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ANTIBIOFILM ACTIVITY OF ETHANOLIC AND ACETONE EXTRACTS OF *EUCALYPTUS GLOBULUS* AGAINST CLINICAL *STAPHYLOCOCCUS AUREUS* ISOLATES

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ABSTRACT

The increasing prevalence of multidrug-resistant *Staphylococcus aureus*, together with its strong biofilm-forming ability, represents a major clinical challenge. This study aimed to evaluate the antibiofilm activity of ethanolic and acetone extracts of *Eucalyptus globulus* leaves against fifteen clinical *S. aureus* isolates.

Antibacterial activity was assessed using agar diffusion and broth microdilution methods, while biofilm formation and antibiofilm effects were evaluated using Congo Red Agar and microtiter plate assays. Phytochemical characterization was performed using liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Both extracts exhibited antibacterial activity, with identical minimum inhibitory and bactericidal concentrations of 3.125 mg/mL and 6.25 mg/mL, respectively. Gallic acid was identified as the predominant compound in both extracts. 86.67% of the isolates were classified as moderate or strong biofilm producers. The ethanolic extract showed significantly greater effect on biofilm biomass than the acetone extract ($p = 0.0006$ for inhibition and < 0.0001 for reduction of preformed biofilm biomass), with inhibition values ranging from 11.14% to 95.68% and reductions in preformed biofilm biomass ranging from 59.40% to 93.75%. In contrast, the acetone extract displayed variable and strain-dependent effects, including increases in crystal violet-stained biofilm biomass in some cases.

These findings suggest that the ethanolic extract of *E. globulus* warrants further investigation as a potential source of natural compounds affecting *S. aureus* biofilm biomass.

Keywords: *Eucalyptus globulus*; *Staphylococcus aureus*; biofilm inhibition; preformed biofilm biomass; ethanolic extract; gallic acid

INTRODUCTION

Since the beginning of the 21st century, multidrug-resistant microorganisms (MDROs) have been increasingly reported, contributing to a growing burden of infections that are difficult to treat **(Uddin *et al*., 2021; Sagar *et al*., 2022)**. The increasing prevalence of antimicrobial resistance has substantially reduced the effectiveness of conventional therapies worldwide. Consequently, antimicrobial resistance has become a major global public health concern, with an estimated 1.27 million deaths directly attributable to bacterial antimicrobial resistance in 2019 **(Marino *et al*., 2025; Ranjbar and Alam, 2023)**.

One of the major bacterial strategies contributing to antimicrobial resistance is biofilm formation **(Uruén *et al*., 2021)**, which represents one of the most widespread and resilient microbial lifestyles **(Liu *et al*., 2024)**. Biofilms consist of structured microbial communities that adhere to surfaces and are embedded within a self-produced extracellular matrix, often referred to as the extracellular polymeric substance (EPS) matrix. This matrix is mainly composed of polysaccharides, proteins, nucleic acids, and lipids, and acts as a protective barrier against antimicrobial agents and host immune defenses, thereby complicating the infection treatment (**Roque-Borda *et al*., 2025; Böhning *et al*., 2024; Karygianni *et al*., 2020)**. In addition, biofilm-associated cells exhibit altered physiological states, including reduced metabolic activity, which further enhances tolerance to chemical and physical stresses **(Liu *et al*., 2024)**.

Biofilms are involved in up to 80% of persistent microbial infections in humans, contributing to prolonged hospital stays, increased healthcare costs, and elevated morbidity and mortality rates **(Assefa and Amare, 2022; Grooters *et al*., 2024)**. Their intrinsic tolerance to antimicrobial agents is further reinforced by factors such as high cell density, quorum-sensing communication, and genetic adaptations, including efflux pump activation and specific mutations, which collectively limit the efficacy of antimicrobial treatments **(Almatroudi, 2025)**. Consequently, the development of alternative and effective strategies for managing biofilm-associated infections has become an urgent priority.

Among the promising alternatives under investigation, natural products—particularly plant-derived extracts—have attracted considerable attention owing to their broad-spectrum antimicrobial and antibiofilm properties **(Elangovan and Mudgil, 2023; Sagar *et al*., 2022; Mezzasalma *et al*., 2025; Shariati *et al*., 2022; Mishra *et al*., 2020)**. *Eucalyptus globulus* Labill. is a well-documented medicinal plant, and its chemical composition and antibacterial activity have been extensively reported. Previous studies have suggested that the antibiofilm activity of *E. globulus* may be associated with interference with bacterial respiration, disruption of membrane integrity, and inhibition of initial bacterial adhesion, which is a critical step in biofilm development **(Mezzasalma *et al*., 2025)**.

Moreover, owing to its high biomass productivity and capacity to synthesize a wide range of bioactive compounds, including terpenoids, polyphenols, phloroglucinol derivatives, flavonoids, and tannins, *E. globulus* represents an important natural source of molecules with potential therapeutic applications **(Saleem *et al*., 2017).**

This study aimed to investigate the inhibitory effects of *Eucalyptus globulus* leaf extracts on biofilm formation and their ability to reduce preformed biofilm biomass in *Staphylococcus aureus*. A comparative approach using two different extraction solvents was employed to assess the influence of solvent polarity on the extraction of bioactive compounds and, consequently, on antibacterial and antibiofilm activities.

MATERIAL AND METHODS

Microorganisms

Fifteen clinical *S. aureus* isolates were recovered from skin infections from different clinical sources (Fig. 1**).** The isolates were cultured on Chapman agar plates and incubated at 35 ± 1 °C for 24 h. For short-term preservation, the strains were maintained on nutrient agar slants at 4 °C.

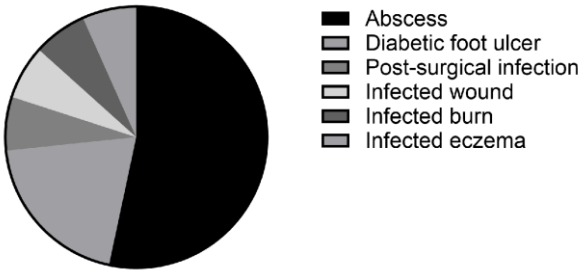


Figure 1 Clinical origins of *Staphylococcus aureus* isolates recovered from skin infections.

Identification and antibiogram

Bacterial identification and antimicrobial susceptibility testing were performed using the VITEK® 2 Compact automated system (bioMérieux, France) in accordance with the manufacturer's instructions. The results were interpreted following the Clinical and Laboratory Standards Institute (CLSI) guidelines (2024).

Modified vancomycin-supplemented BHI agar screening

A modified phenotypic screening assay, adapted from the vancomycin agar screening protocol designed to detect potential vancomycin-intermediate *Staphylococcus aureus* (VISA) phenotypes, was performed on clinical isolates. The original screening approach was based on Brain Heart Infusion (BHI) agar supplemented with vancomycin at 6 µg/mL. In the present study, the protocol was experimentally modified by including four additional vancomycin concentrations (2, 4, 8, and 16 µg/mL), in addition to the standard screening concentration of 6 µg/mL. This modification was intended to further characterize growth patterns associated with different levels of vancomycin susceptibility and to compare these phenotypic observations with the individual vancomycin MIC values obtained using the VITEK® 2 Compact system **(Walters et al., 2015)**. Since reference broth microdilution testing was not performed, the results were interpreted as phenotypic screening observations and were not considered definitive confirmation of vancomycin-susceptible *Staphylococcus aureus* (VSSA), vancomycin-intermediate *Staphylococcus aureus* (VISA), or vancomycin-resistant *Staphylococcus aureus* (VRSA) phenotypes.

***Eucalyptus globulus* extract preparation**

Leaves of *Eucalyptus globulus* were collected from Machtlet El Rayhane (El Hammamet, Tebessa, Algeria; latitude 35.4483° N, longitude 7.9531° E) in September 2021 and identified by botanists from the Faculty of Natural and Life Sciences, Larbi Tebessi University (Tebessa, Algeria). The leaves were air-dried in the dark before extraction.
Ethanolic and acetone extracts were prepared according to previously described protocols, using ethanol, as reported by **Ansari *et al*. (2022)** and acetone, as described by **Müller-Heupt *et al*. (2022)**, without modification. Dried leaves were ground into powder and extracted using a plant material-to-solvent ratio of 1:10 (w/v). The extracts were filtered and concentrated under reduced pressure using a rotary evaporator, then stored at 4 °C until further analysis.

LC-MS/MS chemotyping

Phytochemical profiling was performed using an Agilent 6460 triple quadrupole LC–MS system equipped with a Poroshell 120 EC-C18 column (50 × 4.6 mm, 2.7 µm), maintained at 30 °C. The injection volume was 4 µL, with a flow rate of 0.4 mL/min and a total run time of 40 min.
The mobile phase consisted of solvent A (water containing 0.1% formic acid and 5 mM ammonium formate) and solvent B (acetonitrile containing 0.1% formic acid), delivered using a gradient elution program. Prior to analysis, 50 mg of each extract was dissolved in acetonitrile–methanol–water mixture (1:1:1, v/v/v), vortexed and sonicated when necessary. A hexane clean-up step was applied, followed by centrifugation at 7000 rpm for 5 min. The lower phase was collected, diluted (1:4), filtered through a 0.25 µm, and subjected to LC–MS/MS analysis.
Phenolic compounds were identified by comparing their retention times and mass spectra with those of authentic reference standards.

Miscibility test

To assess miscibility, 1 mL of each extract was mixed with 1 mL of the corresponding solvent in a test tube. The mixture was gently agitated for 30 s and visually examined for phase separation and turbidity. Complete dissolution without phase separation indicates adequate miscibility. This test was performed to ensure homogeneous dispersion of the extracts prior to antimicrobial and antibiofilm assays.

Antimicrobial activity

Agar well diffusion assay

The antibacterial activity of the ethanolic and acetone extracts was evaluated using the agar well diffusion method as described by **Balouiri *et al*. (2016)**, with minor modifications. Bacterial suspensions were adjusted to the 0.5 McFarland standard and evenly spread on Mueller–Hinton agar plates. Wells of 6 mm diameter were aseptically punched and filled with 100 µL of extract solutions at concentrations of 50, 25, 12.5, 6.25, 3.125, and 1.56 mg/mL, prepared from a stock solution of 100 mg/mL.
Dimethyl sulfoxide (DMSO, 1%) served as the negative control, while levofloxacin (5 µg) was used as the positive control. The plates were incubated at 37 °C for 18–24 h, and inhibition zones were measured in millimeters. All assays were performed in triplicate.

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth microdilution method in sterile 96-well microtiter plates, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), with slight modifications.
Bacterial suspensions from overnight cultures were adjusted to the 0.5 McFarland standard (≈ 1.5×10^8 CFU/mL) and diluted in Mueller–Hinton broth to obtain a final inoculum of approximately 5×10^5 CFU/mL per well.
Serial two-fold dilutions of each extract were prepared to obtain concentrations ranging from 100 to 1.56 mg/mL. Each well contained a final volume of 200 µL. Growth and sterility controls were included. Plates were incubated at 37 °C for 18–24 h.
The MIC was defined as the lowest concentration that completely inhibited visible bacterial growth. For MBC determination, aliquots from wells showing no visible growth were subcultured on Mueller–Hinton agar and incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration resulting in no bacterial growth, corresponding to a ≥ 99.9% reduction of the initial inoculum.

Detection of biofilm formation

Qualitative assessment (Congo Red agar method)

The ability of *Staphylococcus aureus* isolates to form biofilms was initially evaluated using the Congo Red Agar (CRA) method, according to the protocol described by **Abdel Halim *et al*. (2018).** The CRA medium was prepared using Brain Heart Infusion broth (37 g/L), agar (10 g/L), sucrose (50 g/L), and Congo red dye (0.8 g/L). The dye was separately prepared in distilled water and added to the molten medium at 55 °C prior to solidification.
The prepared plates were inoculated by streaking or spot inoculation and incubated at 35 ± 2 °C for 18–24 h. After incubation, colony morphology and color were examined. Black, dry, and rough colonies were classified as strong biofilm producers, dark but smooth colonies as moderate producers, and pink, smooth colonies as non-biofilm producers.
CRA was used as a qualitative screening tool, and biofilm formation was further evaluated quantitatively using the microtiter plate assay.

Quantitative Assessment (Microtiter plate Assay)

Biofilm production was quantitatively assessed using the microtiter plate (MTP) method as described by **Cruz *et al*. (2018)** and **Stepanović *et al*. (2007).** A single colony of *Staphylococcus aureus* was inoculated into 10 mL of Brain Heart Infusion broth (BHIB) supplemented with 1% glucose and adjusted to the 0.5 McFarland standard. The suspension was diluted 1:100 and incubated at 35 °C for 17–24 h.
Subsequently, 200 µL of the diluted bacterial culture was dispensed in triplicate into sterile 96-well microtiter plates. Wells containing BHIB were used as negative controls. The plates were incubated for 24 h at 35 °C to allow biofilm formation. After incubation, the wells were gently emptied and washed three times with 300 µL of phosphate-buffered saline (PBS; pH 7.0–7.2) to remove non-adherent cells, then air-dried.
Biofilms were fixed with 200 µL of methanol for 20 min, stained with 0.1% crystal violet for 10 min, and washed three times with PBS. After air-drying, the bound dye was solubilized with methanol, and absorbance was measured at 595 nm (OD595) using a spectrophotometer.
The optical density of each isolate was compared with the cut-off optical density (ODc), calculated as the mean OD of the negative control plus 3 standard deviations (mean + 3SD). Isolates were classified as non-biofilm producers ($OD \leq ODc$), weak producers ($ODc < OD \leq 2 \times ODc$), moderate producers ($2 \times ODc < OD \leq 4 \times ODc$), or strong producers ($OD > 4 \times ODc$).
Crystal violet staining reflects total biofilm biomass by binding to both adherent bacterial cells and the extracellular polymeric matrix. Higher OD values indicate stronger biofilm formation. *Staphylococcus aureus* ATCC 25923 was used as a positive control to validate assay performance.

Biofilm inhibition assay

Biofilm inhibition was evaluated as described by **Bazargani *et al*. (2016)**, with minor modifications. A bacterial suspension prepared from a 0.5 McFarland culture in Brain Heart Infusion broth (BHIB) supplemented with 1% glucose was diluted 1:100. Based on the previously determined minimum inhibitory concentration (MIC = 3.125 mg/mL), this concentration was selected for all biofilm inhibition assays.

For each well, 100 µL of the diluted bacterial suspension was mixed with 100 µL of the plant extract (6.25 mg/mL) to obtain a final concentration of 3.125 mg/mL. Microtiter plates were incubated at 35 °C for 24 h to allow biofilm formation in the presence of the extracts. After incubation, the wells were gently washed three times with phosphate-buffered saline (PBS; pH 7.0–7.2) to remove non-adherent cells and air-dried.

Biofilms were fixed with 200 µL of methanol for 20 min, stained with 0.1% crystal violet for 10 min, rinsed three times with PBS, and air-dried. Bound crystal violet was solubilized with methanol, and absorbance was measured at 595 nm (OD595) using a spectrophotometer.

Biofilm inhibition was calculated by comparing the optical density of treated wells with that of untreated control wells, according to Equation (1):

Equation (1): Inhibition (%) = [(ODcontrol – ODtreated) / ODcontrol] × 100

Reduction of preformed biofilm biomass assay

The effect of plant extracts on preformed biofilm biomass was assessed using the protocol described by **Cruz *et al*. (2018)**. Briefly, 100 µL of a bacterial suspension (1:100 dilution), prepared from a 0.5 McFarland culture in BHIB supplemented with 1% glucose, was dispensed into each well of a sterile 96-well microtiter plate and incubated at 35 °C for 24 h to allow biofilm maturation.

After incubation, non-adherent cells were removed by washing the wells three times with PBS (pH 7.0–7.2). Subsequently, 100 µL of the plant extract solution (6.25 mg/mL) was mixed with 100 µL of fresh BHIB and added to the wells containing preformed biofilms, resulting in a final extract concentration of 3.125 mg/mL. The plates were then incubated for an additional 24 h at 35 °C.

Following treatment, biofilms were fixed with methanol, stained with 0.1% crystal violet, rinsed with PBS, and air-dried. The bound dye was solubilized with methanol, and absorbance was measured at 595 nm (OD595).

The percentage reduction in preformed biofilm biomass was calculated using Equation (2):

Equation (2): Reduction (%) = [(ODcontrol – ODtreated) / ODcontrol] × 100

Classification of antibiofilm activity

For interpretation, antibiofilm activity was classified based on the percentage of biofilm reduction relative to untreated controls. Both biofilm biomass inhibition and reduction of preformed biofilm biomass were classified as strong (≥ 80%), moderate (60–79%), weak (30–59%), and negligible or stimulatory when values were <30% or negative. Negative values were considered separately and indicated an increase in crystal violet-stained biofilm biomass relative to untreated controls.

Statistical analysis

All experiments were performed using three independent biological replicates (n = 3), and results are presented as mean ± standard deviation (SD). Data normality was assessed using the Shapiro–Wilk test. Comparisons of extraction yields were conducted using an unpaired Student's *t*-test. Differences in biofilm biomass and vancomycin susceptibility categories were assessed using the Kruskal–Wallis test. Comparisons between ethanolic and acetone extracts in biofilm biomass inhibition and preformed biofilm biomass reduction assays were performed using the Wilcoxon matched-pairs signed-rank test. The effects of extract type and concentration on antibacterial activity were evaluated using two-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons test. Correlations were assessed using Spearman's rank correlation coefficient. All statistical analyses were performed using GraphPad Prism software (version 10.4.0), and *p*-values < 0.05 were considered statistically significant.

RESULTS

Bacterial isolates and antibiotic susceptibility profiles

The evaluation of the antibiotic susceptibility profiles revealed a multidrug-resistant pattern among the 15 clinical *S. aureus* isolates. High resistance rates were observed to fusidic acid and β-lactams, particularly oxacillin, as well as to commonly used antibiotics such as clindamycin and tetracycline. Moderate resistance was also detected to several other agents, including erythromycin, kanamycin, gentamicin, and daptomycin, reflecting a broad resistance profile. Vancomycin showed a resistance rate of 20.0%, while 40.0% of the isolates were susceptible. Conversely, lower resistance rates were recorded for linezolid, trimethoprim/sulfamethoxazole, nitrofurantoin, and levofloxacin. The overall antibiotic susceptibility profiles and resistance distributions are summarized in Fig. 2.

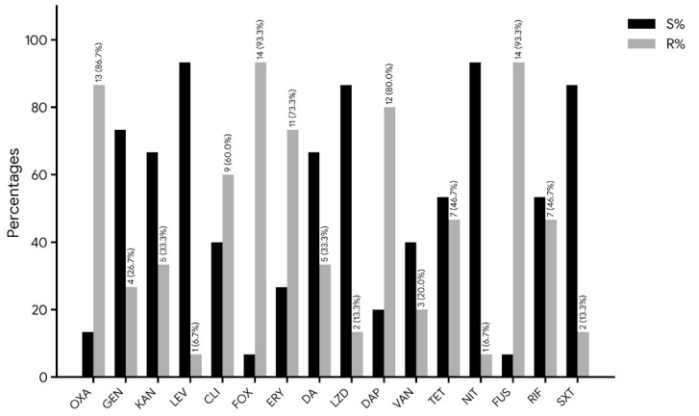


Figure 2 Antibiotic resistance profiles of 15 clinical *Staphylococcus aureus* isolates. Resistance rates were expressed as percentages, with the corresponding absolute numbers of resistant isolates indicated for each antimicrobial agent.

Phenotypic characterization of vancomycin resistance

The modified BHI agar screening assay revealed a concentration-dependent reduction in the number of clinical *S. aureus* isolates capable of growing in the presence of vancomycin. Growth was observed in nine isolates at 2 µg/mL, five isolates at 4 µg/mL, and three isolates at both 6 and 8 µg/mL. Notably, one isolate (S6) maintained growth at the highest concentration tested (16 µg/mL). This isolate was also recorded as having a vancomycin MIC of 32 µg/mL using the VITEK® 2 Compact system. The growth patterns observed on BHI agar were generally consistent with the VITEK® 2 MIC results. However, reference broth microdilution confirmation was unavailable. Therefore, S6 should be considered a presumptive vancomycin-resistant isolate rather than a confirmed VRSA isolate. The representative growth patterns are presented in Fig. 3, while the individual results are summarized in Supplementary Table S3.

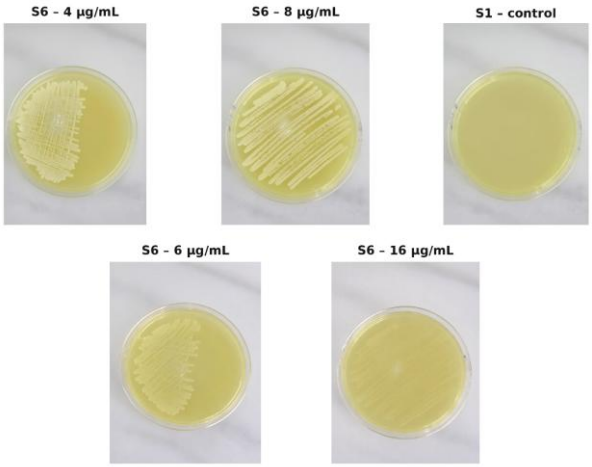


Figure 3 Representative vancomycin growth patterns of clinical Staphylococcus aureus isolates on BHI agar supplemented with increasing vancomycin concentrations, showing isolate S6 (VITEK® 2-classified resistant) and isolate S1 (VITEK® 2-classified susceptible).

Extract yield

The extraction yield of *Eucalyptus globulus* leaves varied depending on the solvent used. The ethanolic extract showed yields ranging from 25.33% to 31.75%, with a mean value of 27.50 ± 3.68%. In contrast, the acetone extract exhibited lower and more consistent yields, ranging from 23.22% to 24.48%, with a mean value of 23.67 ± 0.70% (Tab 1). Statistical comparison between the two solvents showed no significant difference in extraction yield ($p = 0.151$), indicating that the slightly higher mean yield obtained with ethanol was not statistically significant under the experimental conditions (Supplementary Figure S1)

Table 1 Extraction yield (%) of ethanolic and acetone extracts from *E. globulus* leaves

Solvent type	R1	R2	R3	Mean (%)	SD (%)
Ethanolic	25.435	25.325	31.750	27.50	3.68
Acetone	23.217	23.316	24.480	23.67	0.70

Legend: Three independent extraction yields (R1–R3) were reported for each solvent, along with the mean (%) and standard deviation (SD).

Miscibility test

The miscibility test showed that, after 30 s of gentle agitation, both the ethanolic and acetone extracts of *Eucalyptus globulus* were visually homogeneous in their respective solvents. The absence of visible turbidity or phase separation indicated good physical compatibility between the extracts and the corresponding solvents under the tested conditions. This visual homogeneity helped ensure consistent preparation of the extract solutions used in the antimicrobial and antibiofilm assays.

Antibacterial activity in planktonic state

Both ethanolic and acetone extracts produced clear inhibition zones against planktonic *Staphylococcus aureus* isolates. At the highest tested concentration (100 mg/mL), comparable inhibition zone diameters were observed for the ethanolic extract (24.0 ± 0.8 mm) and the acetone extract (24.5 ± 1.2 mm). A progressive decrease in inhibition zone diameters was noted with decreasing extract concentrations, with detectable antibacterial activity observed down to 3.125 mg/mL, whereas no inhibition was detected at 1.56 mg/mL (Fig. 4). Two-way analysis of variance (ANOVA) revealed no significant effect of extract type on antibacterial activity ($p = 0.1112$), whereas a highly significant effect of concentration was observed ($p < 0.0001$). No significant interaction between extract type and concentration was detected ($p = 0.0818$).

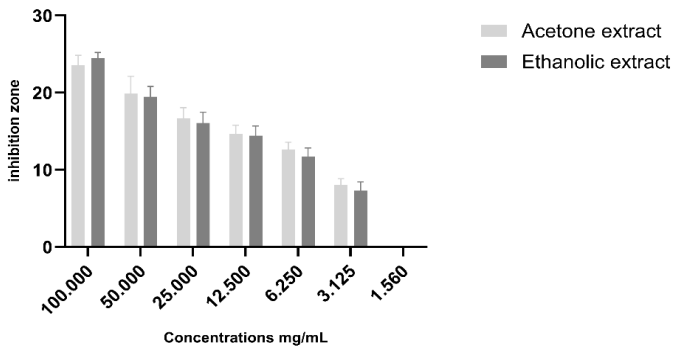


Figure 4 Inhibition zone diameters produced by ethanolic and acetone extracts of *Eucalyptus globulus* against planktonic *Staphylococcus aureus* at different concentrations.

At MIC (3.125 mg/mL), the acetone extract produced a mean inhibition zone of 8.00 ± 0.85 mm, while the ethanolic extract showed a mean diameter of 7.63 ± 1.07 mm, indicating that both extracts maintained detectable antibacterial activity at this concentration. Bonferroni's multiple comparisons test confirmed that inhibition zone diameters did not differ significantly between the ethanolic and acetone extracts at any tested concentration (all adjusted p-values ≥ 0.2490), supporting comparable antibacterial activity between the two extracts.

Determination of MIC and MBC of ethanolic and acetone extracts

The antibacterial activity of the ethanolic and acetone extracts was evaluated by determining their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) using the broth microdilution method.
For the acetone extract, bacterial growth decreased progressively with increasing extract concentrations. The MIC was determined to be 3.125 mg/mL, at which the optical density (OD ≈ 0.021) was comparable to that of the blank (OD ≈ 0.025), indicating complete inhibition of detectable bacterial growth. At lower concentration of 1.56 mg/mL, bacterial growth resumed (OD ≈ 0.058), confirming 3.125 mg/mL as the MIC. The MBC was determined to be 6.25 mg/mL, as no bacterial colonies were observed following subculturing on Mueller–Hinton agar.
Similarly, the ethanolic extract exhibited antibacterial activity. The MIC was also determined to be 3.125 mg/mL, with an OD value (OD ≈ 0.024) comparable to those of the blank (OD ≈ 0.026). At 1.56 mg/mL, bacterial growth was observed (OD ≈ 0.053), indicating a loss of inhibitory activity. The MBC for the ethanolic extract was 6.25 mg/mL, corresponding to the complete absence of bacterial growth after subculturing.
Overall, both extracts exhibited identical MIC and MBC values, with an MBC/MIC ratio of 2, indicating bactericidal effects.

Phytochemical profile of *Eucalyptus globulus* extracts (LC–MS/MS analysis)

Among the identified compounds, the relative abundance of the main phytochemicals (≥ 1%) detected by LC–MS/MS in the acetone and ethanolic extracts of *E. globulus* is illustrated in Fig. 5. Gallic acid was the predominant compound in both extracts, accounting for nearly half of the identified phytochemical profile, with relative abundances of 49.0% in the acetone extract and 50.2% in the ethanolic extract. Chlorogenic acid showed a higher relative abundance in the ethanolic extract (22.3%) than in the acetone extract (17.4%), whereas gentisic acid was more abundant in the acetone extract (13.0%) than in the ethanolic extract (7.4%).
Noticeable qualitative and quantitative differences were observed between the two extracts. Rutin (6.8%), chrysin (1.3%), and vanillin (5.0%) were exclusively detected in the ethanolic extract, whereas biochanin A (6.0%) was identified only in the acetone extract. In addition, trans-ferulic acid showed a higher relative abundance in the acetone extract (10.4%) than in the ethanolic extract (1.8%). Detailed LC–MS/MS data are presented in **Supplementary Table S1.**

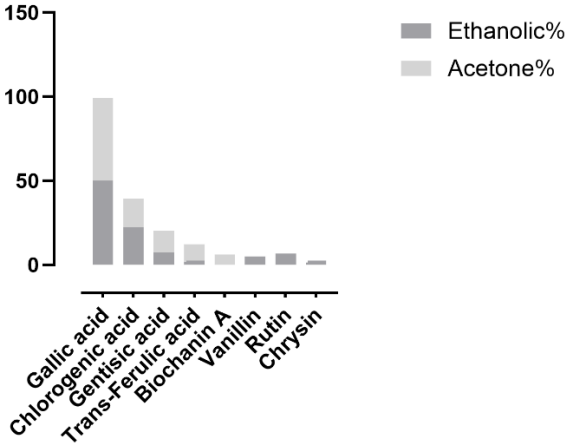


Figure 5 LC–MS/MS phytochemical profiles of acetone and ethanolic *E. globulus* extracts showing qualitative and quantitative differences between the two extracts.

Biofilm-forming capacity of the clinical isolates on CRA

CRA screening revealed a heterogeneous distribution of biofilm-forming phenotypes among the clinical isolates. Strong biofilm producers (++), characterized by black, dry, and rough colonies, accounted for 60.0% of the isolates. An additional 26.7% of the isolates exhibited darker but smoother colonies, corresponding to a moderate biofilm-producing phenotype (±). In contrast, 13.3% of the isolates displayed pink and smooth colonies, indicating a non-biofilm-producing phenotype (−). Overall, these findings demonstrate that biofilm formation is a prevalent trait among this collection of *Staphylococcus aureus* isolates. A representative CRA image illustrating the three observed phenotypes is shown in Fig. 6. The CRA results were subsequently evaluated using a quantitative microtiter plate (MTP) assay.



Figure 6 Congo red agar (CRA) plates showing *Staphylococcus aureus* growth patterns obtained using spot and streak inoculation methods for the assessment of slime (biofilm) production phenotypes.

Biofilm quantification

Quantitative biofilm formation was assessed using a microtiter plate assay under identical experimental conditions. As shown in Fig. 7, most of the tested isolates exhibited high OD595 values, clearly exceeding the cut-off value (ODc = 0.106), indicating their ability to form sessile biomass on polystyrene surfaces. Overall, 53.33% of the isolates were classified as strong biofilm producers, with OD595

values > 0.424. An additional 33.33% of the isolates produced moderate levels of biofilm biomass, corresponding to the moderate biofilm-producing category (OD595 > 0.212 to ≤ 0.424). In contrast, only 6.67% of the isolates exhibited a weak biofilm-forming phenotype (OD595 > 0.106 to ≤ 0.212), while the remaining 6.67% were classified as non-biofilm producers (OD595 ≤ 0.106).
The reference strain, *Staphylococcus aureus* ATCC 25923, used as a positive control, also demonstrated strong biofilm-producing ability. The detailed OD595 values and biofilm categories for each isolate are provided in Supplementary Table S2.

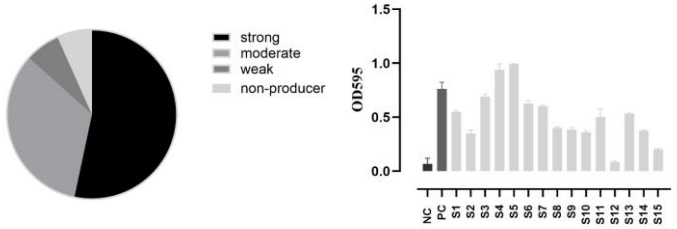


Figure 7 Biofilm biomass quantified by OD595 measurements following crystal violet staining. Isolates were classified as weak, moderate, or strong biofilm producers based on optical density cut-off value (ODc). NC: negative control; PC: positive control (*Staphylococcus aureus* ATCC 25923).

Relationship between biofilm biomass and vancomycin susceptibility

Biofilm biomass was evaluated among isolates classified as vancomycin-susceptible, vancomycin-intermediate, or vancomycin-resistant according to their VITEK® 2 Compact MIC results, interpreted using CLSI breakpoints. These categories represent VITEK® 2-based susceptibility classifications and do not constitute definitive confirmation of VSSA, VISA, or VRSA phenotypes, as reference broth microdilution testing was not performed. As illustrated in Fig. 8, isolates in the intermediate category exhibited higher and more variable OD595 values, reaching approximately 0.99, whereas isolates in the resistant category also showed relatively elevated biofilm biomass. In contrast, isolates in the susceptible category generally displayed lower OD595 values. However, statistical analysis using the Kruskal–Wallis test revealed no statistically significant differences in biofilm biomass among the three susceptibility categories (p = 0.5113).

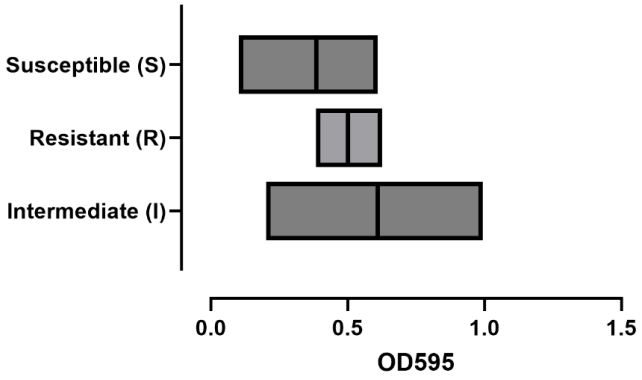


Figure 8 Distribution of biofilm biomass according to vancomycin susceptibility categories (OD595)

Biofilm inhibition and reduction of preformed biofilm biomass

The antibiofilm activity of *Eucalyptus globulus* extracts was evaluated using biofilm inhibition and preformed biofilm treatment assays. The results were expressed as percentage of biomass reduction relative to untreated controls, based on crystal violet staining. The detailed percentages obtained for each isolate and both extracts are presented in (Tab 2)**.** For the ethanolic extract, biofilm biomass varied among the tested strains. Strong inhibition (≥80%) was observed in 20.00% (3/15) of the isolates, while weak inhibition (30–59%) occurred in 33.33% (5/15) of the isolates. Moderate inhibition (60–79%) was not detected, whereas the remaining 46.67% (7/15) of the isolates exhibited negligible inhibition (< 30%). Notably, three isolates and the reference strain exhibited inhibition rates exceeding 90%. For the reference strain, the measured biomass following treatment fell below the threshold used to classify biofilm production.
In the preformed biofilm treatment assay, the ethanolic extract produced a marked reduction in biofilm biomass. Reductions of ≥ 80% were observed in 80.00% of the isolates, while 13.33% exhibited moderate reductions (60–79%) and 6.67% showed weak reductions (30–59%). Substantial reductions were also recorded in isolates with initially low or moderate biofilm-forming capacity. Overall, biofilm biomass inhibition ranged from 11.14% to 95.68% in the inhibition assay, whereas the reduction in preformed biofilm biomass ranged from 59.40% to 93.75% in the preformed biofilm treatment assay.
In contrast, the acetone extract exhibited a highly strain-dependent response, with generally lower effects on biofilm biomass. In the inhibition assay, strong inhibition (≥80%) was observed in only 6.67% (1/15) of the isolates, whereas moderate inhibition (60–79%) was not detected. Weak inhibition (30–59%) occurred in 13.33% (2/15) of the isolates, while the majority of the isolates (80.00%, 12/15) exhibited negligible or negative values, indicating either a limited inhibitory effect or increased biofilm biomass relative to untreated controls. In the preformed biofilm treatment assay, the acetone extract also showed a heterogeneous response. Strong biomass reductions (≥80%) were recorded in 13.33% (2/15) of the isolates, moderate reductions (60–79%) in 46.67% (7/15), and weak reductions (30–59%) in 26.67% (4/15). Negligible reductions or negative values were observed in the remaining 13.33% (2/15) of the isolates. Overall, the acetone extract showed a less pronounced and more variable effect than the ethanolic extract, reflecting its strain-dependent antibiofilm activity.
Spearman correlation analysis for the ethanolic extract revealed no significant association between the percentage reductions observed in the inhibition and preformed biofilm treatment assays (r_s = −0.05, 95% CI: −0.5598 to 0.4873, p = 0.8626) (Supplementary Figure S2). In contrast, a positive correlation was observed between initial biofilm biomass and the reduction in preformed biofilm biomass for the ethanolic extract (r_s = 0.66, 95% CI: 0.2145–0.8816, p = 0.0085). For the acetone extract, a borderline positive correlation was observed (r_s = 0.52, 95% CI: −0.0042 to 0.8213, p = 0.0488). Although the exact two-tailed P value indicated borderline statistical significance, the approximate 95% confidence interval marginally included zero, highlighting the uncertainty of this association.

Table 2 Biofilm inhibition and reduction of preformed biofilm biomass percentage (mean ± SD, n = 3).

Strain	Ethanolic inhibition %	Acetone inhibition%	Ethanolic reduction %	Acetone reduction%
S1	14.78 ± 1.95	-22.51 ± 0.89	83.58 ± 1.66	44.33 ± 1.70
S2	16.18 ± 1.64	-23.50 ± 1.97	76.06 ± 4.76	59.43 ± 4.32
S3	28.14 ± 1.94	12.53 ± 0.65	90.11 ± 1.58	67.44 ± 0.90
S4	29.69 ± 1.88	-12.89 ± 1.81	93.75 ± 2.05	60.87 ± 2.49
S5	14.69 ± 3.05	28.61 ± 0.46	93.34 ± 1.74	88.69 ± 0.07
S6	32.52 ± 3.92	-27.98 ± 1.01	85.60 ± 2.67	78.25 ± 1.22
S7	11.14 ± 0.61	4.48 ± 1.56	89.85 ± 0.62	75.61 ± 0.28
S8	25.16 ± 3.26	8.00 ± 2.38	84.85 ± 2.83	83.42 ± 0.55
S9	37.32 ± 1.05	-18.34 ± 1.69	88.48 ± 1.87	52.51 ± 2.61
S10	55.78 ± 3.01	19.58 ± 4.31	88.59 ± 2.13	72.36 ± 1.45
S11	95.68 ± 1.82	96.48 ± 0.84	90.66 ± 2.28	72.81 ± 4.76
S12	48.35 ± 1.64	44.68 ± 1.46	59.40 ± 1.80	-16.18 ± 1.28
S13	58.75 ± 1.49	37.29 ± 1.66	85.52 ± 2.97	41.72 ± 0.97
S14	95.23 ± 0.53	24.10 ± 1.20	89.97 ± 2.18	69.97 ± 0.33
S15	93.59 ± 0.98	-14.18 ± 2.02	70.00 ± 0.00	-2.99 ± 0.11

Legend: Negative values indicate an increase in crystal violet-stained biofilm biomass relative to the untreated control.

Statistical analysis confirmed significant differences between the two extracts. Comparison of the ethanolic and acetone extracts showed that the ethanolic extract produced significantly greater inhibition of biofilm biomass (p = 0.0006) and a significantly greater reduction in preformed biofilm biomass (p < 0.0001), as determined using the Wilcoxon matched-pairs signed-rank test (n = 15). As the data did not meet the assumptions of normality, a non-parametric paired test was applied. These results are illustrated in Supplementary Figures S3 and S4, which show the inhibition and reduction values for each isolate, respectively.

DISCUSSION

Influence of Extraction Solvents on Phytochemical Composition

The present findings are consistent with previous studies demonstrating that extraction solvents influence the qualitative and quantitative composition of bioactive compounds **(Borges *et al*., 2012)**. Similarly, previous studies on *Eucalyptus globulus* have reported gallic and ellagic acid derivatives as the predominant phenolic groups **(Bey-Ould *et al*., 2016)**.
The predominance of gallic acid in both extracts, together with the detection of biochanin A in the acetone extract and vanillin, rutin and chrysin in the ethanolic extract, indicates a diverse phytochemical profile that may contribute to the observed biological activities. Similar associations between phenolic-rich extracts and antimicrobial or antibiofilm activities have been reported previously **(Atta *et al*., 2023)**.
Differences in biological activity among published studies may reflect variations in extraction procedures, including solvent polarity, extraction conditions, and extraction techniques, which influence both the yield and composition of phytochemicals **(Sun *et al*., 2025; Nawaz *et al*., 2020; Tourabi *et al.*, 2025; Akinmoladun *et al*., 2022).** In addition, the observed biological activities are likely the result of synergistic interactions among multiple phytochemicals rather than the action of individual compounds **(Vaou *et al*., 2022; Usta *et al*., 2025;**

Chaachouay *et al*., 2025). Therefore, the contribution of specific constituents cannot be confirmed without further fractionation or functional validation **(Vaou et al., 2022; Abubakar and Haque, 2020; Abraham *et al*., 2025; Mani *et al*., 2022).**
However, this comparison of extraction yields was based on only three biological replicates per extract, which limits the statistical power to detect subtle solvent-dependent differences; a larger number of replicates would strengthen the reliability of this comparison in future work.
Furthermore, the phytochemical composition of Eucalyptus globulus extracts may vary according to geographical origin, seasonal conditions, and plant maturity, thereby affecting the reproducibility of their composition and, consequently, their biological activities **(Usman *et al*., 2020).**

Biofilm-Forming Ability of *Staphylococcus aureus* Isolates

In this study, the majority of *Staphylococcus aureus* isolates were capable of forming biofilms under the tested conditions. More than half of the isolates (53.33%) were classified as strong biofilm producers, while 33.33% exhibited moderate biofilm-forming capacity and only 6.67% were weak producers, whereas the remaining 6.67% comprised non-biofilm producers.
These findings differ from those of Wang et al. (2023), who observed a predominance of weak biofilm producers (62.5%) and a lower proportion of strong producers (26.0%). Despite the relatively small number of clinical isolates (n = 15), the predominance of moderate and strong biofilm producers supports the biological relevance of the subsequent inhibition and preformed biofilm biomass reduction assays.
Variability in biofilm formation among *S. aureus* isolates has been widely documented and is influenced by multiple factors, including genetic background, surface adhesion properties, and environmental conditions **(Goudarzi *et al*., 2019).** Our results showed numerically higher biofilm biomass among isolates classified as vancomycin-intermediate and vancomycin-resistant, based on VITEK® 2 Compact MIC results, compared with vancomycin-susceptible isolates. However, these differences were not statistically significant ($p = 0.5113$), indicating that no differences in biofilm biomass could be established among the three vancomycin susceptibility categories in the present study. Although previous studies have suggested a possible relationship between reduced vancomycin susceptibility and enhanced biofilm formation, our findings did not demonstrate such a relationship under the experimental conditions of the present study **(Chapman *et al*., 2024; Abd El-Hamid *et al*., 2024)**.

Antibiofilm Activity of the Extracts

Regarding antibacterial activity, the MIC values obtained for both extracts in the present study (3.125 mg/mL) were higher than those reported in previous studies investigating *Eucalyptus globulus* leaf extracts against *Staphylococcus aureus*. **Mezzasalma *et al.* (2025)** reported MIC_{50} and MIC_{90} values of 0.5 and 1.0625 mg/mL, respectively, against bovine mastitis isolates, whereas **Salari et al. (2006)** reported lower values of 0.064 and 0.128 mg/mL, respectively, against clinical isolates. These differences may be attributed to variations in extraction procedures, solvent composition and polarity, phytochemical profiles, and characteristics and origins of the bacterial isolates tested. These factors can influence the antimicrobial activity of plant extracts. Furthermore, the antibacterial activity of crude plant extracts may be influenced by the combined effects of multiple phytochemicals. However, potential synergistic or antagonistic interactions among these compounds remain hypothetical, as no direct functional assays were performed to validate such interactions in the present study **(Vaou *et al*., 2022)**.
The ethanolic extract reduced biofilm biomass in both the inhibition and preformed biofilm treatment assays. Biofilm inhibition values ranged from 11.14% to 95.68%, while reductions in preformed biofilm biomass ranged from 59.40% to 93.75%. These findings indicate a reduction in crystal violet-stained biomass under the experimental conditions used. These findings are consistent with previous reports showing that ethanol-based plant extracts can exhibit antibiofilm effects against different bacterial species **(Ham *et al*., 2025)**. This activity may be related to the overall phytochemical composition of the ethanolic extract. Plant-derived phenolic compounds have been reported to potentially interfere with bacterial adhesion, extracellular polysaccharide production, and quorum-sensing regulation; however, these mechanisms were not directly investigated in the present study. Similarly, **Hamzah *et al.* (2021)** reported that the ethanolic extract of *Oxalis corniculata* leaves exhibited substantial antibiofilm activity against *S. aureus* ($71.32 \pm 0.01\%$) during the mid-phase and $69.33 \pm 0.01\%$ during the maturation phase, further supporting the potential of ethanol-based plant extracts as sources of antibiofilm compounds.
In contrast, the acetone extract exhibited heterogeneous and strain-dependent effects on biofilm biomass. Most isolates exhibited negligible inhibition or negative inhibition values, whereas only a limited number exhibited stronger effects, with inhibition reaching up to 96%. In the preformed biofilm treatment assay, the reduction in biofilm biomass was predominantly moderate, although some isolates showed reductions exceeding 80%. Notably, a small number of isolates exhibited negative inhibition or biomass reduction values, indicating an increase in crystal violet-stained biofilm biomass relative to untreated controls.
Overall, the acetone extract exhibited less consistent and generally weaker antibiofilm effects than the ethanolic extract. This difference may be associated with variations in phytochemical composition and interactions among bioactive constituents, which may result in different responses depending on the bacterial isolate **(Goecke *et al*., 2012; Piekarska-Radzik *et al*., 2025; Vaou *et al*., 2022)**. However, the factors underlying the observed strain-dependent responses remain unclear, as the mechanisms involved were not investigated in this study. Further research incorporating viability assays and mechanistic approaches is needed to better understand these effects. Nevertheless, these support further investigation of *Eucalyptus globulus* extracts, particularly ethanolic extracts, as potential candidates for reducing biofilm biomass under in vitro conditions.
Statistical analysis revealed no significant correlation between biofilm biomass inhibition and the reduction of preformed biofilm biomass by the ethanolic extract, indicating that the magnitude of these two effects was not statistically associated with the present dataset. Additionally, the acetone extract exhibited greater variability in its effects across the tested isolates. These observations are consistent with previous reports suggesting that the effects of plant-derived compounds on biofilm formation and established biofilms may overlap but are not necessarily directly correlated **(Rayan *et al*., 2023; Buchmann *et al*., 2023; Fydrych *et al*., 2025)**.
The present study had some limitations. Biofilm assays were performed at a single extract concentration corresponding to the MIC; therefore, dose–response experiments were not conducted. In addition, MBIC and MBEC values were not determined in the present experimental setting. Moreover, biofilm quantification relies exclusively on the crystal violet assay, which measures total attached biofilm biomass without distinguishing between viable and non-viable bacterial cells **(Stepanović *et al.*, 2007; Azeredo *et al*., 2017)**. Consequently, the observed reductions should be interpreted as decrease in biofilm biomass rather than direct evidence of bacterial killing or complete eradication of viable bacteria within the biofilm. Furthermore, the relatively small number of clinical isolates (n = 15), and the absence of molecular characterization limit the generalizability of the findings and prevent conclusions regarding the molecular mechanisms underlying the observed effects. Future studies should address these limitations through dose–response experiments, standardized MBIC and MBEC determinations, complementary viability assays such as colony-forming unit (CFU) enumeration, microscopy-based approaches, and molecular characterization to further investigate the antibiofilm effects of *Eucalyptus globulus* extracts.

CONCLUSION

This study highlights *Eucalyptus globulus* as a potential source of bioactive compounds with in vitro effects on *Staphylococcus aureus* biofilm biomass. The differences observed between the ethanolic and acetone extracts emphasize the influence of the extraction solvent on phytochemical composition and the resulting biological activity. The greater effects generally observed with the ethanolic extract suggest that its phytochemical profile may contribute to its activity against biofilm biomass, although the specific contribution of individual constituents remains to be elucidated.
These findings also highlight the variability of responses observed among bacterial isolates, suggesting that the effects of plant extracts on biofilm biomass may depend on both their chemical composition and the characteristics of the tested strains. However, as the antibiofilm assessment relied exclusively on crystal violet staining, the observed effects reflect changes in attached biofilm biomass and do not establish bacterial killing, complete eradication of viable bacteria within the biofilm, or specific underlying mechanisms.
Although this study represents a preliminary in vitro assessment, it provides a basis for further investigations involving dose–response experiments, complementary viability assays, molecular characterization, and evaluation across a broader range of bacterial isolates. Such studies are necessary to clarify the mechanisms underlying the observed effects and to assess the reproducibility and potential biological relevance of these findings. Overall, *E. globulus* warrants further investigation as a source of natural compounds that may contribute to the development of future strategies for controlling *S. aureus* biofilm-associated infections.

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