed mannitol fermentation and supported the presence of *Staphylococcus* species. Growth on nutrient agar showed smooth,

circular, opaque colonies with a typical golden-yellow pigment. On blood agar, the isolates exhibited β -haemolysis, seen as clear zones around colonies due to complete red blood cell lysis.

These findings collectively suggest that the isolates are likely *Staphylococcus aureus*, as evidenced by their ability to ferment mannitol, produce characteristic pigmentation, and exhibit β -haemolysis, which are key diagnostic features of this organism.

1.2. Preliminary Identification test

Gram Staining: Gram staining of the isolated colonies revealed Gram-positive cocci arranged in irregular clusters, which described the characteristic of *Staphylococcus aureus*.

Biochemical Test: The Biochemical Test results were shown in Table 1.

Table 1: Biochemical Test Results.

BIOCHEMICAL TESTS	MRSA- 1	MRSA- 2	MSSA	<i>S. aureus</i> ATCC 25923
Catalase	Positive	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative	Negative
Slide Coagulase	Positive	Negative	Positive	Negative
Tube Coagulase	Positive	Negative	Positive	Negative

1.3. Antibiotic Susceptibility Test

The isolated *Staphylococcus aureus* strains were subjected to antimicrobial susceptibility testing using the Kirby–Bauer disc diffusion method with penicillin, erythromycin, vancomycin, cefoxitin, and oxacillin. The results revealed distinct resistance and sensitivity patterns characteristic of the isolates. The result was shown in Table 2.

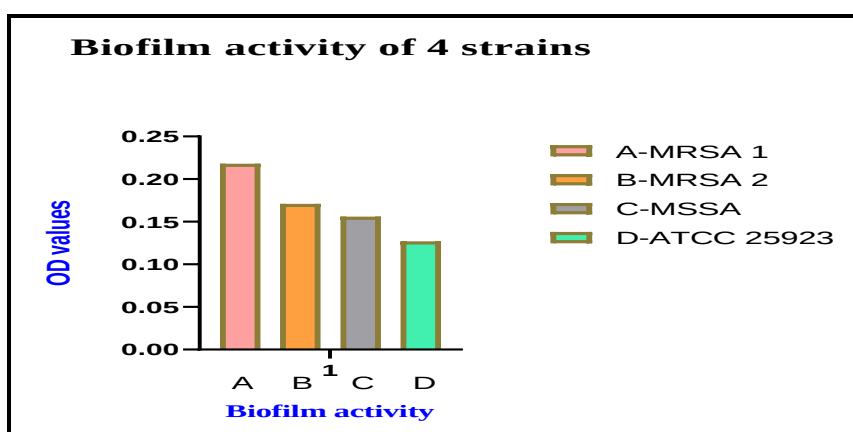
- **Cefoxitin (30 μ g):** Strong activity in MSSA and ATCC, reduced in MRSA isolates useful for resistance detection.
- **Oxacillin(1 μ g) & Penicillin (10 μ g):** Poor activity against MRSA, confirming methicillin resistance and β -lactamase production.
- **Vancomycin (30 μ g):** Retains activity against MRSA, though inhibition zones are smaller, consistent with its role as a last-line therapy.
- **Erythromycin (10 μ g):** Moderate activity, but reduced effectiveness in MRSA compared to MSSA and ATCC.

Table 2: Antibiotic Susceptibility Test (AST) Results.

	Cefoxitin	Oxacillin	Vancomycin	Penicillin	Erythromycin
MSSA	19mm	10mm	15mm	6mm	13mm
ATCC	25mm	19mm	22mm	15mm	18mm
MRSA 1	16mm	8mm	10mm	6mm	12mm
MRSA 2	18mm	12mm	12mm	6mm	10mm

1.4. Biofilm activity of the isolates

The microtiter plate assay was used to evaluate the 4 strains have the capacity to generate biofilms. Dense adhesion to the wells and high optical density values following crystal violet staining confirmed the strains significant biofilm activity. The dense and homogeneous appearance of the biofilm matrix demonstrated all the stains have the capacity for long-term colonization. These results demonstrate the strains have capacity to withstand host defence and antimicrobial treatments, which is in line with the established function of biofilms in MRSA pathogenicity. The results of 4 strains revealed distinct strong and weak adherence activity. The results are shown in Fig 1.

**Fig. 1: Biofilm activity of the isolates.**

2. Methanolic Extraction of Clove

Methanolic extraction of clove buds produced a dark brown colour with a strong characteristic aroma. The strong aroma and viscous nature of the extract indicated the presence of essential oils and phenolic constituents, particularly eugenol, which is the major bioactive compound in clove.

3. Agar well diffusion Method

Agar well diffusion assay was performed using clove extract against 4 strains. Four concentrations (25 µL, 50 µL, 75 µL, 100 µL) of clove extract were tested, with

erythromycin serving as the positive control. The results showed a concentration-dependent increase in the zone of inhibition, with the highest activity observed at 100 μ L. Erythromycin produced a larger inhibition zone compared to clove extract, confirming its effectiveness as a control. The results were shown in **Table 3** and **Fig 2**.

Table 3: Agar well diffusion.

Strains	25 μ l	50 μ l	75 μ l	100 μ l	Control (Erythromycin)
MRSA Strain 1	8mm	12mm	13mm	15mm	24mm
MRSA Strain 2	8mm	12mm	15mm	17mm	25mm
MSSA	8mm	16mm	18mm	19mm	27mm
<i>S. aureus</i> ATCC	8mm	16mm	18mm	19mm	28mm

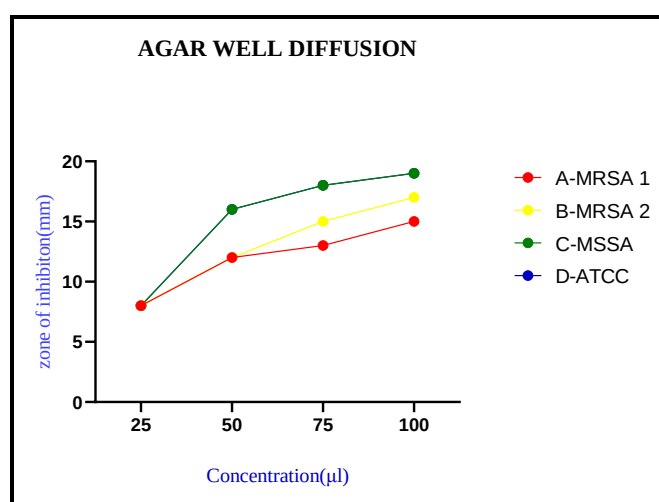


Fig. 2: Agar well diffusion graph.

4. Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of clove extract against *Staphylococcus aureus* strains (MSSA, ATCC strain, MRSA-1 and MRSA-2) was determined using the broth microdilution method in a 96-well microtiter plate. Bacterial growth was assessed by measuring optical density (OD) values, where a lower OD indicates effective inhibition of bacterial growth. Across all tested strains, a gradual increase in OD values was observed from wells 1 to 10, indicating a concentration-dependent response of the organisms to the clove extract. The lowest OD values were observed at higher extract concentrations, demonstrating effective growth inhibition, while increased OD values at lower concentrations reflected reduced antimicrobial activity. Overall, the results indicate that clove extract exhibits concentration-dependent antimicrobial activity against both methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. The ability of the extract to inhibit MRSA strains

suggests that bioactive compounds such as eugenol present in clove may interfere with bacterial cell membrane integrity and metabolic processes.

5. Determination of Antibiofilm Activity

The OD values revealed a concentration-dependent inhibition of MRSA biofilm formation by clove extract. Wells treated with lower concentrations (1.25%) showed relatively higher OD readings, indicating weaker biofilm adherence, whereas higher concentrations (5%) resulted in markedly reduced OD values showing no biofilm adherence. At the concentration of 1.25%, biofilm formation was significantly suppressed, with OD values approaching those of the blank control. These findings confirm that clove extract exhibits potent antibiofilm activity against MRSA. The 4 strains showed distinct stronger, weaker and no adherence activity. The comparative analysis of the biofilm and antibiofilm activity are shown in **Table 4** and **Fig 3**.

Table 4: Comparison of Biofilm and Antibiofilm activity.

Concentration (%)	A	B	C	D
Biofilm	0.468	0.171	0.156	0.127
5%	0.093	0.08	0.079	0.075
2.5%	0.148	0.117	0.101	0.098
1.25%	0.134	0.121	0.115	0.119

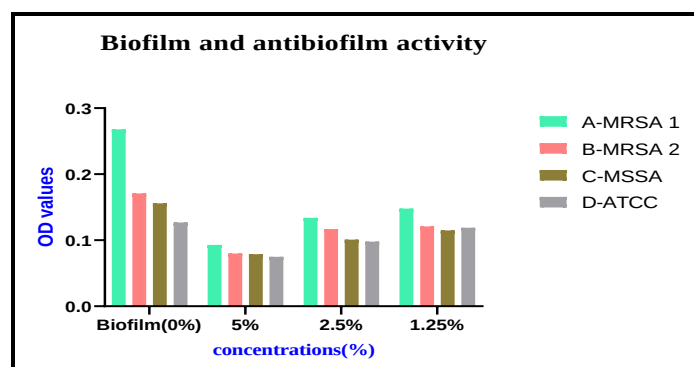


Fig. 3: Biofilm and Antibiofilm activity Comparative analysis.

CONCLUSION

Overall, the study provides strong evidence that clove extract can serve as a promising source of phytochemicals for developing novel antimicrobial agents. Clove, consumed daily as part of diet or traditional medicine, contributes to antimicrobial defence by supplying phytochemicals that weaken MDR bacteria's survival strategies. Its greatest promise lies in synergistic use with antibiotics, offering a natural route to combat resistance. Its ability to

inhibit both planktonic growth and biofilm formation suggests that clove could be integrated into future strategies to manage antibiotic-resistant infections and reduce reliance on conventional antibiotics. Hence, the Clove extract helps combat multidrug resistance by weakening bacterial defence mechanisms (biofilms, quorum sensing), exerting direct antimicrobial effects, and enhancing antibiotic efficacy. Its phytochemicals target pathways that conventional antibiotics often fail to address, making it a promising natural adjunct in managing resistant infections like MRSA.

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