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Zingiber officinale: Hot Aqueous Extract against the Growth and Production of MR Staphylococcus aurous Biofilms

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Abstract

Aims: to determine the efficacy of Zingiber officinale (Ginger) as a medicinal plant containing active compounds against the growth and biofilm production of methicillin-resistant Staphylococcus aureus (MRSA).
Study design: the Case control study.
Place and Duration of Study: Department of Biology Faculty of Education /Kufa University and hospitals of Al-Najaf Al-AShraf /Iraq for the period (September 2 to December 25 /2025)
Methodology: Twenty clinical isolates of MR Staphylococcus aureus and were diagnosed by using VITEK 2 compact system. Its efficiency in production of biofilm was performed by Congo Red Agar and Tissue culture plate methods. Four concentrations (2.5%, 5%, 10%, and 20%) of the hot aqueous ginger extract were prepared, and their efficacy against MR Staphylococcus aureus isolates was tested using both the well-diffusion and drop methods. Additionally, the effect of these concentrations on biofilm formation was evaluated using the tissue culture plate method.
Results: The aqueous ginger extract demonstrated the ability to inhibit the growth of multidrug-resistant Staphylococcus aureus specifically methicillin-resistant isolates achieving a maximum inhibition zone diameter of 35 mm via the agar well diffusion method at a 20% extract concentration. Although the isolates were capable of producing biofilms as evidenced by Congo Red agar and tissue culture plate assays (conducted both with and without glucose) various concentrations of the aqueous ginger extract reduced biofilm production levels, while higher concentrations completely inhibited its formation. Conclusion: Aqueous ginger extract plays active role in inhibiting the growth of MR Staphylococcus aureus and preventing its biofilm formation. Consequently, aqueous ginger extract can be recommended as a safe, natural alternative to address the issue of antibiotic resistance in S. aureus specifically the adverse effects associated with biofilm formation, which pose a major obstacle to treating the infectious diseases caused by this bacterium.

Keywords: Biofilm, MR Staphylococcus aureus, Zingiber officinale, Congo Red, Ginger.

1. Introduction

Staphylococcus aureus are among the leading infectious agents responsible for high mortality rates in 135 countries, according to a study published in The Lancet. The highest mortality rates associated with this bacterium have been observed in individuals over 15 years of age (Liu et al., 2024).

Virulence factors and biofilm formation are key attributes that protect Staphylococcus aureus from antimicrobial agents and the host immune system, while also facilitating bacterial adhesion to host cell surfaces. Implanted medical devices serve as a substrate for Staphylococcus aureus biofilm formation, leading to the development of infections

that are highly resistant to antimicrobial agents. According to the literature, biofilm-forming bacteria are 10 to 1000 times more resistant to antibiotics dispersed (Douglas et al., 2023).

The ability of bacteria to form antibiotic-resistant biofilms composed of extracellular polymeric substances (EPS), a complex mixture of extracellular DNA (eDNA), proteins, and polysaccharides makes eradicating pathogenic bacteria difficult (Idrees et al., 2021). The efficacy of penicillin-binding protein 2 (PBP2) is modulated by the fem gene family via the regulation of peptidoglycan precursor metabolism (Ambade et al .2023; Hou et al. 2023) while the agr quorum-sensing system indirectly influences resistance characteristics (including biofilm formation and antibiotic resistance) by dynamically (Rimi et al. 2024). It has recently been observed that inappropriate antibiotic treatment can have counterproductive results in treating Staphylococcus aureus biofilm-associated infections, as it promotes the manipulation of cellular systems and enhances biofilm growth (He et al., 2023). Other researchers have also noted that biofilm growth in the presence of antibiotics occurs in a dose-dependent manner, resulting in a more robust and adhesive biofilm (Rabin et al., 2015).

Zingiber officinale - Ginger- has long been used as a spice and an herbal medicine. Numerous bioactive compounds have been identified in ginger, such as phenolic and terpenoid compounds. The phenolic compounds in ginger consist primarily of gingerols, shogaols, and paradols, which are responsible for various biological activities (Kieliszek et al., 2020). Many studies have demonstrated that ginger possesses biological properties, including antioxidant, anti-inflammatory (Nile and park (2015) and antimicrobial activities (Akbari et al.2023).

Given the rising antibiotic resistance in Staphylococcus aureus compounded by its virulence factors and biofilm forming capabilities there is an urgent need to develop effective strategies to combat the global challenge posed by the infectious diseases it causes. One significant approach involves utilizing bioactive compounds derived from medicinal plants and herbs, particularly those with a history of safe human consumption in daily diets; Ginger is a key plant highlighted in this study, as it contains bioactive substances believed to play a role in inhibiting or preventing biofilm formation by Staphylococcus aureus.

2. Material and Methods

2.1 Bacterial Isolates

Twenty Clinical isolates of MR Staphylococcus aurous were obtained from the hospitals of Al-Najaf Al-AShraf /Iraq for the period (September 2 to December 25 /2025)

2.2 Identification of Staphylococcus aurous

For the purpose of diagnosing bacterial isolates, samples were initially cultured on manitol salt agar and blood agar media, then incubated at 37oC for 18 hours, and diagnosed according to the cultural characteristics both types of media (De la Maza et al.2020). the diagnosis of bacteria was confirmed by using VITEK compact system according to Manufacturers Instructions bioMe' rieux) ; ID for Gram positive Cocci Cards.

2.3 Collecting and preparing the aqueous extract of Zingiber officinale

The Ginger (Zingiber officinale) rhizomes were purchased from local markets. It was peeled, dried at room temperature and ground with electric grinder to obtain dry powder, the powder was packed I glass bottle until extraction and then prepared hot aqueous different concentrations (2.5, 5, 10, 20) %

2.4 Detection of crud active substances in Ginger Extract

The active crud materials were detected using each of: Meyer’s reagent for flavonoids, Molisch’s reagent Carbohydrates, Ferric chloride for phenols and (Lieberman- Burchard) reagent for identifies terpenoids, For the detection of volatile oils, a sodium hydroxide solution with drops of hydrochloric acid was used. (Harborne, 1998; Anyasor et al., 2010)

2.5 Testing MR Staphylococcus aurous against Antibiotic

The method of Bauer et al. (1996) was used to detect of MR Staphylococcus aurous resistance and sensitivity against different antibiotics resistance and sensitivity (Oxacillin, Amoxyclave, Rifampin, Ciprofloxacin, Ceftriaxone, Erythromycin, Gentamycin, Tigecycline, Fusidic acid). the bacterial isolates were considered sensitive or resistant to these antibiotics used based on international specifications contained in CLSI (2025). then it was confirmed that the isolation were diagnosed using the Vitek2 compact system ID and AST Biomerieux (France) specific card for gram positive bacteria.

2.6 Effectiveness of Zingiber officinale aqueous extract against MR Staphylococcus

The well diffusion method (Piddock, 1990) was used, MRS Staphylococcus aurous isolates (1.5 * 108 cell/ml) were spreading on Muller- Hinton Agar, the wells 8 mm were made by cork poorer, plant extract concentration (2.5, 5, 10, 20) % were added, and the negative control represented by normal saline. incubated at 37oC for 24 hours, The diameters of the inhibition zones were measured using a ruler, and then the well diameter was subtracted from the averages of the inhibition zones, each separately.

The spotting method also used by adding 5 microliter of each concentration on the bacteria grown on Muller- Hinton Agar, then the inhibition zone were measured to be compared with well diffusion.

2.7 Detection of MRS Staphylococcus aurous biofilm formation and impact of Ginger extract.

Six isolates of the most antibiotic-resistant Staphylococcus aurous were selected to test their efficiency in producing biofilms. There are two method were used to determine production of bacterial biofilm, the first was tissue culture plate method described by (Mathur et al., 2006) and the Second was Congo Red agar according to Freeman et al., (1989)

Tubes containing brain heart infusion broth were inoculated with Staphylococcus aurous isolates. A bacterial suspension was then prepared for each isolate at McFarland standard turbidity (1.5 *108) cell/ml. Twenty microliters of the bacterial suspension were taken and placed in individual wells of microtiter plate and incubated at 37oC for 24 hours. after that the plate was washing by phosphate buffer saline (pH=7.2) to remove non-adherent bacteria, then sodium acetate (2%) was added to fix the adhering bacterial cells. It was then stained with (0.1 w/v) crystal violet. washed with distilled water. The absorbance was then measured by ELISA auto reader at a wavelength of 570 nm. The same steps as above were followed when measuring the effect of the Ginger extract on biofilm production by adding equal volumes of different concentrations (2.5, 5, 10, 20) % of the extract to the bacterial isolates individually.

The ability of bacteria to form a biofilm was quantitatively estimated by measuring the optical density of the adhering dye, and the results were recorded according to the following table:

Biofilm formation	**Strong**	**Weak**	**Non produce**
Optical density	≥ 0.240	0.240 - 0.125	< 0.125

2.8 Statistical Analysis

The results were statistically analyzed using (SPSS) software by performing a one-way ANOVA and using the least significant difference (LSD) method with p value ≤ 0.05.

3. Results and Discussion

The results on the active raw compounds in the plant extract of ginger revealed that it contains flavonoids, as evidenced by the extract turning yellow after the addition of Meyer,s reagent. A purple ring also formed in the extract tube after the addition of Molisch,s reagent indicating the presence of sugars in the aqueous extract of the plant. A bluish-green

color is produced when using Lieberman- Burchard reagent to confirm the presence of terpenoids. When the extract was examined for volatile oils, a light oily ring was formed upon the addition of NaOH along with drops of HCl.

Studies have shown that ginger is rich in active compounds, as it contains phenols in various forms (Gingerols, Shogaols, and Paradols) that transform into other forms with changes in temperature and storage. Ginger also contains a number of terpenes, including α-curcumene, zingiberene, α-farnesene, β-bisabolene and β-sesquiphellandrene which are the main components of its volatile oils. (Yeh et al. ,2014). In addition, it contains polysaccharides, organic acids, lipids and crud plant fiber. (Schadich et al., 2016)

The results regarding resistance and sensitivity of the Staphylococcus aureus isolates, shown in Figure (3-1), indicate that the bacteria were 100% resistant to both Oxacillin and Penicillin, The second most common antibiotic was Erythromycin, with a percentage of 80%, Meanwhile, the bacterial isolates exhibited varying degrees of resistance to the remaining antibiotics, namely Ceftriaxone, Tig, clindamycin, fusidic acid, Ciprofloxacin and rifampin, respectively. While the isolates were sensitive to the antibiotic Vancomycin at a rate of 84%.

The intrinsic resistance mechanisms of Methicillin-resistant Staphylococcus aureus (MRSA) are due to the presence of the mecA gene, which is the main molecular basis of resistance. This gene is encoded by penicillin-binding protein 2a (PBP2a), the latter provides a strong defensive barrier against beta-lactam antibiotics by remodeling the cell wall biosynthesis pathway (Abebe and Birhanu 2023) . Several pathogenic bacterial isolates exhibit heterogeneous resistance to antibiotics, including a Subpopulation with a minimum inhibitory concentration (MIC) higher than the minimum inhibitory concentration (CBP), and are expected to better resist the drug concentrations achieved in the treated patient (Heidarian et al.,2024)

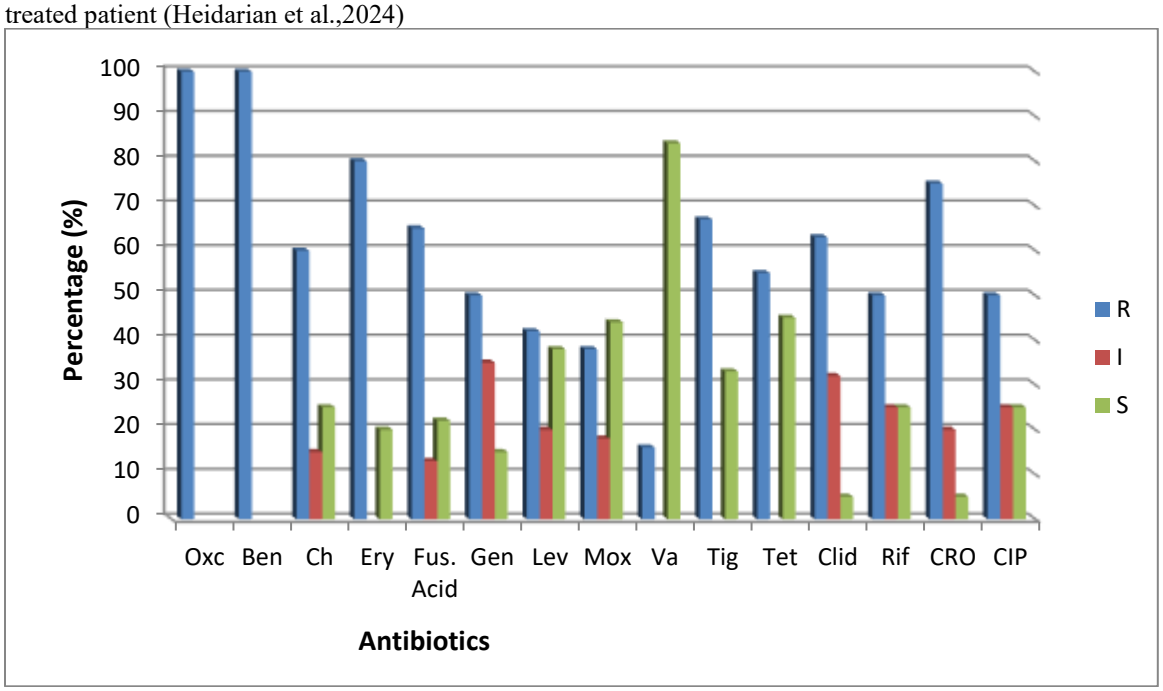


Figure (1): *Resistance and susceptibility of Staphylococcus aureus to the various antibiotics*

Table (1) Shows a comparison of the effect of Zingiber officinale extract on inhibiting the Staphylococcus aureus isolates under investigation using the well diffusion method (Figure 1) and the Spotting method. This finding shown in the table indicate that the average diameters of the inhibition zones increased significantly ($p \leq 0.05$) with increasing concentrations (5, 10,15,20) % of the Zingiber officinale extract, respectively, for both methods. A concentration of 20% gave the highest inhibition rate of bacteria at a diameter of 35 mm using the diffusion method as Figure (2). It is also noted that concentration 2.5% failed to produce any bacterial inhibition.

Molecular weight plays a crucial role in agar diffusion (Flanagan and Steck, 2017). These observations indicate that as the diffusion rate increases with increasing plant extract concentration, the inhibition zone increases proportionally with the depth of the agar layer. The accuracy of this test is affected by the uniformity of the agar depth, which is ideally 4 mm (CLSI, 2013).

Ginger contains gingerol, which is classified according to the length of its unbranched alkaloid chain, 6-gingerol is a bioactive compound with a mild pungent flavor in ginger and has anti-inflammatory and antibacterial properties (Sang et al. 2020). Some pathogenic bacteria, particularly Staphylococcus aureus, encode a unique enzyme, 6-hydroxymethyl-7,8-dihydropyrin pyrophosphate kinase (SaHPPK) (Dennis et al. 2016), against which no antibiotics have been found. Therefore, the active ingredients in the plant extract of ginger are likely to be anti- Staphylococcus aureus agents, especially those that are methicillin-resistant. Rampogu et al. (2018) proposed nominating both shogaol, and gingerenone-A as antibacterial drugs because molecular dynamics simulation results showed their directional stability and strong interactions with catalytic active residues in the catalytic pocket of the SaHPPK enzyme.

Table (1): Antibacterial effectiveness of different concentrations of Zingiber officinale extract on Staphylococcus aureus isolates.

Plant extract conc. (%)	Average of inhibition zone (mm) (main ± Sd)	
	Well diffusion method	Spotting method
2.5	0	0
10	5 ± 0.54	5 ± 0.86
15	15 ± 1.52 *	10 ± 1.45 *
20	35 ± 2.9 **	18 ± 2.08 **

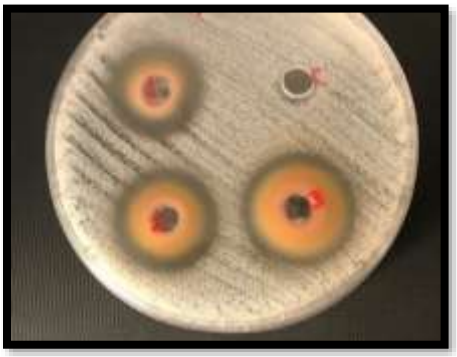


Figure (2): *Inhibition of Staphylococcus aureus using different concentrations of the extract by diffusion method. (the well at the top left is 5% conc., below left is 10 %, but the below right is 20%, while the top right is control)*

After conducting sensitivity and resistance tests on Staphylococcus aureus isolates, the six most efficient isolates were selected based on their multidrug resistance to the antibiotics used in the study. These isolates were then tested for biofilm production using both Congo Red Agar medium and tissue culture plate methods

The results obtained the ability of all six isolates to produce biofilm through their growth on Congo Red medium, as shown in Figure (3), the colonies appeared black after growing on the culture medium overnight, this is due to the presence of glucose, which reacts with Congo red dye through bacterial action, resulting in the production of a black color (Mathur et al., 2006). The Congo red agar method is simple, specific, and 100% sensitive, with an accuracy of 43% compared to other methods used to detect biofilm production (Banerjee et al., 2018)

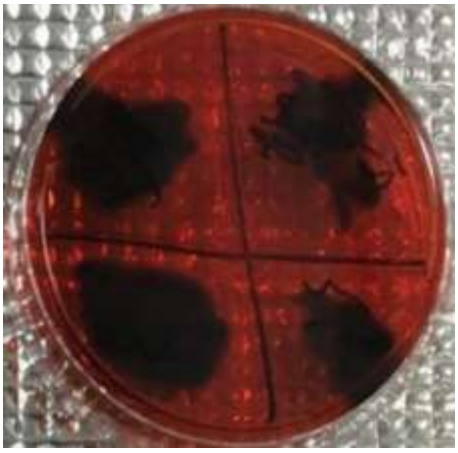


Figure (3): *Detection of Staphylococcus aureus biofilm formation by Congo Red Agar medium (Bacterial colonies appear black after growing on the culture medium for 24 hours)*

Regarding the production of biofilm using tissue culture, it can be observed from Figure (4) that the biofilm production of the isolates varied; some were highly productive, others moderately productive, with the exception of isolate number one, which was weak. Furthermore, there was an increase in production in the presence of glucose compared to its absence in the culture medium. This reinforces the findings of Wieland et al. (2025) who, during their study of two Staphylococcus aureus strains, Newman and Newman D2C, found that they differed significantly in their ability to adhere to non-living surfaces and form biofilms on them in various laboratory tests. However, when tested in a mouse model of foreign body-associated infection, both strains produced similar vegetative growth on the implanted catheter tube.

The culture medium used in bacterial cultivation is of paramount importance in increasing biofilm formation. Furthermore, the increased production of biofilms in the presence of glucose may be attributed to its phosphorylation. Vasu et al. (2017) found that phosphorylation by glucokinase (glkA) increased its activity fourfold, highlighting the important role this compound plays in biofilm formation.

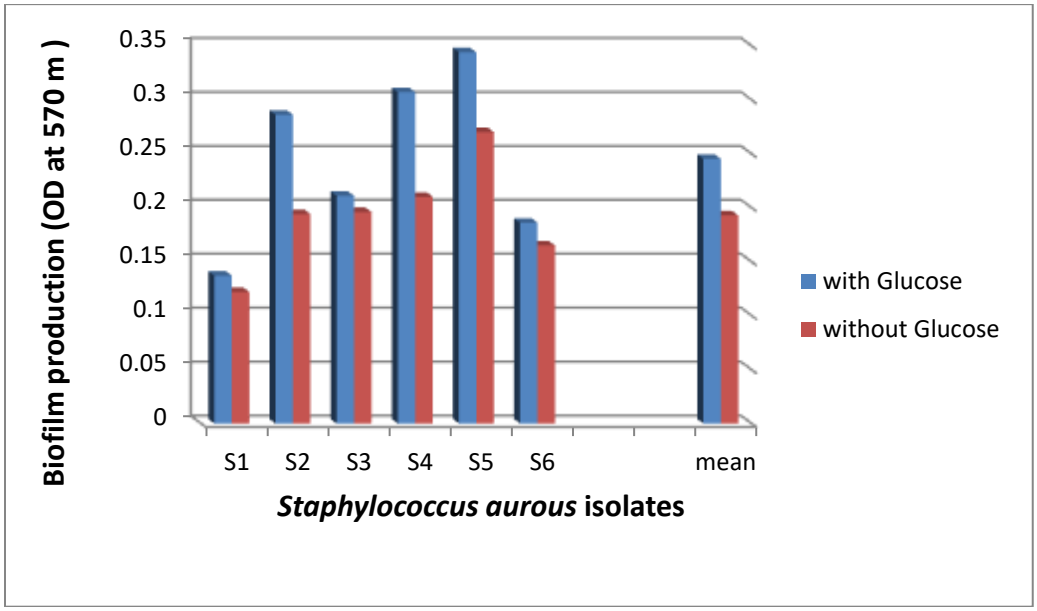


Figure (4): *Staphylococcus aureus biofilm production in the presence and absence of glucose.*

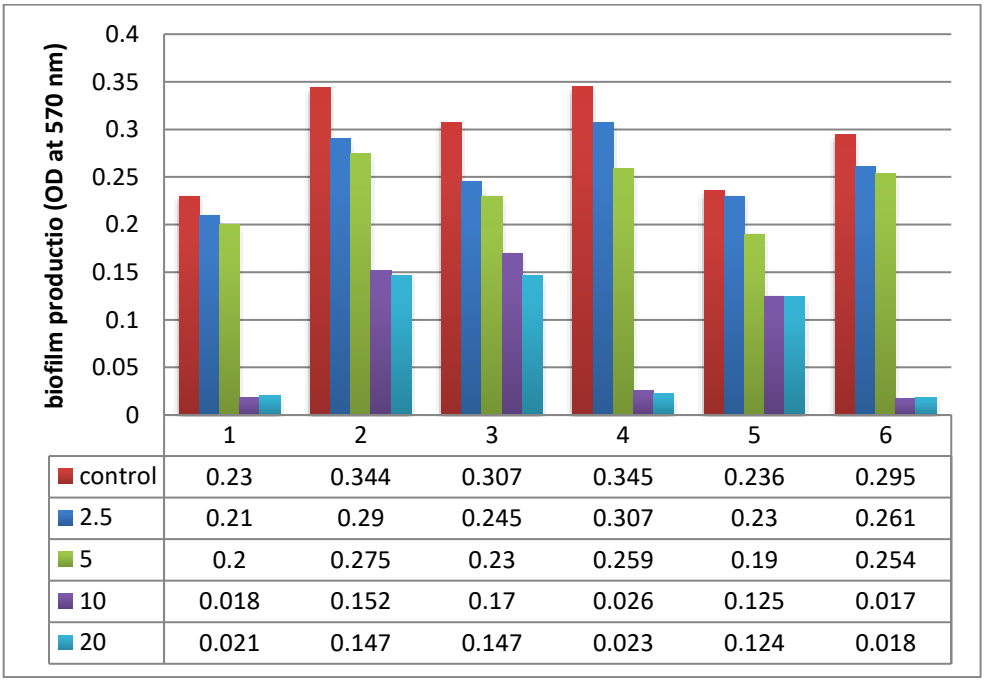
When investigating the effect of Zingiber officinale extract on the biofilm production of Staphylococcus aureus, the data in Figure (5), (6) showed that all isolates exhibited a significant decrease in biofilm production, transforming into non-biofilm-producing bacteria due to the extract, particularly at concentrations of 10% and 20%. This can be explained by Bioactive chemicals rise in tandem with extract concentration, which reduces the production of biofilms.

There are several bioactive substances with antibacterial properties can be found in ginger extracts. Some of these constituents have been identified, including zingerone, 6-gingerol, and 6-shogaol. According to certain research, zingerone is efficient in preventing Pseudomonas aeruginosa from forming biofilms (Kumar et al .2013), The current results also confirm what the researchers (Akbari et al.2023) have concluded: that ginger extract has a greater effect on Gram-positive bacteria, specifically B. subtilis, than on Gram-negative bacteria P. aeruginosa. Furthermore, the crude ginger extract and its methanolic fraction inhibited the adhesion and biofilm formation of Streptococcus mutans and its glucan synthesis by downregulating virulence genes. Consistent with the in vitro study, a reduction in the development of caries induced by this bacterium was observed in a group of treated rats (Hasan et al. 2015).

Initial surface adhesion is the first stage of biofilm formation, which is followed by biofilm growth and maturation and dissemination. It is an intricate and evolving process that encompasses alterations in physiological characteristics, the control of multiple genes, metabolic variability, and the capacity to react to environmental and cellular stimuli (Chai et al., 2024). Therefore, it is expected that the 20% concentration inhibited the adhesion process, which is the initial step in bacterial biofilm formation.

Plant extracts affect bacterial cell membranes through both direct and indirect mechanisms. The direct mechanism involves biologically active components disrupting bacterial cellular structures, destabilizing the biofilm matrix, or inhibiting vital enzymatic activities, thereby reducing bacterial adhesion, aggregation, and viability [Bandyopadhyay et al. (2024); Huang et al. (2025). For instance, ethanolic extracts of Torilis japonica have been found to inhibit the expression of adhesion genes—specifically sarA, icaA, and hla—in Staphylococcus aureus (strain KCTC 1927), thereby suppressing biofilm formation (Kim et al., 2022)

Based on the current results, concentrations of 20% and 15% inhibited biofilm formation in the Staphylococcus aureus isolates under study. This potential effect may be attributed to the higher concentration of active compounds in the ginger extract at these levels, as well as to direct and indirect effects on the bacteria that prevent adhesion and biofilm formation.



	1	2	3	4	5	6
control	0.23	0.344	0.307	0.345	0.236	0.295
2.5	0.21	0.29	0.245	0.307	0.23	0.261
5	0.2	0.275	0.23	0.259	0.19	0.254
10	0.018	0.152	0.17	0.026	0.125	0.017
20	0.021	0.147	0.147	0.023	0.124	0.018

Figure (5): *Impact of varying aqueous ginger extract concentrations on Staphylococcus aureus biofilm formation.*

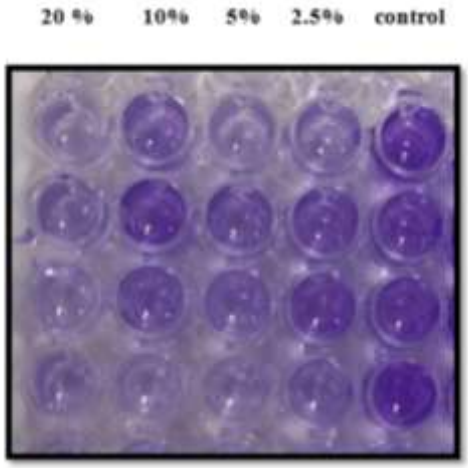


Figure (6): *Reduction of Biofilm Production Levels in Staphylococcus aureus and Inhibition of Its Formation by Various Concentrations of Aqueous Ginger Extract.*

4. Conclusion

The results of the current study indicate that the aqueous extract of ginger, at various concentrations, demonstrates high efficacy in inhibiting the growth of Methicillin resistant Staphylococcus aureus isolates and reducing their biofilm production levels, as well as preventing biofilm formation at higher concentrations. Given its content of active natural compounds, the aqueous ginger extract is considered safe for use and a suitable alternative to antibiotics for combating Methicillin resistant Staphylococcus aureus a bacterium responsible for serious diseases that are sometimes difficult to treat.

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