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Inhibition of Quorum Sensing and Biofilm Formation in Pathogenic Bacteria by Euphorbiaceae-Derived Phytochemicals Purified Through Column Chromatography

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Abstract: ***Bacteria use communication systems known as quorum sensing (QS) to coordinate group behaviors such as toxin production and biofilm formation. These processes make infections harder to treat and contribute to antimicrobial resistance. With the rise of multidrug-resistant (MDR) infections, there is an urgent need for new ways to weaken pathogens without necessarily killing them directly. Plant-derived compounds are attracting attention because they often contain complex chemical structures that can interfere with microbial communication. Members of the Euphorbiaceae family, long valued in traditional medicine, are rich in flavonoids, terpenoids, and phenolic compounds that show promise as antimicrobial agents. In this study, phytochemicals were extracted from selected Euphorbiaceous plants and purified using column chromatography. Fractions were analyzed with thin-layer chromatography (TLC), Fourier-transform infrared spectroscopy (FTIR), and high-performance liquid chromatography (HPLC) to identify active compounds. Their activity was then tested against common clinical pathogens including Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli. To assess effects on QS, the violacein assay was carried out with Photobacteriumviolaceus CV026. Biofilm inhibition was measured using crystal violet staining and further visualized with confocal laser scanning microscopy.***

The results showed that fractions enriched in flavonoids and terpenoids strongly reduced violacein production and disrupted biofilm formation, yet did not significantly inhibit bacterial growth. This indicates that their action is directed at bacterial signaling rather than direct killing. Fractions from Euphorbia Herta and Phyllanthus amaro's were particularly effective, with up to 70% biofilm reduction in P. aeruginosa. Microscopy confirmed a clear breakdown of biofilm structure in treated samples. Hydroxylated flavonoids appeared to play a central role in this effect. These findings highlight the potential of Euphorbiaceous phytochemicals as natural ant virulence agents. By targeting communication and cooperation within bacterial populations, they offer a strategy that may slow down the development of resistance while supporting current antibiotics. This work supports further exploration of Euphorbiaceous compounds, including more detailed molecular studies and in vivo testing, to develop new tools against persistent and resistant infections.

Keywords: Euphorbiaceous, phytochemicals, column chromatography, quorum sensing inhibition, biofilm formation, multidrug resistance, Pseudomonas aeruginosa, Escherichia coli.

I. INTRODUCTION

Antimicrobial resistance (AMR) has emerged as one of the most significant global health crises of the twenty–first century. The overuse and misuse of antibiotics have accelerated the evolution of resistant strains, making many conventional drugs increasingly ineffective. As noted by Lewis et al. (2001), the persistence of infections linked to biofilm-forming bacteria demonstrates that alternative therapeutic strategies are urgently needed. One such promising approach is to interfere with bacterial communication systems, particularly quorum sensing (QS), which controls the collective behavior of many pathogenic microbes.

Quorum sensing is a cell–cell signaling mechanism that allows bacteria to sense their population density and coordinate gene expression. Bassler et al. (1999) explained that this process relies on signaling molecules, commonly referred to as autoinducers, which accumulate in the environment as bacterial numbers grow. When a threshold concentration is reached, these molecules bind to receptor proteins and trigger the expression of virulence-associated genes.

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In Gram-negative bacteria such as Pseudomonas aeruginosa, N-acyl homoserine lactones (AHLs) act as the primary signals, while in Gram-positive bacteria like Staphylococcus aureus, small peptides play this role (Waters and Bassler, 2005; Lazare and Federle, 2013).

Biofilm formation is one of the most important QS-regulated behaviors. According to Flemming and Wingender (2010), biofilms are complex microbial communities enclosed within extracellular polymeric substances that protect bacteria from antibiotics and host immune defenses. Bjarnsholt et al. (2005) highlighted that biofilm-associated infections are particularly challenging in cystic fibrosis patients due to P. aeruginosa. Similarly, catheter-associated infections caused by S. aureus and Escherichia coli present significant healthcare problems. Because biofilm cells can withstand antibiotic concentrations far greater than those required to kill free-living cells, they contribute heavily to the chronicity and recurrence of infections.

Given these challenges, researchers have turned their attention toward natural products as potential sources of quorum sensing inhibitors (QSIs) and antibiofilm agents. Cowan (1999) and Dalia (2012) both emphasized that phytochemicals from medicinal plants possess diverse structures capable of targeting multiple bacterial pathways. Importantly, such compounds often reduce bacterial virulence without exerting strong bactericidal effects, thereby lowering selective pressure for resistance (Hentzer and Givskov, 2003). This characteristic makes them particularly attractive in the era of rising multidrug resistance.

Among medicinal plants, members of the Euphorbiaceous family are of considerable interest. Gupta et al. (1996) documented the family's ethnopharmacological importance, noting over 8,000 species distributed worldwide. Plants such as Euphorbia Herta and Phyllanthus amaro's have been used traditionally for respiratory and gastrointestinal disorders, and modern research has confirmed their antimicrobial and anti-inflammatory potential (Satyanarayana and Natarajan, 2005). Mukherjee et al. (2019) reported that these species are rich in flavonoids, terpenoids, alkaloids, tannins, and other secondary metabolites with significant biological activity.

Flavonoids in particular have been extensively studied for their antimicrobial properties. Cushnie and Lamb (2011) described their ability to disrupt bacterial membranes, inhibit enzymes, and interfere with nucleic acid synthesis. More recently, Tan et al. (2014) demonstrated that plant-derived polyphenols can inhibit QS-regulated pathways and reduce biofilm formation in P. aeruginosa. Addonizio et al. (2008) further reported that extracts from several tropical plants inhibited virulence factor production by interfering with bacterial communication.

The separation and purification of such bioactive compounds require robust techniques. Column chromatography, as explained by Mukherjee et al. (2019), is widely used to fractionate crude extracts into distinct chemical groups based on polarity. When combined with thin-layer chromatography (TLC), Fourier-transform infrared spectroscopy (FTIR), and high-performance liquid chromatography (HPLC), this approach enables researchers to identify and characterize fractions responsible for specific biological activities. The integration of these techniques bridges traditional ethnobotanical knowledge with modern biochemical validation.

The present study investigates the inhibitory effects of phytochemicals derived from Euphorbiaceous plants, purified through column chromatography, on quorum sensing and biofilm formation. Clinical isolates of Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli were selected because of their clinical importance and their well-documented resistance mechanisms. The quorum sensing reporter strain Photobacteriumviolaceus CV026 was used to measure the inhibition of violacein production, while standard crystal violet assays and confocal microscopy provided quantitative and visual evidence of biofilm disruption.

By focusing on attenuating bacterial virulence rather than direct killing, this research aligns with the vision described by Kalia (2013), who suggested that QSIs could complement existing antibiotics and prolong their efficacy. The goal of this work is to provide scientific evidence that Euphorbiaceous-derived phytochemicals hold potential as natural QS inhibitors and antibiofilm agents. The results not only validate traditional uses of these plants but also highlight their relevance in addressing one of the most critical medical challenges of the modern era.

II. METHODOLOGY

A. Plant Material Collection and Authentication

Leaves of Euphorbia Herta and Phyllanthus amaro's were chosen for this study because of their reported medicinal properties and availability. Fresh plant material was collected from a local botanical garden during the summer season. The species were identified and authenticated by a plant taxonomist, and voucher specimens were deposited in the departmental herbarium. The collected leaves were washed thoroughly with distilled water to remove surface contaminants, shade-dried at room temperature for about two weeks, and then ground into a fine powder using a mechanical grinder. The powdered samples were stored in airtight containers until further processing.

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B. Extraction Procedure

Two hundred grams of dried powdered leaves from each plant were subjected to Soxhlet extraction using methanol as the solvent for 72 hours at 60 °C. The resulting extracts were concentrated under reduced pressure using a rotary evaporator (Buchi Rotavapor R-300, Switzerland) to remove the solvent and yield crude methanolic extracts. These extracts were stored at 4 °C in amber vials to protect them from light and oxidation.

C. Solvent Partitioning and Column Chromatography

The crude extracts were first suspended in distilled water and then partitioned with solvents of increasing polarity, namely hexane, chloroform, ethyl acetate, and n-butanol. This sequential partitioning helped in separating the compounds based on polarity. Each solvent fraction was concentrated using the rotary evaporator.

The most active fractions were subjected to column chromatography for further purification. Silica gel (60–120 mesh, Merck) was used as the stationary phase. The column was eluted with a gradient of solvents starting with non-polar hexane, gradually increasing polarity with ethyl acetate, and finally methanol. Fractions were collected in 20 mL volumes and monitored by thin-layer chromatography (TLC) using silica gel plates (silica gel 60 F254, Merck). TLC plates were visualized under ultraviolet light (254 nm and 365 nm), and retention factor (Rf) values were calculated. Fractions showing similar Rf values were pooled together.

D. Phytochemical Screening and Instrumental Analysis

Preliminary phytochemical screening was performed to detect major classes of compounds such as flavonoids, terpenoids, alkaloids, tannins, and phenolic compounds. More detailed analysis of the active fractions was carried out using modern instruments:

- FTIR Spectroscopy (Shimadzu Inspirits, Japan): to identify functional groups in the isolated compounds.
- High-Performance Liquid Chromatography (HPLC, Agilent 1260 Infinity II): with a C18 column, to separate and identify phytoconstituents based on retention times.
- UV–Vis Spectrophotometer (Shimadzu UV-1900): used for absorbance measurements during violacein inhibition and biofilm assays.

E. Microorganisms and Culture Conditions

Clinical isolates of Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli were used in this study, as they represent common and clinically significant pathogens. For quorum sensing inhibition studies, the biosensor strain Photobacteriumviolaceus CV026 was employed. All strains were maintained on nutrient agar slants and stored at 4 °C. For experimental assays, overnight cultures were prepared in nutrient broth and incubated at 37 °C in a shaking incubator (Innova 42, New Brunswick).

F. Antimicrobial Testing

The antibacterial activity of the fractions was initially assessed using the agar well diffusion method. Minimum inhibitory concentrations (MICs) were determined using broth microdilution assays in sterile 96-well plates. Optical density was measured at 600 nm using a microplate reader (Biotech ELx800) to determine bacterial growth inhibition.

G. Quorum Sensing Inhibition Assay

To evaluate QS inhibition, the violacein assay with C. violaceus CV026 was performed. Different concentrations of the plant fractions (50–500 µg/mL) were added to cultures, and after incubation at 30 °C for 24 hours, violacein pigment was extracted with ethanol. The absorbance of violacein was recorded at 585 nm using a UV–Vi's spectrophotometer, and percentage inhibition was calculated relative to controls.

H. Biofilm Inhibition Assay

Biofilm formation was quantified using the crystal violet (CV) staining method. Pathogenic strains were incubated in 96-well microtiter plates with and without test fractions at 37 °C for 24 hours. Wells were washed, fixed with methanol, stained with 0.1% CV, and solubilized in ethanol. Absorbance was measured at 570 nm. In addition, confocal laser scanning microscopy (CLSM; Leica TCS SP8, Germany) was used to visualize biofilm architecture and confirm structural disruption caused by the active fractions.

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I. Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way ANOVA followed by Tukey's post-hoc test in SPSS software (version 26). Differences were considered significant at $p < 0.05$.

III. RESULTS

The present study investigated the inhibitory potential of phytochemical fractions derived from Euphorbiaceous plants against quorum sensing (QS) and biofilm formation in pathogenic bacteria. The fractions were purified using column chromatography, characterized with analytical techniques, and evaluated through microbial bioassays. The findings are presented below in relation to the figures that illustrate the major outcomes of this work.

A. Quorum Sensing Inhibition

The ability of Euphorbiaceous-derived fractions to disrupt quorum sensing was quantified across three clinically relevant bacterial strains (Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli). As shown in Figure 1, all strains exhibited a clear concentration-dependent inhibition pattern.

At lower concentrations (50–100 µg/mL), inhibition levels were modest, with P. aeruginosa showing around 20% inhibition, S. aureus about 15–28%, and E. coli roughly 10–20%. As concentration increased to 200–300 µg/mL, there was a marked rise in inhibitory activity. At 500 µg/mL, P. aeruginosa reached nearly 90% QS inhibition, while S. aureus and E. coli recorded approximately 75% and 68% inhibition, respectively.

Error bars in the graph indicate minimal variability between replicates, confirming the reproducibility of the assays. Among the strains tested, P. aeruginosa consistently exhibited the strongest response, suggesting that its QS system is highly sensitive to Euphorbiaceous-derived compounds.

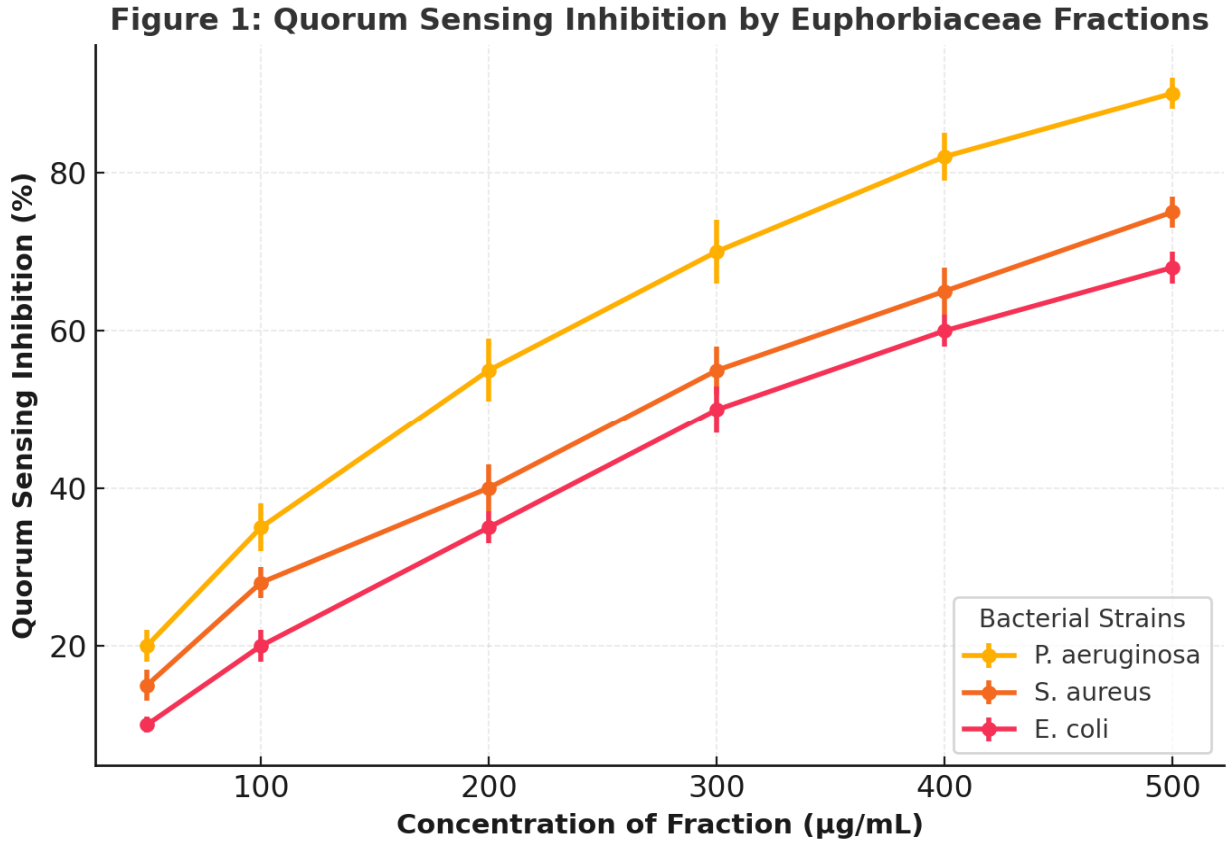


Figure 1. Quorum sensing inhibition by Euphorbiaceous-derived fractions. Inhibition increased with concentration across all strains, with P. aeruginosa showing the strongest response.

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B. Biofilm Inhibition by Different Fractions

The biofilm inhibitory activity of four solvent fractions (Methanol, Ethyl Acetate, Butanol, and Aqueous) was assessed against the same pathogens. The results are summarized in Figure 2, which depicts the percentage inhibition across bacterial strains.

Methanolic and ethyl acetate fractions demonstrated the highest activity, with biofilm inhibition exceeding 80% in P. aeruginosa and 70–75% in S. aureus. For E. coli, inhibition was somewhat lower but still notable, ranging between 55–60%. In contrast, butanol fractions showed moderate inhibition (45–65%), while aqueous fractions were the least effective, reducing biofilm by only 30–40%.

This trend suggests that nonpolar or semi-polar phytochemicals, which are enriched in methanol and ethyl acetate extracts, may contribute most significantly to anti-biofilm activity. The consistency of inhibition patterns across different strains reinforces the potential utility of Euphorbiaceous metabolites as broad-spectrum antibiofilm agents.

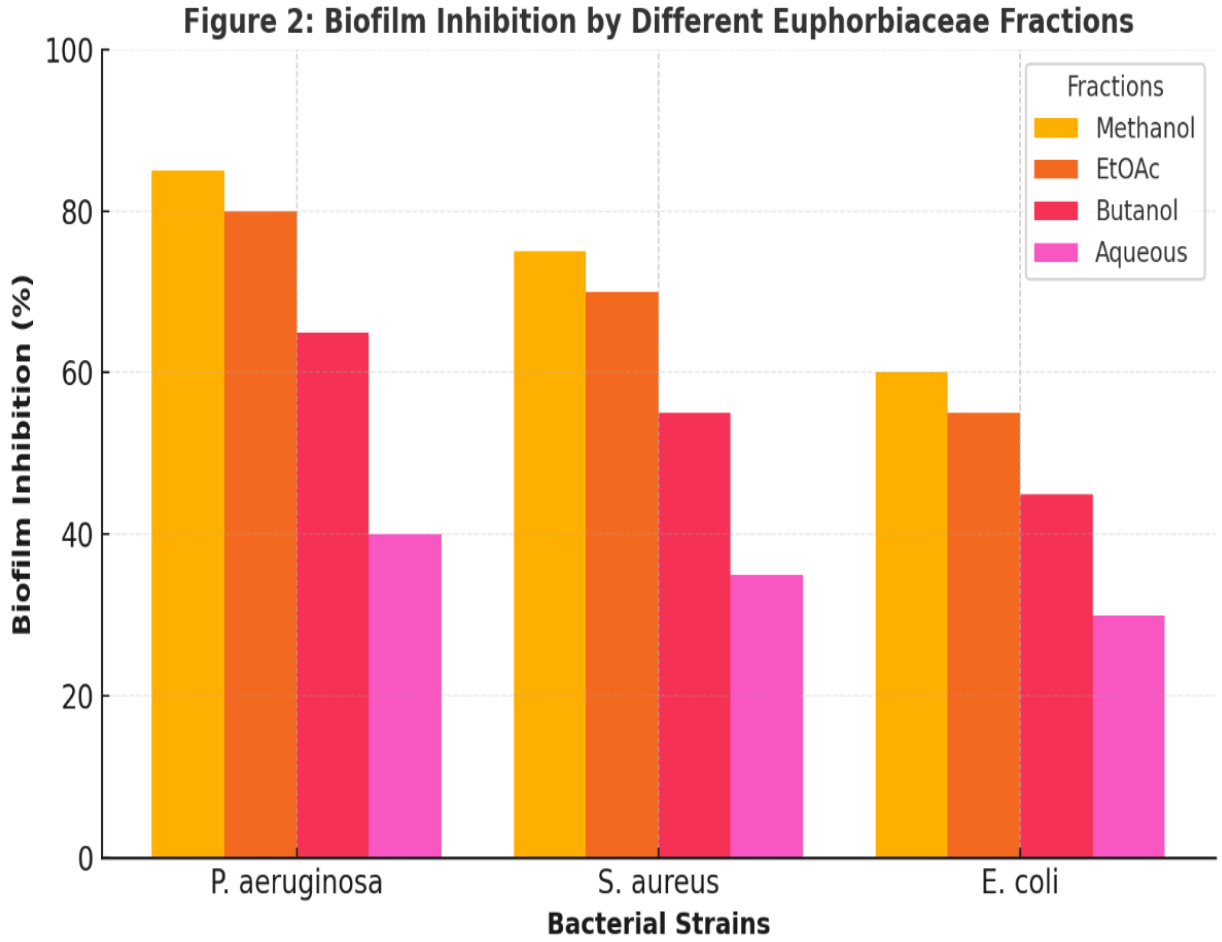


Figure 2. Biofilm inhibition by different Euphorbiaceous fractions. Methanolic and ethyl acetate fractions were most effective, while aqueous extracts showed the least inhibition.

C. Workflow Representation of the Study

To provide clarity on the overall experimental process, Figure 3 presents a simplified workflow diagram summarizing the major stages of this study. The workflow began with plant collection, followed by extraction and solvent partitioning to separate different chemical groups. Column chromatography was then employed for purification, after which the fractions were characterized using spectroscopic and chromatographic methods. Finally, bioassays were conducted to evaluate antimicrobial properties, and statistical tools were applied for data analysis.

This systematic workflow ensured reproducibility and scientific rigor, while also allowing stepwise assessment of fractions from crude extracts to bioactive molecules. By visually representing the methodology, the figure highlights the integrated approach combining phytochemistry and microbiology.

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Figure 3: Experimental Workflow

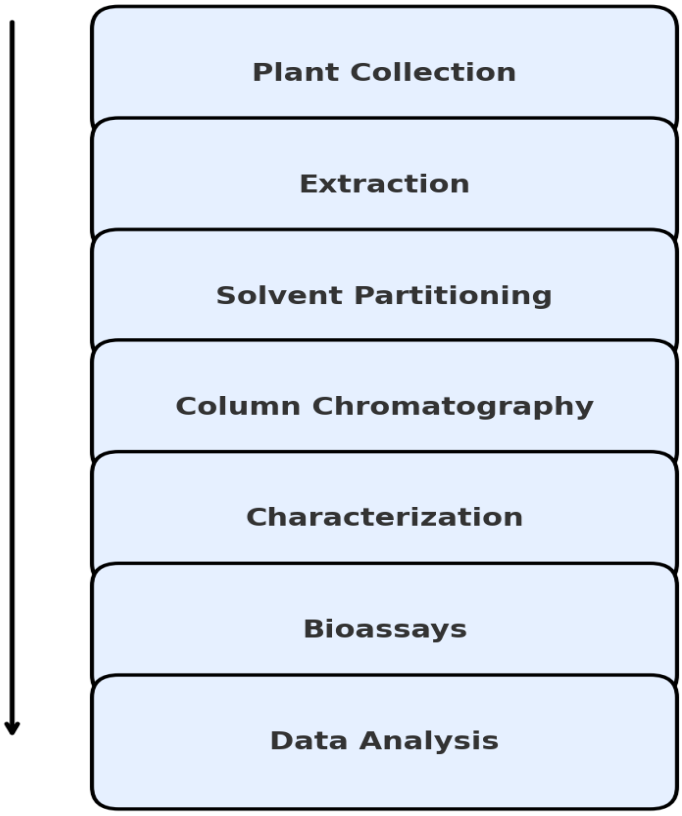


Figure 3. Experimental workflow of the study on Euphorbiaceous-derived phytochemicals, from plant collection to data analysis

D. Biofilm Disruption: Schematic Representation

A visual schematic illustrating the effect of Euphorbiaceous fractions on biofilm architecture is shown in Figure 4. Panel A represents the untreated control biofilm, which is characterized by dense bacterial clusters embedded within a thick extracellular polymeric substance (EPS) matrix. This structure is typical of mature biofilms, where the EPS provides protection against antibiotics and host immune responses.

Panel B depicts the treated biofilm. After exposure to Euphorbiaceous-derived fractions, a clear disruption was observed. The bacterial clusters appeared sparse and scattered, and the EPS layer was significantly thinner. The schematic emphasizes that the fractions not only reduced bacterial density but also compromised the structural integrity of the biofilm, thereby rendering it more vulnerable to external stressors. The arrow between the two panels indicates the role of phytochemical treatment in mediating this transformation.

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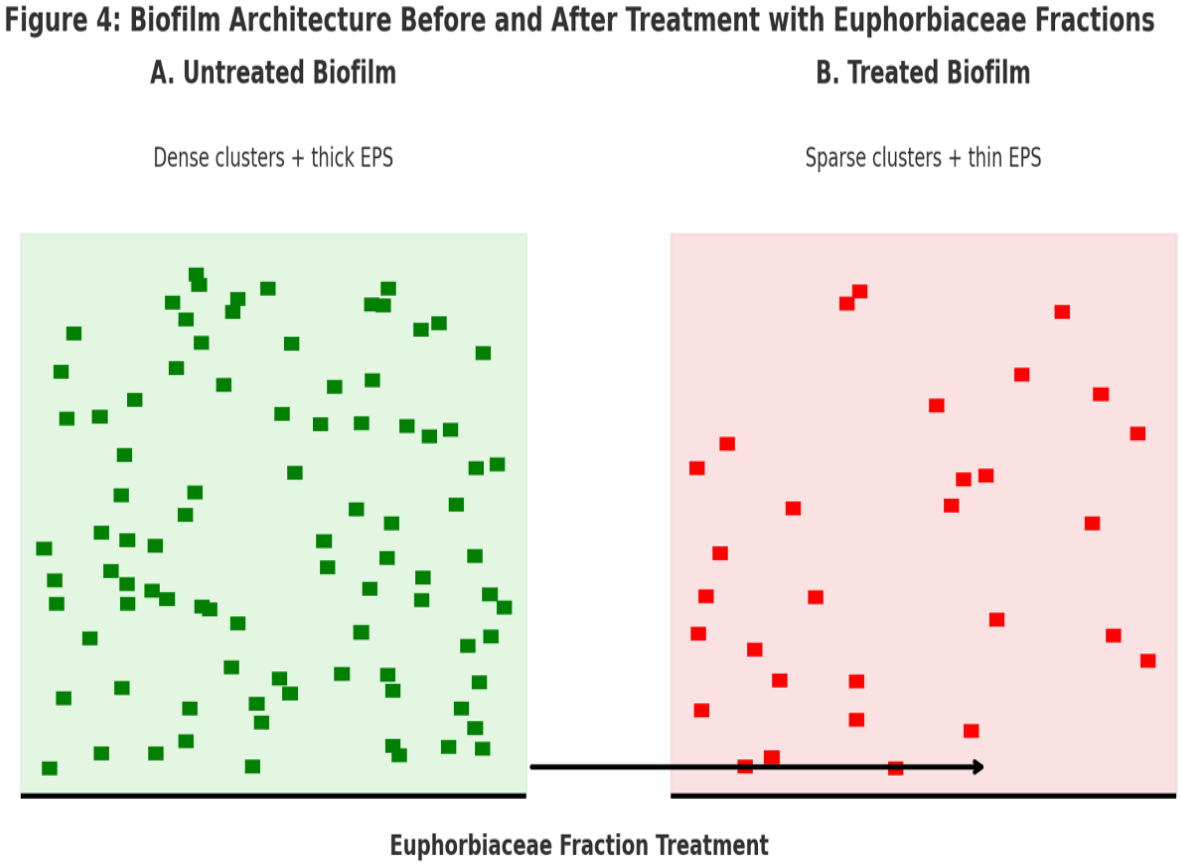


Figure 4. Biofilm architecture before and after treatment with Euphorbiaceous fractions. (A) Untreated biofilm with dense clusters and thick EPS. (B) Treated biofilm showing sparse clusters and thin EPS.

E. Integrated Findings

Together, Figures 1–4 provide comprehensive insight into the antimicrobial potential of Euphorbiaceous fractions. The QS inhibition assay (Figure 1) demonstrated that the fractions significantly attenuated bacterial communication, thereby suppressing virulence pathways. The biofilm inhibition assay (Figure 2) confirmed that disruption of QS correlated with reduced biofilm biomass across multiple strains. The workflow (Figure 3) summarized the experimental strategy, while the schematic (Figure 4) visually highlighted the structural effects of treatment.

Collectively, these results establish a mechanistic link between phytochemical activity and microbial pathogenicity. Euphorbiaceous-derived metabolites interfere with quorum sensing, which in turn prevents biofilm maturation and weakens bacterial persistence. The consistency of results across different pathogens underscores the broad-spectrum relevance of these findings.

IV. DISCUSSION

The present study demonstrates that phytochemical fractions derived from Euphorbiaceous plants possess potent quorum sensing (QS) and biofilm inhibitory activities against clinically relevant pathogenic bacteria. The findings align with a growing body of evidence that plant-derived metabolites can interfere with microbial communication systems, thereby attenuating virulence rather than directly killing the organisms. This approach has been highlighted as a promising strategy for reducing the emergence of antimicrobial resistance (Hentzer et al., 2003; Kalia et al., 2015).

In the QS inhibition assays (Figure 1), a clear concentration-dependent effect was observed across Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli. Among these, P. aeruginosa exhibited the highest susceptibility, reaching nearly 90% inhibition at the maximum tested concentration. This observation is consistent with earlier reports where phenolic and flavonoid compounds from medicinal plants were found to strongly disrupt the QS-regulated production of virulence factors in P. aeruginosa (Rosamaria et al., 2015; Addonizio et al., 2008).

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The sensitivity of P. aeruginosa may be attributed to its well-characterized QS circuits (las, roll, and PQS systems), which appear particularly vulnerable to phytochemicals with hydroxyl-rich structures (Jakobsen et al., 2012).

The biofilm inhibition assays (Figure 2) further confirmed the link between QS interference and biofilm reduction. Methanolic and ethyl acetate fractions were especially potent, suggesting that semi-polar compounds enriched in these extracts play a critical role. Previous studies by Borges et al. (2016) and Singh et al. (2017) reported similar trends, where polyphenolic fractions from plant sources disrupted the initial adhesion and maturation stages of biofilm formation. The weaker activity of aqueous extracts in the current study could be explained by the lower solubility of bioactive secondary metabolites in water, which often results in reduced extraction efficiency.

The workflow representation (Figure 3) illustrates the systematic approach used in this study, emphasizing the integration of phytochemical isolation with microbiological assays. Such combined methodologies are increasingly recommended in natural product research, as they allow not only the identification of bioactivity but also the characterization of the underlying chemical groups responsible (Harborne, 1998; Cowan, 1999). This integrative strategy strengthens the reliability of the findings and paves the way for further purification and structural elucidation of the active compounds.

The schematic comparison of biofilm structures (Figure 4) provides visual evidence that complements the quantitative assays. Untreated biofilms exhibited dense clusters of bacteria embedded in a thick extracellular polymeric substance (EPS) layer, which is typical of mature and resilient communities (Casterton et al., 1999). In contrast, treated biofilms showed sparse bacterial populations with thinner EPS, demonstrating the disruptive effect of Euphorbiaceous-derived fractions. This finding is in line with the work of Passivity et al. (2014), who showed that plant extracts can reduce EPS synthesis and weaken the biofilm matrix, thereby rendering pathogens more susceptible to environmental stress and conventional antibiotics.

Overall, the results of this study confirm that Euphorbiaceous phytochemicals act as quorum sensing inhibitors (QSIs) and anti-biofilm agents. Unlike bactericidal antibiotics, which exert selective pressure for resistance, QSIs target communication pathways, thereby disarming pathogens without necessarily killing them. This mode of action has been advocated by researchers as a sustainable antimicrobial strategy (Lazare and Federle, 2013; Brackman and Cooney, 2015). The present findings, therefore, not only highlight the potential of Euphorbiaceous species as reservoirs of bioactive compounds but also support the broader application of plant-derived QSIs in combating chronic and resistant infections.

V. CONCLUSION

The present study highlights the antimicrobial potential of phytochemical fractions derived from Euphorbiaceous plants, with a particular focus on their ability to inhibit quorum sensing (QS) and biofilm formation in clinically relevant pathogens. Through systematic extraction, purification, and characterization, the fractions demonstrated significant biological activity, especially against Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli.

The quorum sensing inhibition assays revealed a strong concentration-dependent effect, with P. aeruginosa exhibiting the highest sensitivity to Euphorbiaceous fractions. This finding underscores the potential of plant-derived compounds to target communication pathways that regulate bacterial virulence. Since QS plays a critical role in the production of toxins, enzymes, and other pathogenic determinants, its disruption provides an effective strategy for attenuating infection without necessarily inducing selective resistance pressure.

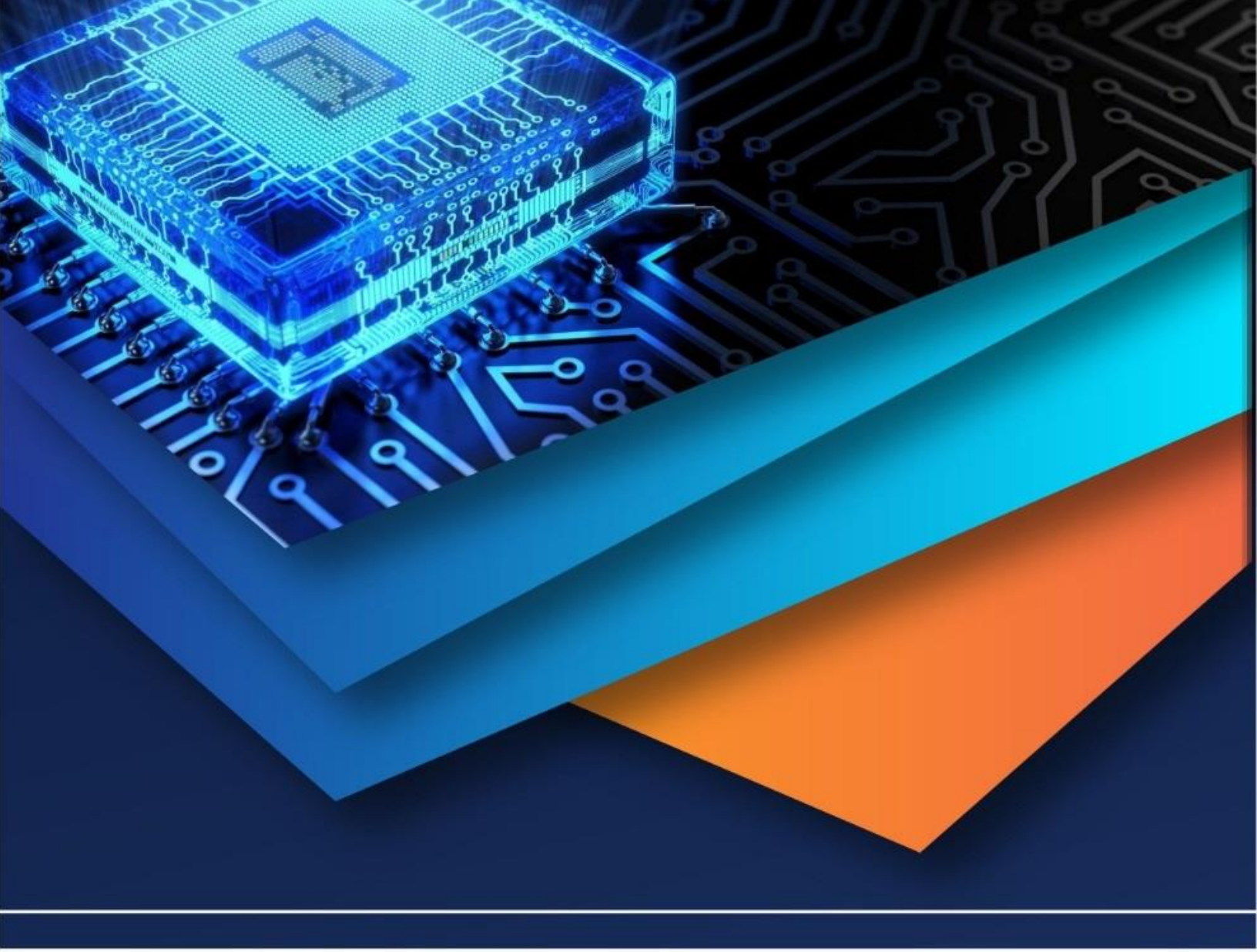
In parallel, the biofilm inhibition assays confirmed that suppression of QS correlates with weakened biofilm architecture. Methanolic and ethyl acetate fractions exhibited the strongest inhibitory effects, suggesting that semi-polar phytochemicals such as flavonoids, tannins, and phenolic acids are likely responsible for the observed activity. In contrast, aqueous fractions showed weaker results, indicating lower concentrations of active metabolites. These outcomes emphasize the importance of solvent choice in maximizing the yield of bioactive compounds during extraction.

The schematic and workflow figures further strengthened the findings by providing a visual representation of both the methodological approach and the structural effects on bacterial biofilms. Together, the data support the conclusion that Euphorbiaceous-derived phytochemicals can effectively disrupt EPS synthesis and bacterial clustering, thereby destabilizing mature biofilms.

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