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Medicinal Plant-Based Inhibitors of Quorum Sensing and Biofilm in Gram-Negative Pathogens: An Integrated Experimental and In Silico Study

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ABSTRACT

Multidrug resistance (MDR) in pathogenic bacteria has emerged as a significant clinical challenge. Targeting quorum sensing (QS), which regulates bacterial virulence, represents a promising strategy for combating MDR infections. In this study, we aimed to evaluate the anti-quorum sensing potential of selected medicinal plants against bacterial pathogens, alongside in silico interactions of their bioactive phytocompounds with QS and biofilm-associated proteins. Based on ethnopharmacological relevance, 18 plants were selected, and methanolic extracts were tested against Chromobacterium violaceum 12472. Among them, Acacia nilotica exhibited the highest activity and was further fractionated using solvents of increasing polarity (n-hexane, chloroform, and ethyl acetate) to assess anti-QS activity. The ethyl acetate fraction, identified as the most active, was evaluated at sub-MIC levels for its effect on QS-regulated virulence factors in Pseudomonas aeruginosa PAO1 and Serratia marcescens MTCC 97. Biofilm inhibition was quantified using microtiter plate assays and visualized through light microscopy. Phytochemical profiling of the ethyl acetate fraction was performed using GC/MS and LC/MS analyses. Molecular docking studies were conducted with AutoDock Vina to investigate interactions of the identified phytocompounds with QS proteins (LasI, LasR, CviR, RhlR) and biofilm-associated proteins (PilY1 and PilT). The MIC of the ethyl acetate fraction was determined as 250, 500, and 1000 µg/ml against C. violaceum 12472, P. aeruginosa PAO1, and S. marcescens MTCC97, respectively. At sub-MIC concentrations, the fraction significantly inhibited QS-regulated virulence factors and reduced biofilm formation by over 50%. GC/MS analysis revealed benzoic acid, 3,4,5-trihydroxy-, methyl ester (61.24%) as the major bioactive compound, while LC-MS detected retronecine in A. nilotica pods for the first time. In silico analysis showed that dehydroabietic acid occupied the CviR ligand-binding domain similarly to its antagonist, while betulin and epicatechin gallate interacted with PilY1 and PilT, disrupting biofilm formation. Overall, the findings indicate that phytochemicals from A. nilotica pods hold potential as anti-QS agents against Gram-negative pathogens. Further studies are warranted to evaluate the therapeutic efficacy of standardized extracts or isolated compounds in vivo.

Keywords: Medicinal plant, Quorum sensing, Virulence factors, Biofilm inhibition, Phytochemistry

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Introduction

Antimicrobial resistance (AMR) in pathogenic bacteria has emerged as a major global health threat. Overuse of antibiotics in clinical settings is a well-recognized driver of bacterial resistance. Beyond classical resistance mechanisms, the ability of pathogenic bacteria to form biofilms enhances their tolerance to high antibiotic concentrations and contributes to their virulence. Biofilm formation is therefore considered an important virulence factor [1, 2]. Numerous strategies have been proposed to address multidrug resistance (MDR) [3, 4], and targeting quorum sensing (QS)—the bacterial cell-to-cell communication system—has gained attention as a potential approach to suppress virulence and pathogenicity [5, 6]. QS enables bacteria to sense population density through small signaling molecules called autoinducers, which regulate gene expression and orchestrate phenotypes that enhance survival in specific environments [7].

Previous studies have highlighted the therapeutic potential of medicinal plants and herbal medicines in both traditional medicine and drug discovery due to their diverse bioactive compounds [8, 9]. India, a global biodiversity hotspot, has a rich traditional medical system utilizing a wide range of plants to treat infectious and non-infectious diseases [10]. Recently, there has been increasing interest in identifying non-toxic, broad-spectrum anti-QS agents from plants and microbes [11–13]. Medicinal plants produce a variety of secondary metabolites, including phenolics, terpenoids, flavonoids, quinones, catechins, and alkaloids, some of which mimic or interfere with acyl-homoserine lactone (AHL) QS signals [14, 15]. Screening plants for bioactive compounds is justified due to their vast chemical diversity [16]. Quorum-modulatory agents are considered promising anti-infective candidates because they disrupt QS-mediated pathogenicity with a lower likelihood of resistance development compared to conventional antibiotics [17, 18]. Nevertheless, some studies indicate that bacteria may develop resistance against anti-virulence compounds, as evolutionary pressures can drive adaptation even under QS-targeted interventions [19–21].

Biofilm formation is a key pathogenic trait in many bacteria, providing protection against antibiotics, the host immune system, and environmental stresses [22]. However, the multiplicity of QS signaling pathways and molecules complicates the identification of broad-spectrum anti-QS compounds from plants. For example, Pseudomonas aeruginosa can form biofilms even when one QS system is inactive, highlighting that biofilm development is multifactorial and may not be fully suppressed by QS inhibition alone [23]. Therefore, evaluating plant extracts for both QS-inhibitory and anti-biofilm activity is crucial to identify effective anti-infective agents. Several medicinal plants have previously been screened for QS inhibition, and numerous phytochemicals have been shown to impair pathogenicity by interfering with QS signaling [17, 24–27]. Despite India's rich plant diversity and traditional usage, many species, including Acacia nilotica, have yet to be systematically evaluated for their anti-QS and anti-biofilm potential.

Acacia nilotica Lam. (Mimosaceae), commonly known as 'Babul' or 'Kikar,' is a medium-sized tree distributed widely across tropical and subtropical regions [28]. Various parts of the plant—including leaves, fruits, roots, seeds, bark, flowers, gum, and immature pods—have been reported to exhibit diverse pharmacological activities, such as anticancer, antimutagenic, spasmogenic, vasoconstrictive, antipyretic, anti-asthmatic, cytotoxic, anti-diabetic, anti-platelet, anti-plasmodial, molluscicidal, antifungal, and antiviral effects against Hepatitis C virus [29–31]. However, its potential anti-QS and anti-biofilm activities remain largely unexplored.

The present study aimed to identify plant-derived bioactive compounds with promising anti-infective properties and to explore their mechanisms of action through in silico analyses. Eighteen Indian medicinal plants were initially screened using Chromobacterium violaceum 12472 as a reporter strain. The most active plant extract and its active fraction were further evaluated for anti-QS and anti-biofilm effects against Pseudomonas aeruginosa PAO1 and Serratia marcescens MTCC 97. The phytochemicals present in the most active extract were identified using GC/MS and LC/MS, and in silico studies were conducted to predict their interactions with QS and biofilm-associated proteins.

Materials and Methods

Chemical and reagents

All reagents and chemicals employed in this study were of high analytical quality. Growth media, including Luria Bertani (LB) and Pseudomonas broth, along with TTC (2,3,5-Triphenyltetrazolium chloride), crystal violet, and glutaraldehyde, were purchased from Hi-Media, Mumbai. Protein substrates azocasein and Elastin Congo Red were obtained from Sigma-Aldrich Pvt. Ltd. Solvents such as methanol, chloroform, n-hexane, ethyl acetate, ethanol, and hydrochloric acid (all ≥99.9% purity) were supplied by Thermo Fisher Scientific India Pvt. Ltd., Mumbai.

Bacterial strains and growth conditions

The study employed three bacterial strains: Chromobacterium violaceum ATCC 12472, which naturally synthesizes a purple pigment in response to its C6 AHL quorum sensing molecules (ATCC, USA); Serratia marcescens MTCC 97 (MTCC, India); and Pseudomonas aeruginosa PAO1, kindly provided by Prof. R. J. C. McLean (USA). Cultures were maintained on Luria Bertani (LB) agar, containing 0.5% yeast extract, 15 g/L tryptone, 0.5% NaCl, and 20 g/L agar. Incubation was performed at 30 °C for C. violaceum and 37 °C for the other strains

Plant material used

Plant materials used in this study were either generously provided by the Himalaya Drug Company (Dehradun, India) or collected from local sites in Aligarh, India. Their botanical identities were authenticated with assistance from the Department of Botany at Aligarh Muslim University (AMU), and representative voucher specimens were archived at the Department of Agricultural Microbiology, AMU.

Preparation of plant extracts and their fractionation

Shade-dried plant materials, including leaves or fruits/pods, were first ground into a fine powder using an electric grinder. For extraction, 100 g of this powdered material was immersed in 500 mL of methanol (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India) and left for five days with occasional stirring. After extraction, the mixture was filtered through Whatman No. 1 paper and centrifuged at 10,000 rpm for 15 minutes (Sigma 2-16KL, High-Speed Tabletop Centrifuge). The supernatant was then concentrated at 40 °C under reduced pressure using a rotary evaporator (RE-2000A, Associated Scientific Technologies, Delhi, India) to obtain the crude extract.

The crude extract was re-dissolved in hot double-distilled water and partitioned sequentially with solvents of increasing polarity—n-hexane, chloroform, ethyl acetate—leaving an aqueous fraction as the final layer. Each fraction was concentrated under vacuum as described above and stored at 4 °C. Prior to bioassays, the dried fractions were dissolved in ≤1% (v/v) DMSO to prepare stock solutions.

Screening of plant extract for anti-QS activity against Chromobacterium violaceum 12472

Methanolic extracts from all 18 medicinal plants were evaluated for their ability to suppress pigment production in C. violaceum 12472, following the procedure described previously [32]. In brief, 100 µL of an overnight culture of C. violaceum 12472 (10^6 CFU/mL) was mixed with 3 mL of molten LB agar (0.5% w/v) and overlaid onto an LB agar plate, which was then allowed to solidify. Plant extracts were tested at a concentration of 1 mg/mL to assess anti-quorum sensing activity. Plates were incubated overnight at 30 °C, and inhibition of violacein production and/or bacterial growth was determined by measuring the diameter of the corresponding zones. The extract demonstrating the highest inhibitory effect was chosen for further investigation.

Determination of minimum inhibitory concentration (MIC) of Acacia nilotica

The minimum inhibitory concentration (MIC) of the most active plant fraction, the ethyl acetate extract of Acacia nilotica (ANEa), was determined to establish sub-inhibitory concentrations (sub-MICs) for subsequent experiments. MIC determination was performed using the broth microdilution method with TTC dye, as described previously [33]. In brief, the ethyl acetate fraction of the methanolic extract was prepared in twofold serial dilutions ranging from 8 mg/mL to 0.125 mg/mL in LB broth, with a final volume of 200 µL per well in a 96-well microtiter plate. The bacterial inoculum was adjusted to 0.5 McFarland standard (~10^8 CFU/mL) and further diluted 1:100. Wells containing medium with bacterial culture served as the positive control, while sterile medium without bacteria was used as the negative control. Plates were incubated at 37 °C for 18 h. After incubation, 20 µL of 2 mg/mL TTC (Hi-Media, India) was added to each well and incubated for an additional 10 min at 37 °C. The reduction of TTC to its formazan product, indicated by the development of a pink color, signified bacterial growth. The MIC was defined as the lowest concentration at which no color change occurred. Doxycycline was included as a reference antibiotic. To further confirm the absence of growth or partial inhibition, 100 µL from wells showing no color change was streaked onto LB agar plates.

Analysis of bacterial growth curve

To evaluate whether the ANEa fraction affected bacterial proliferation, the highest sub-inhibitory concentration was tested against Pseudomonas aeruginosa PAO1, Serratia marcescens MTCC 97, and Chromobacterium violaceum 12472. Cultures were prepared in 100 mL LB broth with or without the extract and incubated at 37 °C for a full day. Bacterial growth was monitored at two-hour intervals by measuring the optical density at 600 nm, and the resulting data were used to construct growth profiles.

Quantitative estimation of violacein

The effect of ANEa on violacein production by *C. violaceum* 12472 was evaluated using a modified protocol from a previous study [34]. Overnight cultures were incubated in LB broth with or without sub-MIC levels of the extract

(125, 62.5, and 31.25 µg/mL) at 30 °C under constant shaking at 220 rpm. Following incubation, cells were pelleted by centrifugation at 10,000 rpm for 10 minutes (Sigma 2-16KL). The pellet was resuspended in 1 mL DMSO and vortexed for 30 seconds to solubilize violacein. Cellular debris was removed via a second centrifugation at 10,000 rpm for 10 minutes. The absorbance of the extracted violacein in the supernatant was measured at 585 nm using a spectrophotometer (LABMAN LMSP-V3205). All assays were performed in triplicate, and the percentage inhibition of violacein relative to the untreated control was calculated as:

$$\%\text{Inhibition} = \frac{A_c - A_t}{A_c} \times 100\% \quad (1)$$

where AcA_cAc and AtA_tAt correspond to the absorbance values of the control and treated samples, respectively.

Assays for determination of QS-linked virulence factor in Pseudomonas aeruginosa PAO1

Assay for pyocyanin production

Quantitative analysis of pyocyanin production by Pseudomonas aeruginosa PAO1 was carried out in Pseudomonas broth (PB; 1.4 g/L $MgCl_2$, 10 g/L K_2SO_4, 20 g/L peptone) following a previously reported method with minor modifications [35]. Cultures were grown in the presence or absence of sub-inhibitory concentrations of the ANEa fraction (500, 250, and 125 µg/mL) at 37 °C under shaking for 18 h. After incubation, cultures were centrifuged at 10,000 rpm for 10 min (Sigma 2-16KL High-Speed Tabletop Centrifuge). Five milliliters of the supernatant were extracted with 3 mL chloroform, and 1 mL of 0.2 M HCl was added to the organic layer. Following a second centrifugation at 10,000 rpm for 10 min, the absorbance of the resulting pink to deep-red aqueous layer was measured at 520 nm using a LABMAN LMSP-V3205 spectrophotometer. Pyocyanin concentration (µg/mL) was calculated by multiplying the OD_{520} by 17.072

Assay for pyoverdine production

Pyoverdine production by Pseudomonas aeruginosa PAO1 was assessed following a previously reported protocol with minor modifications [36]. Cell-free supernatants were obtained from cultures grown in the presence or absence of sub-inhibitory concentrations of ANEa (500, 250, and 125 µg/mL). To each 100 µL of supernatant, 900 µL of 50 mM Tris-HCl buffer (pH 7.4) was added, and the fluorescence was measured using a Shimadzu RF-5301PC spectrofluorometer at an excitation wavelength of 400 nm and emission wavelength of 460 nm.

Assay for inhibition of proteolytic activity

Exoprotease activity of Pseudomonas aeruginosa PAO1 was measured using the azocasein degradation assay as described by Kessler *et al*. [37]. In brief, 100 µL of cell-free supernatants from treated or untreated cultures were mixed with 1 mL of 0.3% (w/v) azocasein prepared in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.5 mM $CaCl_2$ and incubated at 37 °C for 15 minutes. The reaction was stopped by adding 500 µL of 10% (w/v) trichloroacetic acid, followed by centrifugation at 12,000 rpm for 10 minutes (Sigma 2-16KL High-Speed Tabletop Centrifuge). The absorbance of the resulting supernatant was recorded at 400 nm using a LABMAN LMSP-V3205 spectrophotometer.

Swarming motility assay

The impact of the ANEa fraction on swarming motility of Pseudomonas aeruginosa PAO1 was assessed following a previously described method [38]. LB agar plates (0.5% agar) were supplemented with different concentrations of ANEa (500, 250, and 125 µg/mL), prepared on the day of the experiment, and allowed to air-dry at room temperature for 1 h. Five microliters of bacterial culture (~10^8 CFU/mL) were spot-inoculated at the center of each plate, while untreated plates served as controls. Plates were incubated upright at 37 °C for 18 h, and the diameter of the swarming zones was measured from the inoculation point.

Attenuation of QS-linked virulence factor in Serratia marcescens MTCC 97

Quantitative estimation of prodigiosin

Prodigiosin (2-methyl-3-phenyl-6-methoxyprodigiosin) production, a quorum sensing-regulated virulence factor of *Serratia marcescens* MTCC 97, was evaluated for inhibition by the ANEa fraction following a previously

described method [39]. Briefly, bacterial cultures were incubated in the presence or absence of sub-MIC concentrations of ANEa (250, 125, and 62.5 µg/mL). Cells were harvested by centrifugation at 10,000 ×g for 15 minutes, and prodigiosin was extracted from the pellet using an acidified ethanol solution (4 mL 1 M HCl in 96 mL ethanol). The pigment concentration was determined by measuring absorbance at 534 nm with a LABMAN LMSP-V3205 spectrophotometer. The percentage inhibition of prodigiosin relative to the untreated control was calculated using the formula:

$$\%\text{Inhibition} = \frac{A_c - A_t}{A_c} \times 100\%\text{Inhibition} = \text{AcAc} - \text{At} \times 100 \quad (2)$$

where AcA_cAc and AtA_tAt represent the absorbance of the control and treated samples, respectively.

Swarming motility

The effect of the ANEa fraction on swarming motility of Serratia marcescens MTCC 97 was evaluated following the same procedure as described previously for P. aeruginosa [38]. Swarm agar plates (0.5% agar) containing ANEa at concentrations of 250, 125, and 62.5 µg/mL were freshly prepared and allowed to air-dry at room temperature. Control plates without the extract were included for comparison. Five microliters of S. marcescens culture were spot-inoculated at the center of each plate, which were then incubated at 30 °C for 16 h. Swarming behavior was assessed by measuring the spread from the inoculation point to determine inhibitory effects.

Inhibition of exo-protease activity

Exoprotease activity of Serratia marcescens MTCC 97 was assessed using the azocasein assay as described previously. Briefly, 100 µL of culture supernatant, either treated with ANEa (250, 125, and 62.5 µg/mL) or untreated, was mixed with 1 mL of 0.3% azocasein prepared in 0.05 M Tris-HCl buffer containing 0.5 mM $CaCl_2$ (pH 7.5). The reaction mixture was incubated at 37 °C for 15 minutes and then terminated by adding 500 µL of 10% (w/v) trichloroacetic acid. Samples were centrifuged at 12,000 rpm for 10 minutes (Sigma 2-16KL High-Speed Tabletop Centrifuge), and the absorbance of the resulting supernatant was recorded at 400 nm.

Quantitative assessment of biofilm formation

Biofilm formation was quantified using a 96-well microtiter plate assay, following a previously described method with modifications [34]. Overnight bacterial cultures were inoculated into wells containing LB medium. Wells designated for treatment received sub-MIC concentrations of the ANEa fraction, while untreated wells served as controls. Plates were incubated statically at 37 °C for 24 h. After incubation, planktonic cells and excess medium were removed by gently washing the wells three times with sterile phosphate buffer (0.1 M, pH 7.4), and the plates were air-dried for 20 minutes at room temperature. Adherent biofilms were stained with 0.1% (w/v) crystal violet for 15 minutes, followed by gentle washing to remove unbound dye. The bound dye was solubilized in 90% ethanol, and the absorbance of the resulting solution was measured at 620 nm using a microplate reader (Thermo Scientific, Multiskan EX, India). The percentage inhibition of biofilm formation relative to the untreated control was calculated as:

$$\%\text{Inhibition} = \frac{A_c - A_t}{A_c} \times 100\%\text{Inhibition} = \text{AcAc} - \text{At} \times 100 \quad (3)$$

where AcA_cAc and AtA_tAt represent the absorbance values of the control and treated samples, respectively.

Microscopic evaluation

Biofilm development on glass surfaces was assessed using 12-well tissue culture plates containing LB medium and sterile coverslips. Overnight bacterial cultures were added to the wells, with some wells receiving the ANEa treatment and others serving as untreated controls. After 24 h incubation, the coverslips were carefully rinsed with phosphate buffer (0.1 M, pH 7.4) to remove non-adherent cells. The remaining biofilm was stained with 0.1% crystal violet, air-dried, and examined under a light microscope (Olympus BX60, Model BX60F5, Japan) equipped with a digital camera (Sony SSC-DC-58AP, Japan) at 40× magnification.

Detection of major phytocompounds in extract

The crude methanolic extract of Acacia nilotica and its solvent fractions were analyzed for the presence of key bioactive phytochemicals, including flavonoids, alkaloids, saponins, and glycosides, using standard qualitative colorimetric assays [40].

Fourier transform of infrared spectroscopy (FTIR) analysis of A. nilotica

Fourier-transform infrared (FTIR) spectroscopy was employed to detect functional groups and chemical bonds present in the ANEa fraction. A small portion of the extract was finely ground with potassium bromide and loaded into the FTIR spectrometer (Shimadzu, Japan). Spectra were recorded over a range of 4000–400 cm^{-1} using a PerkinElmer spectrophotometer (Version 10.4.00). Functional groups were identified based on the characteristic absorption peaks observed in the IR spectra.

Gas chromatography-mass spectrometry (GC-MS) analysis

The ethyl acetate fraction of Acacia nilotica was analyzed using a gas chromatography–mass spectrometry (GC-MS) system equipped with a mass selective detector (Shimadzu, Kyoto, Japan) at the Advanced Instrumentation Research Facility (AIRF), JNU, New Delhi, India. Helium was used as the carrier gas at a constant flow rate of 1.21 mL/min, with an injection temperature of 260 °C and a split ratio of 1:10. Separation was achieved using a temperature program starting at 50 °C, increasing to 280 °C at 10 °C/min, with a final hold at 280 °C for 25 min. Data were acquired in scan mode over an m/z range of 40–650. Compounds were identified by comparing the mass spectra of detected peaks to entries in the NIST Mass Spectral Library. Relative abundances of the compounds were expressed as percentages based on peak areas in the total chromatogram, with retention times and a hold time of 2 min considered for each peak.

Liquid chromatography-mass spectrometry (LC-MS) analysis

Polar constituents in the ethyl acetate fraction of Acacia nilotica were analyzed using LC-ESI-Q-TOF MS (Agilent G6550A) at SAIF, IIT Bombay, Mumbai, India. The chromatographic separation employed a binary solvent system, with 0.1% formic acid in water as solvent A and 90% acetonitrile with 0.1% formic acid as solvent B, using a 5 µL injection. The gradient began at 95% A and 5% B, reached 100% B at 25 min, and returned to the starting composition within 1 min, holding for an additional 5 min at a flow rate of 0.3 mL/min under 1200 bar pressure. Mass spectrometric detection was carried out in both positive and negative ESI modes over an m/z range of 150–1000. Instrument settings included a capillary voltage of 3500 V, nozzle voltage 1000 V, fragmentor 175 V, skimmer 65 V, octupole RF peak 750 V; gas flow 13 L/min at 250 °C; sheath gas flow 11 at 300 °C; and nebulizer pressure 35 psig. Compound profiling, identification, and quantification were performed using Mass Hunter software, with both high-resolution MS and MS/MS analyses.

In silico molecular docking studies

The phytochemicals identified in the ANEa fraction via GC/MS and LC/MS were further investigated through computational docking against quorum-sensing and biofilm-related proteins from C. violaceum 12472 and P. aeruginosa PAO1. Crystal structures for CviR, LasI, LasR, PilY1, and PilT were retrieved from the Protein Data Bank, while the RhlR structure was obtained from UniProt (https://www.uniprot.org/uniprot/P54292
) due to its unavailability in PDB. Three-dimensional structures of the selected ligands—including methyl gallate, pyrogallol, betulin, phloroglucinol, oleic acid, 5-hydroxyvanillin, 2,3-dihydro-benzofuran, dehydroabietic acid, pyrocatechol, homomangiferin, and retronecine—were downloaded from PubChem in SDF format and converted to PDB format using Chimera 1.10.2. Receptor and ligand files were prepared in PDBQT format with MGLTools 1.5.6. Protein–ligand interactions, including hydrogen bonds and hydrophobic contacts, were analyzed with LigPlot+, and final visualizations were generated using PyMOL and BIOVIA Discovery Studio 4.2.

Statistical analysis

All experiments were performed in triplicate, and the results are presented as mean ± standard deviation (SD). Statistical significance was considered at $P \leq 0.05$. P-values were calculated using a t-test in Microsoft Excel 2019 to assess the significance of differences between groups.

Results and Discussion

Screening of some plants for their anti-QS activity

Inhibition of quorum sensing (QS)-regulated purple pigment production in C. violaceum 12472 serves as an indicator of QS interference. Among the 18 medicinal plants screened for anti-QS activity using this reporter strain, only eight—Acacia nilotica, Andrographis paniculata, Cinnamomum verum, Gymnema sylvestre, Murraya koenigii, Myristica fragrans, Punica granatum, and Syzygium aromaticum—demonstrated varying levels of pigment inhibition. While methanolic extracts of Andrographis paniculata, Gymnema sylvestre, Murraya koenigii, and Myristica fragrans exhibited primarily antibacterial activity, they showed only marginal QS inhibition at the tested concentrations. Acacia nilotica displayed the highest QS inhibitory effect, with a 14 mm pigment inhibition zone, followed by Cinnamomum verum (8 mm), Syzygium aromaticum (5 mm), and Punica granatum (4 mm) **(Table 1).** Due to its strong anti-QS activity, the methanolic extract of A. nilotica was fractionated sequentially using n-hexane, chloroform, and ethyl acetate, with the ethyl acetate fraction showing the most pronounced pigment inhibition. This fraction was subsequently evaluated for broad-spectrum QS inhibition, including its effects on QS-regulated virulence factors in Pseudomonas aeruginosa PAO1 and Serratia marcescens MTCC 97.

Table 1. Inhibition of QS mediated violacein production in the presence active plant extracts against *C. violaceum* 12472.

Plants Name	Parts used	Zone of inhibition against *C. violaceum* 12472 (mm)		
		Growth + Pigment inhibition or Total inhibition (r_1)	Growth inhibition (r_2)	QS Inhibition (r_1-r_2)
Acacia nilotica	Pods	32	18	14
Andrographis paniculate	Leaves	14	11	3
Cinnamomum verum	Bark	22	14	8
Gymnema sylvestre	Leaves	15	11	4
Murraiyya koenigii	Fruit	12	10	2
Myrisrtica fragrance	Fruit	16	14	2
Punica granatum	Rind	20	16	4
Syzygium aromaticum	Flower buds	33	28	5

Values are the round off to the nearest whole number of mean values of three independent experiments.
r_1 = radius of total inhibition (growth + pigment in mm).
r_2 = radius of growth inhibition (mm).
r_1-r_2 = radius of QS inhibition (mm).

Minimum inhibitory concentration (MIC)

To establish sub-inhibitory concentrations for evaluating anti-infective activity, the MIC of the ethyl acetate fraction of Acacia nilotica (ANEa) was determined against three bacterial strains. The fraction inhibited C. violaceum 12472 at 250 µg/mL, S. marcescens MTCC 97 at 500 µg/mL, and P. aeruginosa PAO1 at 1000 µg/mL. In comparison, the standard antibiotic doxycycline exhibited MICs of 8 µg/mL for C. violaceum and S. marcescens, and 16 µg/mL for P. aeruginosa.

Quantitative estimation of violacein

Quantitative analysis of QS-inhibitory activity of the ANEa fraction demonstrated a concentration-dependent suppression of violacein production compared to the untreated control **(Figure 1).** Treatment with sub-inhibitory concentrations of 125, 62.5, and 31.2 µg/mL resulted in pigment reductions of 94.7%, 89.2%, and 81.2%, respectively, relative to the control.

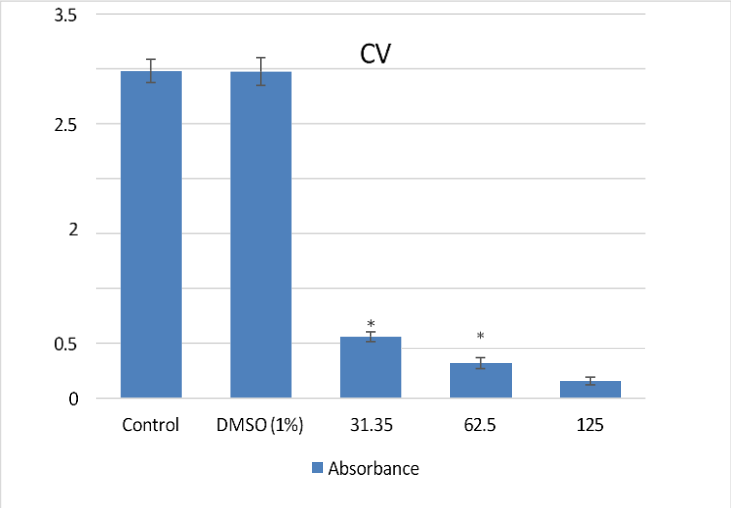


Figure 1. Effect of sub-MIC concentrations (125, 62.5, and 31.25 µg/mL) of Acacia nilotica ethyl acetate fraction (ANEa) on violacein production in C. violaceum 12472. The secondary y-axis represents percentage inhibition. Data are shown as mean ± SD from three independent experiments. Statistical significance relative to the untreated control was assessed using p-values, with ∗ indicating $p \leq 0.05$.

Analysis of growth curve

The impact of the highest sub-inhibitory concentrations of ANEa on bacterial growth was assessed for P. aeruginosa PAO1 (500 µg/mL), S. marcescens MTCC 97 (250 µg/mL), and C. violaceum 12472 (125 µg/mL). No notable changes in growth patterns were observed for any of the tested strains.

Attenuation of QS-linked virulence factor in P. aeruginosa PAO1

The ANEa fraction was evaluated for its effects on key QS-regulated virulence traits of P. aeruginosa PAO1, including pyocyanin, pyoverdine, and swarming motility. Treatment with ANEa caused a dose-dependent reduction in pyocyanin production, with the highest sub-MIC (500 µg/mL) resulting in 51.7% inhibition, while pyoverdine production decreased by 36.5% at the same concentration **(Table 2).** Lower sub-MICs produced correspondingly smaller reductions. Assessment of extracellular protease activity using the azocasein assay revealed decreases of 49.3%, 43.8%, and 26.8% at 500, 250, and 125 µg/mL, respectively, compared to untreated controls. Swarming motility was also markedly impaired, with the zone diameter decreasing to 11.6 ± 0.5 mm at 500 µg/mL, compared to 19.3 ± 1.1 mm in the control **(Table 2).**

Table 2. Effect of sub-MICs of *A. nilotica* ethyl fraction (ANEa) on the virulence factors of *P. aeruginosa* PAO1.

ANEa concentrations	Pyocyanin[a]	Pyoverdine[b]	Total protease[c]	Swarming motility[d]
Control	16.25 ± 0.01	747.6 ± 8.13	0.81 ± 0.02	19.3 ± 1.1
125 µg/ml	10.87 ± 0.04∗∗ (33.1%)	630.1 ± 1.85∗∗ (15.7)	0.59 ± 0.003∗∗ (26.8%)	15 ± 1∗ (22.27)
250 µg/ml	8.78 ± 0.01∗∗ (46%)	525.8 ± 4.68∗∗ (29.66)	0.45 ± 0.001∗∗ (43.8%)	14.3 ± 0.5∗ (25.90)
500 µg/ml	7.84 ± 0.004∗∗∗ (51.7%)	474.2 ± 11.7∗∗∗ (36.57)	0.41 ± 0.004∗∗∗ (49.3%)	11.6 ± 0.5∗ (39.89)

Data represent mean and standard deviation of three independent experiments. Values mentioned in parentheses represent percent reduction over control. $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.001$.

a Pyocyanin concentrations were represented as µg/ml of culture supernatant.

b Pyoverdine were expressed as fluorescent intensity at 460 nm (arbitrary unit).

c Total proteases activity is expressed as absorbance at 400 nm.

d Swarming motility is expressed in mm.

Attenuation of QS-linked virulence factor in Serratia marcescens MTCC 97

In the prodigiosin inhibition assay, the ANEa fraction (62.5–250 µg/mL) effectively disrupted QS-regulated virulence in S. marcescens MTCC 97, resulting in a 10–79% decrease in pigment production **(Figure 2).**

Swarming motility was also significantly impaired in a concentration-dependent manner, with zone diameters reduced to 34.2, 18.6, and 10.9 mm at 250, 125, and 62.5 µg/mL, respectively, compared to 61 mm in the untreated control **(Figure 2).** Additionally, extracellular protease activity, measured via azocasein degradation, was inhibited by 69.9%, 52.6%, and 34.5% at the same concentrations of ANEa.

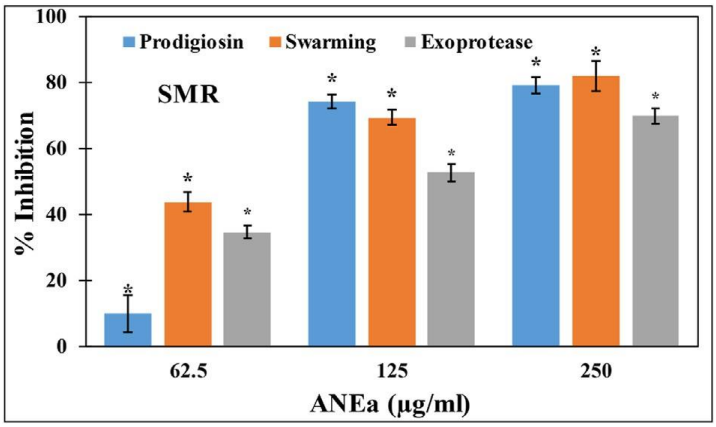


Figure 2. Effect of sub-MIC concentrations (250, 125, and 62.5 µg/mL) of Acacia nilotica ethyl acetate fraction (ANEa) on prodigiosin production, swarming motility, and exoprotease activity of S. marcescens MTCC 97. Data represent the mean ± SD of three independent experiments. Statistical significance relative to the untreated control was determined using p-values, with ∗ indicating $p \leq 0.05$.

Quantitative assessment of biofilm inhibition in gram-negative pathogens

Biofilm formation, a QS-regulated trait in *S. marcescens* MTCC 97 and *P. aeruginosa* PAO1, was significantly inhibited by the ANEa fraction in a concentration-dependent manner **(Figure 3)**. In *C. violaceum* 12472, treatment with ANEa at 125 and 62.5 µg/mL reduced biofilm formation by 74.1% and 64.5%, respectively. Against *P. aeruginosa* PAO1, reductions of 57.1%, 38.9%, and 24.4% were observed at 500, 250, and 125 µg/mL, respectively. Similarly, biofilm formation in *S. marcescens* MTCC 97 decreased by 61.1% at 125 µg/mL **(Figure 3)**. These quantitative findings were further validated using light and scanning electron microscopy. In *P. aeruginosa* PAO1, a dose-dependent reduction in cell density and microcolony formation was evident compared to the dense biofilm of untreated controls **(Figures 4a–4d)**. Comparable reductions in biofilm architecture were observed in *S. marcescens* MTCC 97 **(Figures 4e–4h)**. In *C. violaceum* 12472, treatment with ANEa resulted in scattered and smaller bacterial aggregates relative to the control **(Figures 4i–4l).**

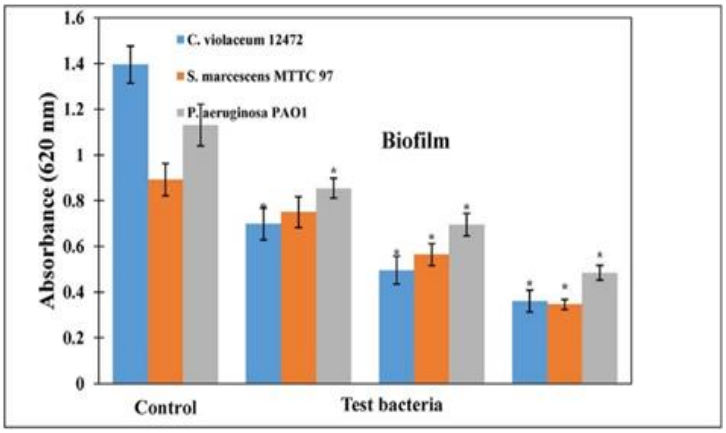


Figure 3. Effect of sub-MIC concentrations of *Acacia nilotica* ethyl acetate fraction (ANEa) on biofilm formation by *C. violaceum* 12472, *S. marcescens* MTCC 97, and *P. aeruginosa* PAO1. For *C. violaceum* 12472, α, β, and γ correspond to 31.25, 62.5, and 125 µg/mL; for *S. marcescens* MTCC 97, α, β, and γ represent 62.5, 125, and 250 µg/mL; and for *P. aeruginosa* PAO1, α, β, and γ indicate 125, 250, and 500 µg/mL. The secondary y-axis shows percentage inhibition. Values represent the mean ± SD of three

independent experiments. Statistical significance relative to the untreated control was assessed by p-values, with ∗ indicating $p \leq 0.05$.

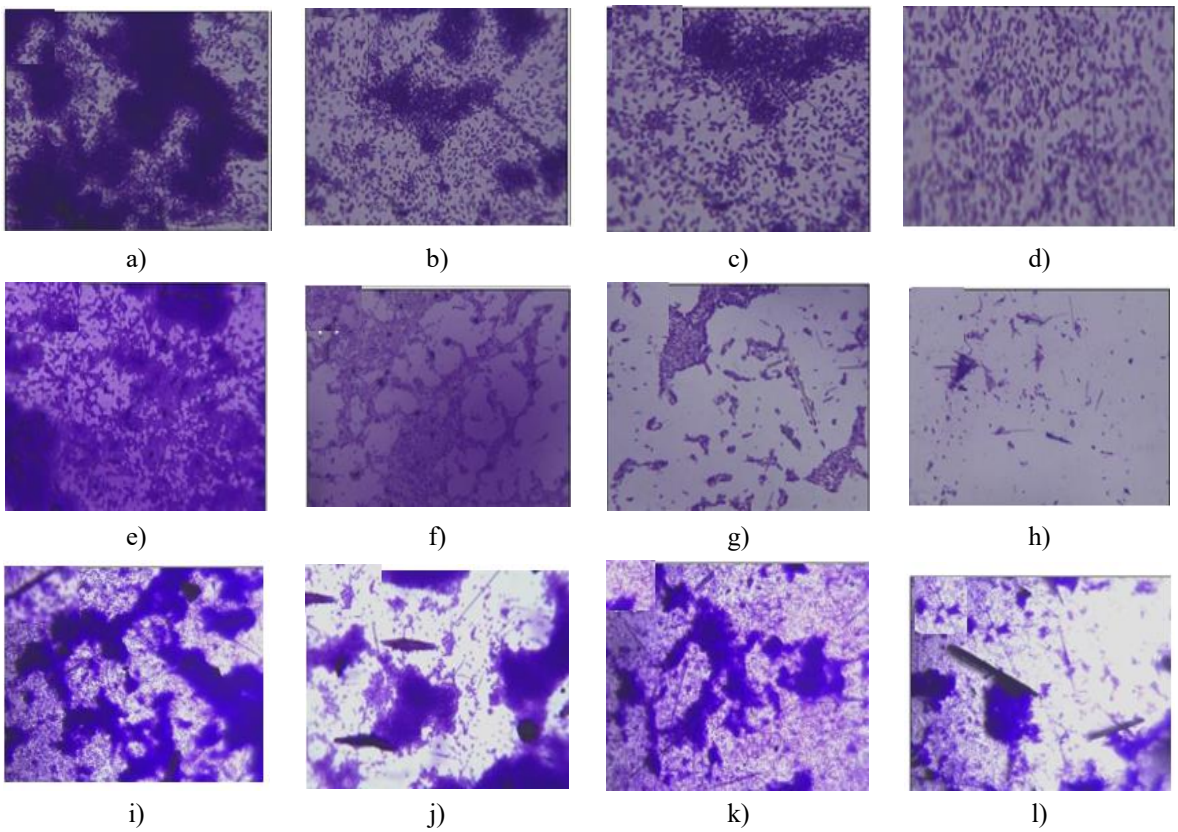


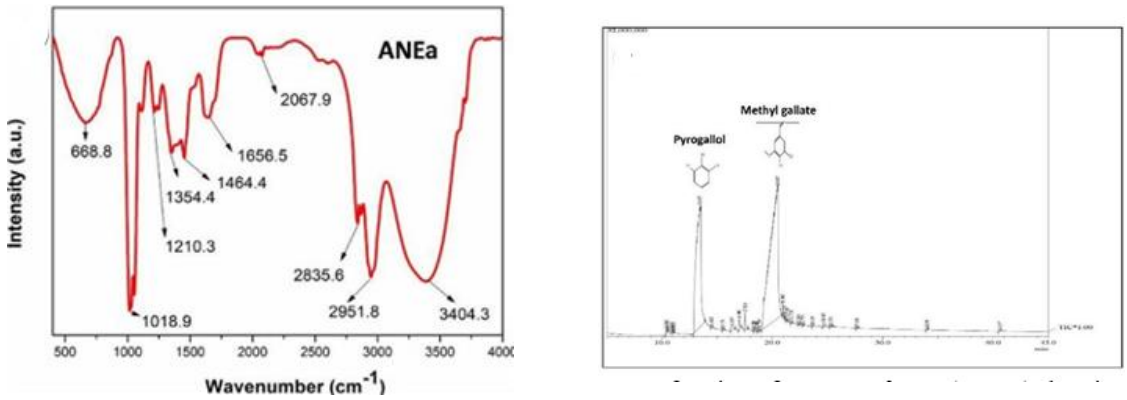
Figure 4. Representative light microscopy images (40× magnification) illustrating the impact of *Acacia nilotica* ethyl acetate fraction (ANEa) on biofilm formation by *C. violaceum* 12472, *S. marcescens* MTCC 97, and *P. aeruginosa* PAO1. Panels: (a) untreated *P. aeruginosa* PAO1; (b) 125 µg/mL ANEa; (c) 250 µg/mL ANEa; (d) 500 µg/mL ANEa; (e) untreated *S. marcescens* MTCC 97; (f) 62.5 µg/mL ANEa; (g) 125 µg/mL ANEa; (h) 250 µg/mL ANEa; (i) untreated *C. violaceum* 12472; (j) 31.25 µg/mL ANEa; (k) 62.5 µg/mL ANEa; (L) 125 µg/mL ANEa.

Preliminary phytochemical screening of extracts

Alkaloids were detected in both the crude extract and its fractions. The bioactive ethyl acetate fraction of *A. nilotica* indicated the probable presence of tannins, phenolics, flavonoids, and saponins, while glycosides were not detected.

Fourier transform of infrared spectroscopy (FTIR) analysis

FTIR analysis was performed to identify the functional groups present in the ANEa fraction, with the results summarized in **Figure 5a**. In the higher wavenumber region, peaks at 3000–3600 cm^{-1} corresponded to O–H stretching of phenols, while C–H stretching of alkanes appeared at 2850–3000 cm^{-1}. A prominent band at 1672 cm^{-1} was attributed to C=C–C(O)–OH and C=O stretching of –COOH groups. Additional peaks between 1000–1300 cm^{-1} indicated –OH in alcohols, and other bands were assigned to C–O–C stretching of ethers. Overall, the FTIR spectra confirmed the presence of phenols, alcohols, ethers, tannins, aromatic compounds, alkynes, and alkyl halides in the ANEa fraction.



a) b)

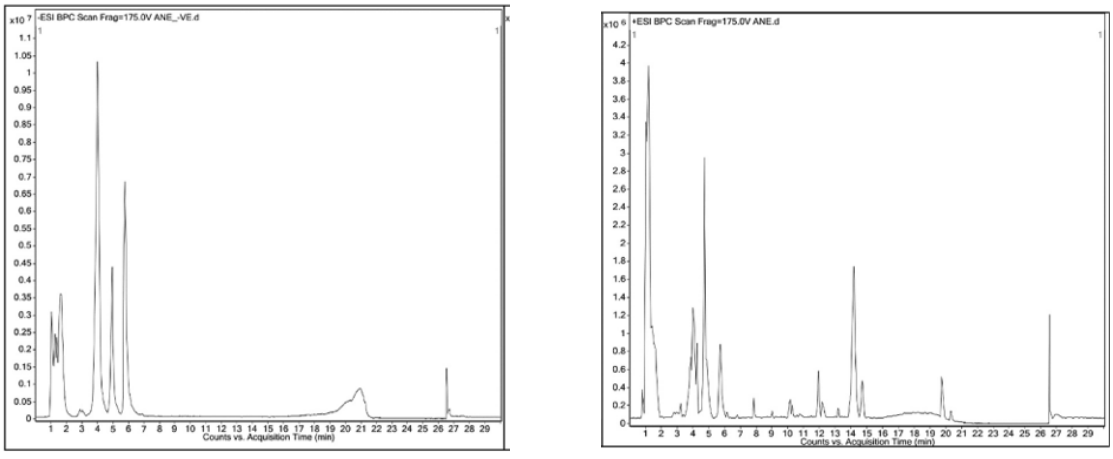
Figure 5. (a) FTIR spectrum of the ethyl acetate fraction of *Acacia nilotica* (ANEa) showing characteristic functional groups. (b) GC–MS chromatogram of ANEa, with peak numbers corresponding to retention times.

GC-MS analysis of ANEa fraction

The GC–MS profiling of ANEa fraction revealed diverse classes of phytochemicals, including esters, alkanes, fatty acids, aldehydes, and ketones. A total of 23 peaks were detected in the chromatogram, each representing a different bioactive molecule. Identification was performed by comparing retention times, relative peak areas, and mass fragmentation patterns with entries in the NIST database **(Figure 5b)**. The predominant compounds in the fraction included methyl gallate (benzoic acid, 3,4,5-trihydroxy-, methyl ester; 61.24%), 1,2,3-benzenetriol (35.05%), benzene methanol (0.73%), 3,4-dihydroxy-5-methoxybenzaldehyde (0.67%), oleic acid (0.49%), 6-benzene-1,2-diol (0.29%), 2-cyclohexen-1-one (0.19%), 9,12-octadecanoic acid (0.12%), and hexadecenoic acid (0.05%).

LC-MS analysis of ANEa fraction

The ANEa fraction was analyzed using LC-ESI-Q-TOF MS, leading to the tentative identification of 90 compounds, with 80 detected in positive ionization mode and 10 in negative mode. The corresponding chromatograms are presented in **Figures 6a** and 6B. The identified constituents included phenolics, flavonoids, and various alkaloids, such as epicatechin gallate (ECG, m/z 443.09), epigallocatechin (m/z 307.08), gallic acid (m/z 171.02), gallic acid 3-O-(6-galloylglucoside) (m/z 507.0), retronecine (m/z 156.1), anhalonidine (m/z 223.1), and dihydrocaffeic acid 3-O-glucuronide (m/z 381.0).

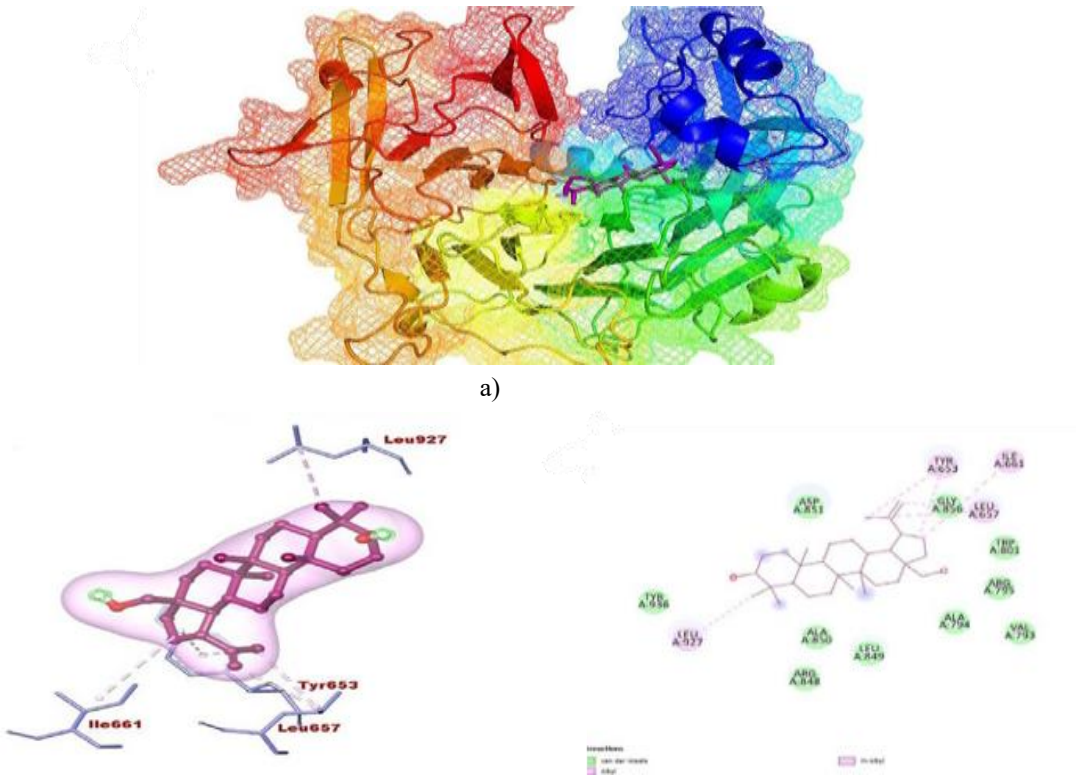


a) b)

Figure 6. (a) LC-MS chromatogram obtained in a positive mode of ionization of *A. nilotica* ethyl acetate fraction (ANEa). (b) LC-MS chromatogram obtained in a negative mode of ionization of *A. nilotica* ethyl acetate fraction (ANEa).

In silico studies

Molecular docking studies were performed to evaluate the potential interactions of selected phytocompounds identified from GC/MS analysis with the ligand-binding domains of quorum sensing (QS) and biofilm-associated proteins, including PilY1 and PilT **(Table 3).** Pyrogallol exhibited strong binding affinities with LasI, LasR, RhlR, and CviR, with docking scores of −7.6, −6.9, −6.7, and −7.8 kcal/mol, respectively. Similarly, dehydroabietic acid displayed favorable binding to LasI, interacting exclusively through hydrophobic contacts with residues Thr145, Val148, Phe105, Trp33, Val143, Thr144, Phe27, Arg30, and Val26. Pyrocatechol and 5-hydroxyvanillin were also found to interact with key active-site residues Ser135 and Ala44 of RhlR, along with additional hydrophobic contacts. Notably, dehydroabietic acid demonstrated a strong binding affinity of −9.7 kcal/mol with CviR, indicating potential occupancy of the catalytic site. Docking analysis of betulin with PilY1 resulted in a high-affinity complex with a binding score of −8.5 kcal/mol, the most significant among the tested ligands **(Figure 7).**



a) b) c)

Figure 7. Docking analysis of Betulin with PilY1. (a) Surface representation showing the binding conformation of Betulin. (b) Ball-and-stick model highlighting Betulin in magenta and the interacting PilY1 residues in blue. (c) 2D schematic illustrating the interactions between Betulin and PilY1.

Table 3. Binding affinities of different phytocompounds of *Acacia nilotica* ethyl fraction (ANEa) with QS and biofilm associated proteins obtained by molecular docking using AutoDock vina.

Ligands (Phytocompounds)	Receptors					
	LasI (1R05)	LasR (3JPU)	RhlR	CviR (3QP5)	PilY1 (3HX6)	PilT (3JVV)
Methyl gallate	−5.7	−5.0	−5.2	−5.6	−5.9	−5.8
Pyrogallol	−7.6	−6.9	−6.7	−7.8	−8.4	−7.9
Betulin	−7.2	−6.8	−6.7	−6.7	−8.6	−6.9
Phloroglucinol	−4.5	−4.2	−4.5	−5.3	−4.5	−4.9
Oleic acid	−4.7	−4.7	−5.0	−5.3	−5.0	−4.6
5-hydroxyvanillin	−5.1	−4.9	−5.4	−5.9	−5.7	−5.7
2,3-dihydro-benzofuran	−5.2	−4.5	−5.4	−6.3	−4.7	−5.1
Dehydroabietic acid	−7.5	−6.7	−6.6	−9.7	−7.6	−7.1
Pyrocatechol	−4.5	−4.1	−4.6	−5.4	−5.0	−5.2
Retronecine	−4.6	−4.3	−4.2	−4.1	−4.9	−4.6
Homomangiferin	−6.9	−7.1	−8.0	−7.5	−7.5	−7.1
ECG/Epicatechin gallate	−7.0	−6.3	−6.2	−6.6	−7.5	−7.6

The screening of selected Indian medicinal plants revealed that several possess both antibacterial and anti-quorum sensing (QS) properties. Among these*, Acacia nilotica* exhibited the highest QS inhibitory activity, with a maximum inhibition zone of 14 mm. Fractionation studies demonstrated that the ethyl acetate fraction (ANEa) was the most potent. Growth kinetics analysis showed that sub-inhibitory concentrations of ANEa did not significantly affect the growth of *C. violaceum 12472, P. aeruginosa PAO1*, or *S. marcescens MTCC* **97**, indicating that the observed effects were not due to growth suppression.

ANEa fraction effectively suppressed QS-regulated violacein production in the biosensor strain *C. violaceum 12472,* providing initial confirmation of its anti-QS activity. This finding is consistent with previous reports, such as the hexane extract of *Amphipterygium adstringens*, which inhibited violacein production by over 90% at tested concentrations [41]. In *P. aeruginosa PAO1,* ANEa significantly inhibited key QS-regulated virulence factors, including pyocyanin, pyoverdine, and swarming motility, in a concentration-dependent manner. Pyocyanin contributes to pathogenicity through redox-active mechanisms and indirectly promotes biofilm formation [42], while pyoverdine, a siderophore, chelates iron from host iron-binding proteins such as transferrin and ferroproteins, aiding bacterial survival and virulence [43–45]. These observations align with previous studies, for example, the acetone extract of *Senna alexandrina* inhibited pyocyanin and protease production by approximately 58% [46]. Similarly, ANEa attenuated QS-associated virulence factors in *S. marcescens MTCC 97*, comparable to sub-MIC streptomycin, which reduced prodigiosin production by 82.7% [47].

ANEa also strongly inhibited biofilm formation in all tested pathogens (>50% inhibition). Biofilm formation, a hallmark of *P. aeruginosa*, is closely linked to AHL-mediated QS, and QS-interfering compounds are known to disrupt this process [48, 49]. QS inhibitors are particularly valuable in managing *P. aeruginosa* infections due to their role in limiting inflammatory damage and mitigating antimicrobial resistance [50]. Previous studies have reported broad-spectrum QS and biofilm inhibition by extracts of *Mangifera indica* and *Carum copticum* against multidrug-resistant enteric bacteria [32, 33]. In this study, activity-guided fractionation of *A. nilotica* confirmed that ANEa exerts potent anti-QS and anti-biofilm effects against drug-resistant Gram-negative bacteria [34].

Phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, and saponins, which was further supported by FTIR analysis. The FTIR spectrum of ANEa showed peaks characteristic of phenols, alcohols, esters, and tannins, consistent with previous analyses of *A. nilotica* leaves and bark [51, 52]. GC-MS analysis identified a range of compounds, including 9,12-octadecadienoic acid, oleic acid, and 2-cyclohexen-1-one, which have been previously reported in the *Acacia* genus [53, 54]. Notably, methyl gallate and pyrogallol, previously reported in *Nymphaea tetragona*, were detected in *A. nilotica* pods for the first time in this study. Pyrogallol has been shown to inhibit QS in *Vibrio harveyi* through peroxide radical generation, while methyl gallate exhibits antimicrobial activity against drug-resistant intestinal bacteria [55–57]. Together, these compounds constitute approximately 96.3% of the ANEa fraction and likely account for its observed anti-QS activity.

LC-ESI-Q-TOF MS analysis further confirmed the presence of phenolic and flavonoid compounds, including ECG/Epicatechin gallate (m/z 441.0), 8-demethyltetracenomycin C (m/z 457.0), and methyl 2,4,6-trihydroxybenzoate (m/z 83.02) in negative ionization mode. These results are consistent with earlier reports and indicate the presence of catechin/gallate derivatives contributing to the bioactivity of the extract [58, 59].

Acacia nilotica is known to contain high levels of phenolic and flavonoid compounds, with different solvent extracts exhibiting varying antioxidant capacities [54, 60]. Although the ANEa fraction demonstrated pronounced inhibition of QS-regulated virulence factors and biofilm formation, this activity is likely not solely attributable to nonpolar constituents; polyphenols and flavonoids may also contribute significantly. Singh *et al*. previously reported that the hydrolyzed ethyl acetate fraction (HEF) of green *A. nilotica* pods exhibited strong antioxidant and anti-QS activity against *C. violaceum 12472* [61]. To our knowledge, this study represents the first report demonstrating broad-spectrum anti-QS and antibiofilm effects of the ethyl acetate fraction from *A. nilotica* pods.

Molecular docking studies suggested that the phytochemicals present in ANEa can act as antagonists, binding to the active sites of QS and biofilm-associated proteins and potentially inhibiting their interaction with natural ligands. The docking results indicated varying binding affinities across the tested proteins, with the strongest interaction observed against the biofilm pilus assembly protein PilY1 (−8.6 kcal/mol). These findings are consistent with reports where betulin derivatives exhibited strong binding to viral proteins (−9.2 kcal/mol) [62] and demonstrated inhibition of *P. aeruginosa* biofilm formation while interacting with LasI and RhlR both in vitro and in vivo [63]. Betulin also showed high affinity for the Autocrine Motility Factor receptor (AMF) at −7.26 kcal/mol against multidrug-resistant tumor cells [64]. In addition, dehydroabietic acid displayed strong

interactions with PilY1 and PilT, followed by betulin and pyrogallol. Previous in vitro studies demonstrated that dehydroabietic acid effectively reduced viability and biomass of established *Staphylococcus aureus* biofilms [65]. Based on these observations, it can be hypothesized that betulin may interfere with pilus assembly, contributing to the enhanced antibiofilm activity of ANEa.

Conclusion

In summary, the screening revealed several Indian medicinal plants with promising anti-QS potential. Among them***, A. nilotica*** showed broad-spectrum activity, significantly attenuating QS-regulated virulence factors and biofilm formation in *C. violaceum 12472, P. aeruginosa PAO1*, and *S. marcescens MTCC 97*. Phytocompounds identified through GC/MS and LC/MS demonstrated moderate to strong binding affinities to QS and biofilm-associated proteins, supporting the in vitro findings. Further in vivo studies are needed to evaluate the therapeutic potential of these plant extracts and their bioactive compounds. Moreover, these compounds may serve as adjuncts to conventional antibiotics, offering a potential strategy to combat drug-resistant bacterial infections.

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Ethics Statement: None

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