



Antibacterial and Antibiofilm Efficacy of *Aloe vera* Saponins in Wound and Burn Infections

Saba Mejdhab Badr^{1*} , Rana A. H. Al-Lami² , Hanady S. Al-Shmgani³ 

^{1,2}Department of Biology, College of Science for Women/ University of Baghdad, Baghdad, Iraq

³Biology Department, College of Education for Pure Science (Ibn Al-Haitham), University of Baghdad, Baghdad, Iraq

*Corresponding Author

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Abstract

Antibiotic-resistant bacteria, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (MRSA), pose a significant threat to global health. An immediate investigation into alternative antimicrobial and anti-biofilm agents is necessary. This study evaluated the antibacterial and anti-biofilm properties of *Aloe vera* saponin extract against multidrug-resistant *S. aureus* and *P. aeruginosa*. The main objective of the research is to propose saponin, extracted from *Aloe vera* leaf gel, as an alternative therapeutic agent to antibiotics. After collecting 150 samples, 28 *S. aureus* and 30 *P. aeruginosa* isolates were identified. Standard antibiotic tests classified these strains as XDR or MDR. The MIC measured antibacterial effectiveness, and sub-inhibitory doses evaluated anti-biofilm effects. The results demonstrated that the saponin extract effectively inhibited bacterial growth and biofilm formation. Most *S. aureus* isolates had a MIC of 62,500 µg/mL, while *P. aeruginosa* isolates were more susceptible, with MIC values as low as 15,625 µg/mL. Additionally, the extract significantly reduced biofilm formation, decreasing it by up to 69.71% in *S. aureus* and 86.46% in *P. aeruginosa*. The conclusion is that *Aloe vera* saponin extract could potentially replace traditional treatments, especially for resistant infections in burns and wounds. Its proven ability to inhibit growth and biofilm formation of resistant *S. aureus* and *P. aeruginosa* makes it a valuable tool against antibiotic resistance.

Keywords: *Aloe vera* saponin, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, biofilm.

1.Introduction

Humanity appears to be moving toward what is known as the post-antibiotic era. This implies that a simple bacterial species can become increasingly fatal to humans¹. Antibiotic-resistant bacteria, commonly referred to as “superbugs,” pose a significant global public health threat, particularly for critically ill or hospitalized patients. Methicillin-resistant *Staphylococcus aureus* MRSA, Carbapenem-resistant Enterobacterales CRE, Vancomycin-resistant Enterococci VRE, and multidrug-resistant *Pseudomonas aeruginosa* and *E. coli* are examples. Since novel drugs may acquire resistance, new therapeutic routes are needed now. In the post-antibiotic era, medicinal plants with potent secondary metabolites may be utilized to develop antibacterial medications². *Staphylococcus aureus*, a common pathogen, has evolved pathogenicity, especially since the early 2000s, with the advent of CA-MRSA. These strains are methicillin-resistant and pathogenic³. The ability of *S. aureus* to form biofilms enhances its resistance to treatment and may lead to fatal infections in organs⁴. For immunocompromised individuals, *Pseudomonas aeruginosa* can cause severe diseases, including wound and burn infections, which can increase morbidity and mortality⁵. Increasing resistance of *P. aeruginosa* to various pharmaceutical classes and the advent of extensively drug-resistant (XDR) strains have become a public health

problem, necessitating alternative therapies. Biofilm is an organized bacterial population, and the membrane-like extracellular matrix (ECM) formed by bacterial colonies adhering to extracellular polymeric substances (EPS), such as polysaccharides, nucleic acids, and proteins, generated during growth⁶. The interaction between EPS and bacterial aggregation contributes to the biofilm's cohesiveness and viscoelasticity⁷. Thus, germs can adhere to both biotic and abiotic surfaces. Pathogenic biofilms cause chronic infections⁸, which increase antibiotic resistance and enable them to evade the immune system, leading to chronic, difficult-to-treat diseases⁹. The search for new antibacterial agents is necessary due to the limited availability of effective treatments to treat bacterial infections¹⁰. *Aloe vera* has been used medicinally and cosmetically in dermatology for its various benefits¹¹. Flavonoids, phenylpropanoids, coumarins, and several derivatives of phenylpyrone, phenol, chromone, anthraquinone, and phytosterols are among the 110 potentially bioactive compounds in this plant¹². Saponins are essential due to their attractive chemical structures and their antibacterial, antioxidant, and anti-inflammatory properties. Saponins may increase ion permeability in microbial cell membranes, allowing critical internal components to leak, disrupting enzymatic systems, restricting respiration, and inhibiting protein synthesis¹³. These properties make saponins an attractive extract for treating *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections in wounds and burns.

2. Materials and Methods

2.1. Collection and identification of bacterial isolates

In this study, 150 burn and wound samples were collected from patients treated at Baghdad Medical City's Burns Hospital and Ghazi Al-Hariri Hospital, Iraq, between October 2024 and February 2025. The isolates included MDR and XDR strains of *S. aureus* and *P. aeruginosa*. Bacterial identification was performed using Gram staining and biochemical tests (i.e., catalase, oxidase, coagulase, and API 20E/20NE systems). The Vitek 2 method was used at the hospital labs to detect the bacteria. Antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method (CLSI guidelines) was performed to classify MDR/XDR strains.

2.2. Biofilm detection

Microorganisms were grown in Brain-Heart Infusion (BHI) broth supplemented with 1% glucose. The culture was incubated at 37 °C for 18 to 24 hours. Subsequently, 180 µl of BHI broth and 20 µl of the bacterial culture were added to sterile 96-well microtiter plates. 200 µl of uninoculated BHI broth in separate wells served as negative control wells (blanks). After sealing, the plates were incubated at 37°C for 18-24 hours. After incubation, sterile distilled water was used to gently wash the wells to remove non-adherent cells. The wells were then left to air dry at room temperature for 15 minutes. 200 µl of ethanol was added to fix the adherent cells, and the plate was allowed to air-dry again. The adherent biofilm was then stained by adding 200 µl of crystal violet solution (concentration unspecified, but typically 0.1%) and allowed to sit for 15 minutes. The dish was rinsed with distilled water to remove excess color, then left to air-dry for 30 minutes. To quantify the biofilm, 200 µl of 95% methanol was added to dissolve the stained adhering cells, and the mixture was left for 10 minutes¹⁴. An ELISA microplate reader was used to measure the optical density (OD) at 580 nm. Blanks (uninoculated wells) are used to establish the background absorbance (C-). Biofilm growth is confirmed in isolates that exhibit OD values greater than those of the blank control. An equation is used to calculate the cutoff value (O.D.C.) to categorize the isolates. A standard method was then used to measure the optical density (OD) of each isolate to identify potent biofilm isolates. Compared OD values to a cutoff value, O.D.C., to see whether biofilm was growing. Weak production meant $OD < 2 \text{ ODC}$, moderate production meant $2 < OD < 4$, and robust production meant $OD > 4$.

2.3. Antibacterial susceptibility testing

Adhered to CLSI 2024 standards for Kirby-Bauer disk diffusion testing, added 3 mL of normal saline to the overnight nutrient agar-grown bacterial colonies. Each suspension was adjusted to a 0.5 McFarland standard (approximately 1.5×10^8) for turbidity—colony-forming units per

milliliter by density. Four antibiotic disks were carefully placed on Mueller-Hinton agar plates using sterile, calibrated forceps. The disks were far enough apart to prevent zone-crossing during incubation. Inverted plates were held at 37°C for 18–24 hours. Areas of inhibition were assessed after incubation to determine the antibiotic sensitivity of the isolates based on the degree of growth inhibition. MDR and XDR were then distinguished by counting the number of antibiotics they resisted.

2.4. Prepare the saponin extracts

To extract gel from *Aloe vera*, Fresh leaves served as the plant material. Fresh leaves from the plant were rinsed twice with running water, air-dried, and saponins were extracted from the leaf gel through multiple procedures. The *Aloe vera* gel was extracted from the skin, dried in a heat chamber at 45°C for 24 hours, and then pulverized into a powder¹⁶. To obtain saponin, 10 g of powder was mixed with 50 ml of 20% ethyl alcohol and agitated for three hours in a water bath at 55°C. After filtration, 100 ml of 20% aqueous ethanol was applied to extract the residue. The entire filtrate was reduced to 40 ml in a water bath at 90°C. This concentrated solution was transferred to a separating funnel containing 20 ml of diethyl ether. The aqueous layer containing saponin was preserved, while the ether layer was discarded¹⁷. Note: After preparing the saponin extract, it is in a solid state and must be diluted with a suitable amount of DMSO (Dimethyl sulfoxide), to facilitate its use with a micropipette for testing.

2.5 Antibacterial activity assay of extracts

The Microtiter Plate Method (MTP) was used to assess the antibacterial activity of the produced extract. Assays using the Minimum Inhibitory Concentration (MIC) and Sub-Minimum Inhibitory Concentration (Sub-MIC) applied the resazurin-assisted microdilution method in Mueller-Hinton Broth (MHB) on 96-well microtiter plates to test the Saponin Extract's efficacy to kill bacteria against *S. aureus* and *P. aeruginosa*. The test employed a two-fold serial dilution to achieve final concentrations ranging from 10 to 1 in each well. The first well had a starting concentration of 125,000 mL. After making the serial dilutions with 100 µl of MHB in each well, 100 µl MHB in each well, 100 µl of a standardized bacterial suspension (equal to McFarland no. 0.5 (1.5×10^8 CFU/ml)) was added to all of the test and positive control wells. The uninoculated MHB was used as the negative control. The plate was then incubated at 37 °C for 24 hours. After incubation, each well received 30 µl of 0.015 resazurin solution and was incubated again for 2–4 hours. The MIC was the lowest concentration that didn't change color (resazurin remained blue), indicating that bacterial growth was inhibited. The next pink well was the Sub-MIC¹⁸.

2.6 Biofilm-Prevention Effectiveness

A microtiter plate (MTP) assay was employed to evaluate the biofilm-forming capabilities of test materials against *S. aureus* and *P. aeruginosa* isolates. Added 10 µL of the sub-MIC concentrations of the saponin extract to the wells. Positive control wells contained solely bacterial suspension, whereas negative wells contained Brain Heart (BHI) medium only. The plates were incubated at 37°C for 24 hours. Carefully cleaned each well to remove planktonic bacteria after incubation, and stained the residual biofilm in each well with crystal violet (1%). Stained biofilm absorbance was measured at 580 nm using an ELISA Reader¹⁹. Biofilm inhibition % was obtained using the following **Equation (1)**²⁰:

$$\% \text{ Biofilm inhibition} = [(\text{OD Control} - \text{OD Sample}) / \text{OD Control}] \times 100 \quad (1)$$

2.7. Evaluation using statistical methods

A statistical package for the social sciences, SPSS (v.16), was used for data analysis. The means and standard errors (SE) of each experimental group were computed for descriptive statistics. After doing a one-way ANOVA, we used the Least Significant Difference (LSD) test for post hoc comparisons.

3. Results

3.1. Identification of bacteria:

The characterization of 150 clinical isolates. The Vitek-2 method accurately identified 28 *S. aureus* isolates and 30 *P. aeruginosa* isolates, demonstrating a 99.99% accuracy rate according to the system's analytical profile index. Furthermore, biochemical assays, as shown in **Table 1**, were used to characterize the isolates, which were cultivated on selective media.

Table 1. Characteristics of *S. aureus* and *P. aeruginosa*

Characteristics	<i>S. aureus</i>	<i>P. aeruginosa</i>
Gram stain	Positive	Negative
Shape	Cocci	Rods
Capsule	Non-Capsulated	Non-Capsulated
Spore	Non-Sporing	Non-Sporing
Motility	Negative (Non-Motile)	Positive (Motile)
Hemolysis	Positive Beta	Positive Beta
Catalase	Positive	Positive
Coagulase	Positive	Negative
Indole	Negative	Negative
Oxidase	Negative	Positive
Urease	Positive	Negative

3.2 Biofilm detection

A sensitive microtiter plate test was used to quantify biofilm formation in multidrug-resistant bacterial isolates. As shown in **Tables 2** and **3**, *S.aureus* exhibited biofilm formation in 85.71% of isolates. The chi-squared test revealed a statistically significant difference in biofilm formation levels ($P \leq 0.0001$). In *P. aeruginosa*, 53.33% of isolates exhibited moderate biofilm formation, while 40% exhibited strong biofilm formation. The chi-squared test showed no statistically significant difference in the distribution of biofilm formation levels ($P = 0.07$).

Table 2. *S. aureus* isolates according to biofilm formation.

Biofilm produce	No. of <i>S. aureus</i> isolates	Percentage (%)
Strong	12	85.71%
Moderate	1	7.14%
Weak	1	7.14%
Non adhere	0	0%
Total	14	100%
Chi (χ^2)	17.28	
P value	<0.001**	

Table 3 *P. aeruginosa* isolates according to biofilm formation

Biofilm produce	No. of <i>P. aeruginosa</i> isolates	Percentage (%)
Strong	6	40%
Moderate	8	53.33%
Weak	1	6.67%
Non adhere	0	0%
Total	15	100%
Chi (χ^2)	5.2	
P value	0.07 NS	

3.3. Antibiotic susceptibility testing

S. aureus isolates exhibited 100% resistance to Oxacillin and Penicillin, and 75% resistance to Cefoxitin. High resistance rates were also observed for Erythromycin, Ciprofloxacin, and Levofloxacin, while Gentamicin resistance ranged from 68% to 96%. In contrast, the isolates showed high susceptibility to Chloramphenicol (96.43%), Nitrofurantoin (100%), Clindamycin (92.86%), and Rifampin (92.86%). Linezolid susceptibility was 89.29%. as shown in **Figures 1** and **2**.

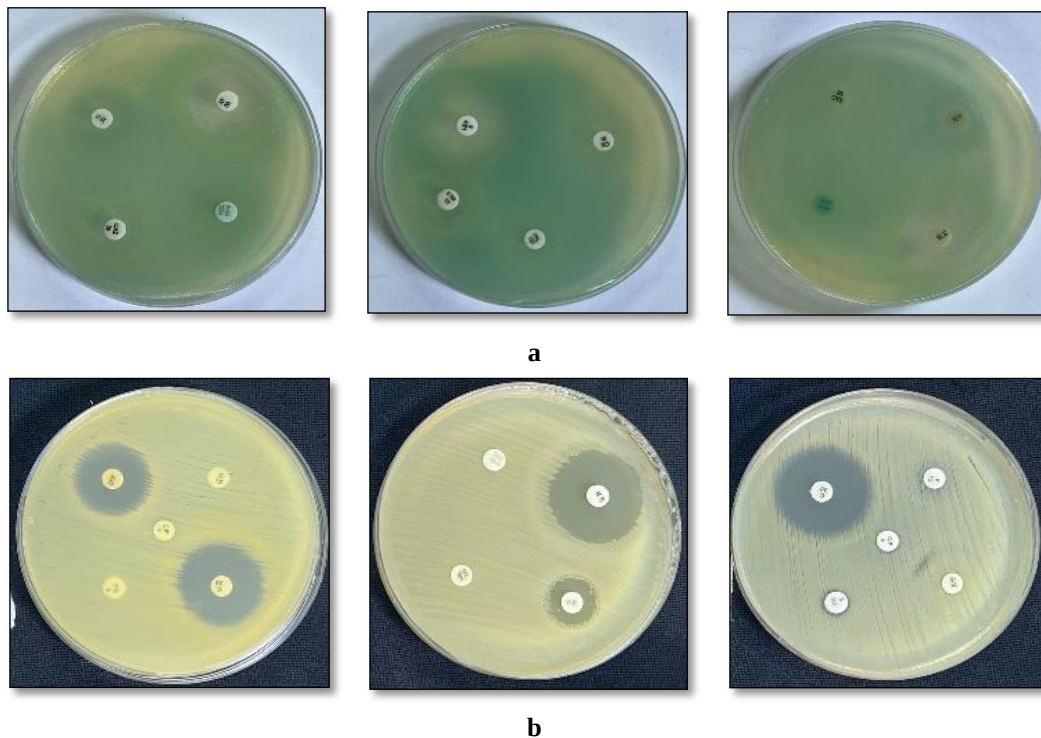


Figure 1. Antibiotic Susceptibility Testing (Kirby-Bauer Method): a. *Pseudomonas aeruginosa*, b. *Staphylococcus aureus*

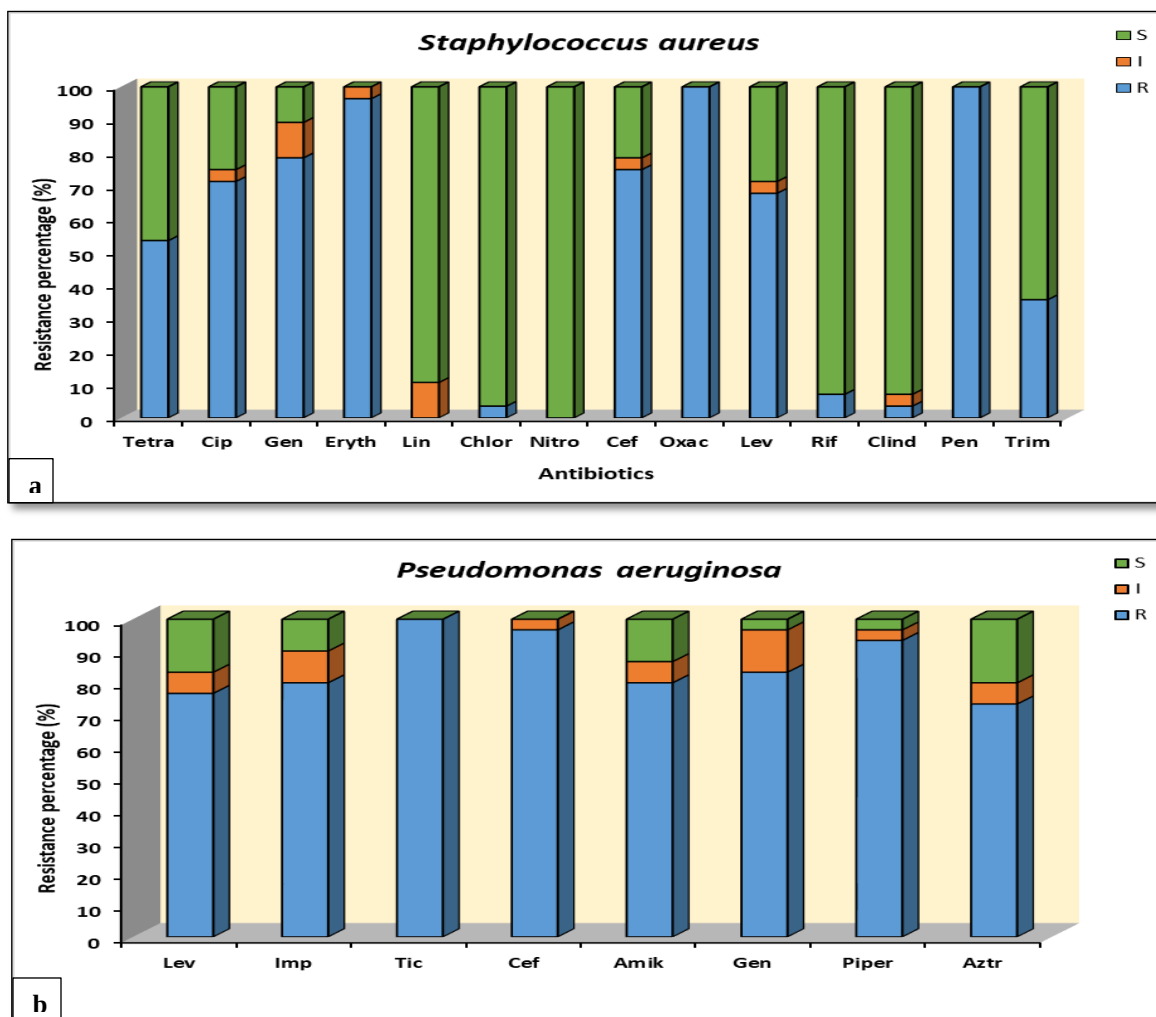


Figure 2. Antibiotic susceptibility testing a. *S. aureus*, b. *P. aeruginosa*.

All *P. aeruginosa* isolates exhibited resistance to Ticarcillin, while resistance rates were 96.67% for Ceftazidime, 93.33% for Piperacillin, 83.33% for Gentamicin, 80% for Imipenem, 80% for Amikacin, and 76.67% for Levofloxacin. The resistance rate to Aztreonam was 6.67%. Antibiotic susceptibility patterns varied significantly ($P \leq 0.001$). However, overall resistance, intermediate susceptibility, and susceptibility did not differ significantly among the tested antibiotics.

3.4. Determination of Minimum Inhibitory Concentration using the MTP method for antibacterial action of saponins

The resazurin assay was used to evaluate the antibacterial activity of the saponin extract by comparing color changes after incubation with those of the control (**Figure 3**). The saponin extract exhibited a minimum inhibitory concentration (MIC) of 62.500 $\mu\text{g/mL}$ against *S. aureus*, although one isolate showed a higher MIC of 125.000 $\mu\text{g/mL}$. For *P. aeruginosa*, two isolates exhibited an MIC of 62.500 $\mu\text{g/mL}$, while other isolates showed lower MIC values of 31.250 $\mu\text{g/mL}$ and sub-MIC values of 15.625 $\mu\text{g/mL}$.

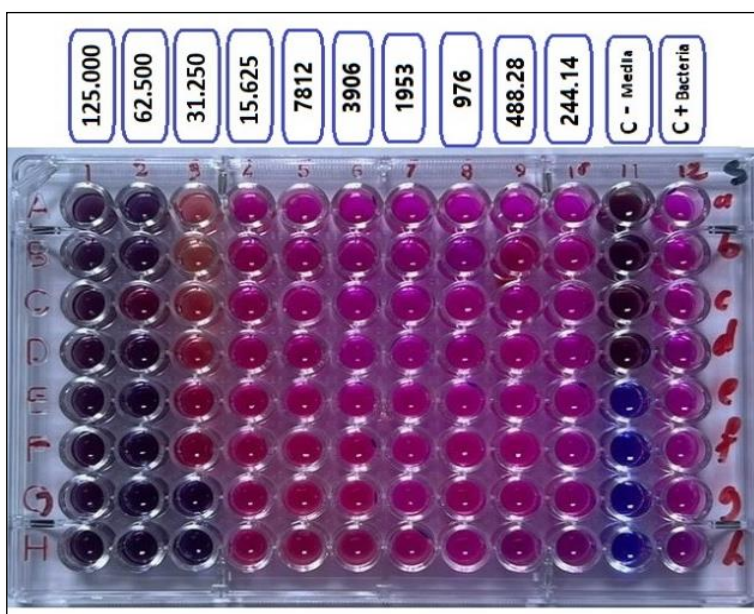


Figure 3. Determination of Minimum Inhibitory Concentration (MIC) of Saponin Extracts using Microtiter Plate Assay with Resazurin dye on *S. aureus* and *P. aeruginosa*

3.5.1. Effect of saponin extract on biofilm formation of *S. aureus*

S. aureus Biofilms have been observed in nearly all the examined isolates, although their amounts varied. As shown in **Table 4** and **Figure 4**, the extract most effectively inhibited isolate two, with a rate of 69.71 ± 1.81 . This result was highly significant ($P = 0.004$), with the most notable difference (LSD) of 7.77.

Table 4. Percentage of biofilm inhibition of Saponin Extract on *S. aureus*

Isolates No.	Percentage of biofilm inhibition (%Mean $\pm$ S.E) for Sub-MIC		LSD	P Value
	Control	Saponin		
1	100	50.32 $\pm$ 0.90	3.86*	<0.001
2	100	69.71 $\pm$ 1.81	7.77*	0.004
3	100	56.65 $\pm$ 15.26	42.65 NS	0.1
4	100	44.96 $\pm$ 14.76	40.99*	0.02
LSD	-	34.78	-	-
P Value	-	0.4 NS	-	-

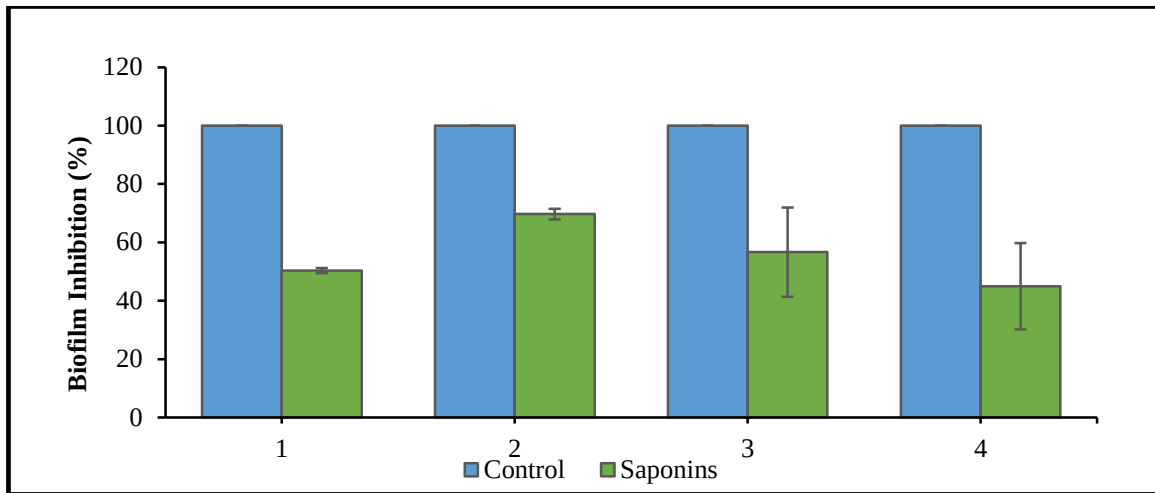


Figure 4. Saponin Extract Sub-MIC Effect on biofilm formation of *S. aureus*

Next, isolate one showed a considerable decrease at 50.32 ± 0.90 ($P < 0.001$; $LSD = 3.86^*$). Isolate four also demonstrated significant suppression, measuring 44.96 ± 14.76 ($P = 0.02$, $LSD = 40.99^*$). The fundamental standard error (15.26) suggests ($P = 0.1$ and $LSD = 42.65$, NS). The average inhibition rates across the four isolates were not statistically different ($P = 0.4$, NS), despite three isolates showing significant inhibition individually. However, this outcome was not statistically significant when considering its power ($P = 0.1$). Post-hoc comparisons revealed that saponins affected the biofilms in isolates 1, 2, and 4 differently compared to the controls³².

3.5.2. Effect of saponin extract on biofilm formation of *P. aeruginosa*:

Responses of the four isolates to the saponin extract at sub-MIC concentrations varied in their biofilm inhibition of *P. aeruginosa*, as shown in **Table 5** and **Figure 5**. Compared to the control, isolate one had the highest inhibition percentage (86.46 ± 5.13), but this difference was not statistically significant ($P = 0.1$, $LSD = 14.26$). Meanwhile, isolate 3 showed an apparent saponin effect, with an inhibition rate of 47.80 ± 2.8 , which was statistically significant ($P < 0.001$, $LSD = 12.16^*$). At 51.89 ± 4.53 ($P=0.009$ with $LSD=12.59$), isolate four also exhibited substantial inhibition. Conversely, isolate 2 had the lowest inhibition rate (45.80 , 17.41).

Due to the significant variance (Standard Error = 17.41), the difference from the control was not statistically significant ($P = 0.09$ with $LSD = 48.33$, NS). With an overall LSD value of 30.85, the analysis of variance across the four isolates' mean total inhibition rates showed a statistically significant difference ($P = 0.05$). This suggests that saponins may prevent *P. aeruginosa* from forming biofilms, even at concentrations lower than those required to inhibit planktonic growth.

Table 5. Percentage of biofilm inhibition of saponin Extract on *P. aeruginosa*

Isolates No.	Percentage of biofilm inhibition (%Mean±S.E) for Sub-MIC		LSD	P Value
	Control	Saponin		
1	100	86.46±5.13	14.26	0.1
2	100	45.80±17.41	48.33 NS	0.09
3	100	47.80±2.83	12.16*	<0.001
4	100	51.89±4.53	12.59	0.009
LSD	-	30.85*	-	-
P Value	-	0.05	-	-

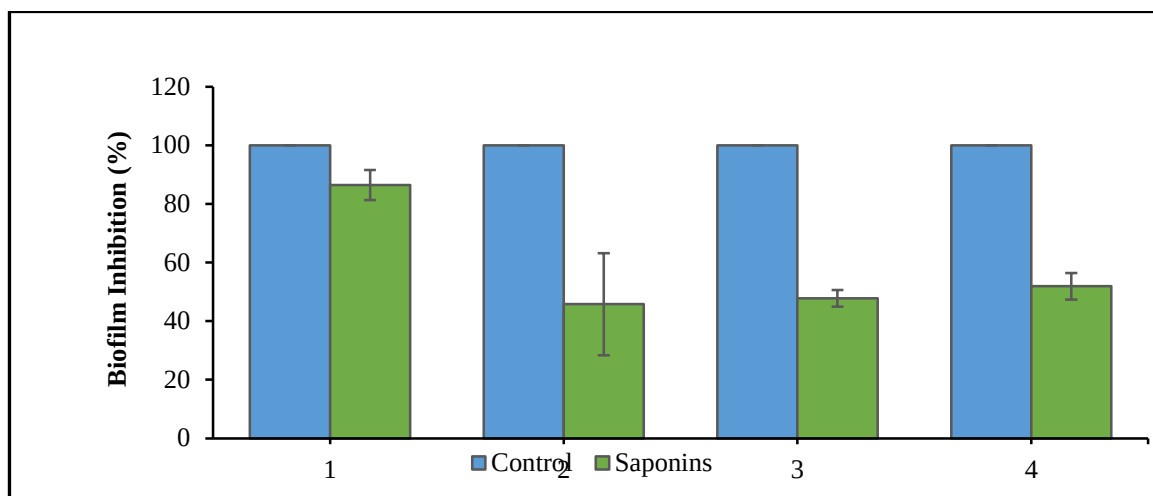


Figure 5. Saponin extract sub-MIC Effect on biofilm formation of *P. aeruginosa*

4. Discussion

The high prevalence of biofilm formation among *Staphylococcus aureus* isolates suggests that this ability may contribute to bacterial persistence and pathogenicity, particularly in multidrug-resistant strains. These findings are consistent with a previous study²¹. The significant chi-squared result indicates that the observed distribution of biofilm formation levels differed significantly from the expected distribution, provided that the assumptions of the statistical test were satisfied. In contrast, *Pseudomonas aeruginosa* isolates exhibited considerable variability in biofilm formation, with moderate and strong biofilm production being the predominant patterns. This variation may be associated with differences in strain-specific genetic characteristics or responses to environmental conditions²². Factors such as quorum-sensing activity and nutrient availability may also influence biofilm development. However, the non-significant result ($P = 0.07$) does not provide sufficient statistical evidence of differences in the distribution of biofilm formation levels among the tested *P. aeruginosa* isolates. These findings highlight the need for further investigation into the factors regulating biofilm development in this species^{23,24}.

Antimicrobial susceptibility testing revealed substantial resistance among both bacterial species, highlighting the challenges associated with treating infections caused by multidrug-resistant and extensively drug-resistant pathogens²⁵. The complete resistance of *S. aureus* isolates to Oxacillin and Penicillin, together with the 75% resistance to Cefoxitin, raises concern regarding the prevalence of methicillin-resistant *S. aureus* (MRSA) and the associated therapeutic challenges^{22,26}. Resistance to Erythromycin and fluoroquinolones, including Ciprofloxacin and Levofloxacin, further emphasizes the challenges associated with antimicrobial resistance. Nevertheless, the high susceptibility observed to Chloramphenicol, Nitrofurantoin, Clindamycin, and Rifampin indicates that these antibiotics retained activity against most of the tested isolates. The observed susceptibility to Linezolid is also consistent with its recognized role in treating infections caused by resistant Gram-positive bacteria, including MRSA²⁷. The significant variation in susceptibility patterns highlights the importance of continuous antimicrobial susceptibility surveillance and evidence-based treatment strategies²⁸.

Similarly, *P. aeruginosa* exhibited high resistance to several tested antibiotics, including Ceftazidime, Piperacillin, Gentamicin, Imipenem, Amikacin, and Levofloxacin, while all isolates were resistant to Ticarcillin. The resistance pattern also included Aztreonam, although the reported resistance rate was considerably lower than those observed for several other antibiotics. These findings are consistent with the challenges associated with treating infections caused by resistant *P. aeruginosa* strains²⁹⁻³¹. The presence of intermediate susceptibility may indicate reduced antimicrobial activity, but it does not independently demonstrate the emergence of resistance. The widespread resistance observed in both bacterial species reinforces the need for

appropriate antimicrobial stewardship, continued susceptibility monitoring, and the investigation of alternative therapeutic approaches^{32,33}.

The present study also investigated the antibacterial and antibiofilm activities of *Aloe vera* saponin extract against *S. aureus* and *P. aeruginosa*. The extract exhibited antibacterial activity against both species, although susceptibility varied among individual isolates. The minimum inhibitory concentration (MIC) was 62.500 µg/mL for most of the tested *S. aureus* isolates, while one isolate exhibited a higher MIC of 125.000 µg/mL. This variation may be associated with differences in cell wall architecture³⁴ or efflux pump activity³⁵, which can influence bacterial susceptibility to antimicrobial compounds. In contrast, some *P. aeruginosa* isolates exhibited lower MIC values, suggesting greater susceptibility to the extract under the experimental conditions. However, differences in MIC values alone cannot establish whether membrane permeability, compound uptake, efflux activity, or other bacterial defense mechanisms account for the observed variation. Therefore, the suggestion that saponins are more effective against *P. aeruginosa* than against *S. aureus* requires confirmation through an appropriate comparative analysis.

Beyond their antibacterial effects, *Aloe vera* saponins may also interfere with biofilm development. The extract reduced biofilm formation at sub-MIC concentrations in isolates of both bacterial species, although the magnitude of inhibition varied among strains^{36,37}. In two of the four *P. aeruginosa* isolates examined, the extract markedly reduced biofilm development³⁸. These findings suggest that the antibiofilm activity of the extract may occur at concentrations below those required to inhibit planktonic bacterial growth. Nevertheless, the extent of this effect appears to depend on the bacterial strain and the concentration of the extract. The observed variability emphasizes the complexity of biofilm development and the need to investigate the mechanisms through which saponins may interfere with biofilm formation. Accordingly, *Aloe vera* saponins may represent promising natural compounds for further investigation as potential antibiofilm agents. Additional studies are required to clarify their mechanisms of action, establish effective concentrations, assess their safety, and evaluate their potential therapeutic applications *in vivo*.

5. Conclusion

MDR and XDR clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were resistant to various conventional antibiotics in this investigation. The saponin extract from *Aloe vera* gel showed considerable antibacterial and anti-biofilm efficacy against these difficult pathogens. At inhibitory concentrations (MICs), saponins inhibited biofilm growth across various isolates, suggesting that they may disrupt bacterial persistence mechanisms without killing bacteria. The observed heterogeneity in inhibitory efficiency across bacterial strains highlights the complexity of biofilm biology and the necessity for tailored interventions. Due to antibiotic resistance, innovative antimicrobial and anti-biofilm techniques are needed. *Aloe vera* saponins are attractive natural compounds for therapeutic development, warranting further investigation into their biofilm-fighting mechanisms and *in vivo* use as standalone or combination therapies.

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Conflict of Interest

The authors declare that they have no conflicts of interest

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Ethical Clearance

This study was conducted in accordance with ethical guidelines and was approved by the Sciences College for women/University of Baghdad, Baghdad, Iraq (document number 22/7269, dated November 5, 2024).

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