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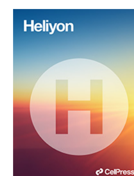
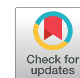


Research article

Anti-quorum sensing and biofilm inhibitory effect of some medicinal plants against gram-negative bacterial pathogens: *in vitro* and *in silico* investigations



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ABSTRACT

Multidrug resistance (MDR) in pathogenic bacteria have become a major clinical issue. Quorum sensing regulated bacterial virulence is a promising key drug target for MDR infections. Therefore, the aim of the present work was to assess the anti-quorum sensing properties of selected medicinal plants against bacterial pathogens as well *in silico* interaction of selected bioactive phytocompounds with QS and biofilm-associated proteins. Based on the ethnopharmacological usage, 18 plants were selected using methanolic extract against *Chromobacterium violaceum* 12472. The most active extract (*Acacia nilotica*) was fractionated in increasing polarity solvents (n-hexane, chloroform and ethyl acetate) and tested for anti-QS activity. The most active fraction i.e. ethyl acetate fraction was evaluated for their activity at sub-MICs against QS-associated virulence factors of *Pseudomonas aeruginosa* PAO1 and *Serretia marcescens* MTCC 97. Microtiter plate assay and light microscopy was used to determine inhibition of biofilm. Phytochemicals of the ethyl acetate fraction were analysed by GC/MS and LC/MS. Phytocompounds were docked with QS (LasI, LasR, CviR, and rhlR) and biofilm proteins (PilY1 and PilT) using Auto dock vina. The MIC of ethyl acetate fraction determined was 250, 500, and 1000 μg/ml against *C. violaceum* 12472, *P. aeruginosa* PAO1, and *S. marcescens* MTCC97 respectively. At sub-MICs QS regulated virulence factors production and inhibited biofilms broadly (more than 50 percent). GC/MS detected the major bioactive compound benzoic acid, 3,4,5-trihydroxy-, methyl ester (61.24 %) and LC-MS detected Retronecine for the first time in *A. nilotica* pods. *In silico*, dehydroabietic acid occupied the same cavity as its antagonist in the CviR ligand binding domain. Also, betulin and epicatechin gallate interact with biofilm proteins PilY1 and PilT, preventing biofilm formation. The findings suggest that the phytochemicals of *A. nilotica* pod could be exploited as an anti-QS agent against Gram-negative pathogens. To discover therapeutic efficacy of standardised bioactive extract/phytochemicals must be tested under *in vivo* condition.

1. Introduction

Antimicrobial resistance (AMR) in pathogenic bacteria has become a major global threat to human health in pathogenic bacteria. Overuse of antibiotics in human medicine is a well-known cause of bacterial resistance. In addition to well-established drug resistance mechanisms in bacteria, biofilm development by pathogenic bacteria contributes to their ability to tolerate high levels of antibiotics. Biofilm forming ability is also considered a virulence factor [1, 2]. Several strategies have been proposed to combat MDR problem by many authors [3, 4]. Quorum sensing, or bacterial cell-to-cell communication, is being investigated as a novel drug target for inhibiting the virulence and pathogenicity of the test pathogens [5, 6]. QS enables the perception of population density through the action of small signalling molecules known as auto-inducers, which alter gene expression in the population, and the resulting phenotype will be capable of sustaining the activities that allow them to survive in their new surroundings [7]. Research in the past indicated that medicinal plants/herbal medicine have a wide range of applications in both traditional medicine and the discovery of new drugs and bioactive compounds [8, 9]. India is one of the world's megadiversity hotspots,

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with a well-established traditional medical system that employs a diverse range of medicinal plants to treat infectious and non-infectious diseases in a variety of formulations [10].

In recent years, the pursuit of non-toxic, broad-spectrum anti-quorum sensing drugs from microbes and plants has gained greater importance [11, 12, 13]. Medicinal plant extracts possess the potential to combat bacterial infection by producing a diverse array of secondary metabolites like phenolics, terpenoids, flavones, quinones, catechins and alkaloids, which are homologous to those of acyl-homoserine lactones (QS signals) [14] and their ability to degrade signal receptors [15]. Screening plants for medicinally significant compounds seems rational due to the vast diversity of plants and their immense chemical heterogeneity [16]. Quorum-modulatory agents can be expected to have an antipathogenic/anti-infective effect on bacterial cells (interfering with QS signalling molecules), imposing a lower risk of resistance development as compared to antibiotics [17, 18]. However, certain reports have indicated potential resistance development against anti-virulence compounds [19, 20]. Evolution is a natural process, and every antibacterial/anti-virulence therapy will eventually generate resistance. Furthermore, this evolution could be directed or exploited in order to develop more powerful antibacterial/anti-virulence strategies. Although quorum quenching (QQ) therapies may have lower rates of resistance than antibiotics, developing an effective resistance-proof therapy to treat bacterial infections is far more difficult, and our fight against infectious diseases may be a never-ending process [21]. In many pathogenic bacteria, the ability to form biofilms is considered to be one of their pathogenic characteristics because it confers multiple levels of antibiotic resistance as well as protection against the host immune system and stressful environments [22]. However, due to occurrence of diverse of QS-signalling mechanisms and signal molecules, it has become extremely difficult to find broad-spectrum anti-QS compounds from medicinal plants for appropriate use in the treatment of MDR bacterial infection. Bacteria such as *Pseudomonas aeruginosa* deficient in one QS system could also form biofilms, indicating that biofilm formation is a complex process that cannot be completely inhibited by QS inhibition [23]. As a result, when evaluating plant extracts, both properties should be considered to obtain a promising active plant extract. Various medicinal plants have been screened for their QS-inhibiting abilities [24, 25, 26]. Several phytochemicals have been shown to impair pathogenicity expression by disrupting the bacterial QS system [17, 27]. India has a rich diversity of medicinal plants with promising therapeutic potential and proven safety in traditional system of medicine. Several medicinal plants have not yet been systematically evaluated including *Acacia nilotica* for anti-infective efficacy targeting QS and biofilms of pathogenic bacteria.

Acacia nilotica Lam (Mimosaceae), often known as 'Babul' or 'Kikar,' is a medium-sized tree that is widely distributed in tropical and subtropical nations [28]. Different parts of this plant, such as the leaves, fruits, roots, seeds, bark, flowers, gum, and immature pods, have been reported for anti-cancer, antimutagenic, spasmogenic, vasoconstrictor, anti-pyretic, anti-asthmatic, cytotoxic, anti-diabetic, anti-platelet agregatory, anti-plasmodial, molluscicidal, anti-fungal, inhibitory activity against Hepatitis-C virus [29, 30, 31]. However, anti-QS and anti-biofilm activity of *Acacia nilotica* is poorly understood.

Therefore, the aim of the current study was to obtain the plant bio actives/extract with promising anti-infective property and to understand its mode of action through *in silico* studies. In this study 18 Indian medicinal plants were screened using the *Chromobacterium violaceum* 12472 (reporter strain). The anti-pathogenic potential of the most active plant extract and its active fraction was further evaluated for its anti-QS and anti-biofilm activity against *Pseudomonas aeruginosa* PAO1 and *Serratia marcescens* MTCC 97. Further, phytocompounds of most active extract identified through GC/MS and LC/MS were analysed for *in silico* studies to decipher the possible mode of interaction with QS and biofilms associated proteins.

2. Materials and methods

2.1. Chemical and reagents

All the chemicals and reagents used throughout the study were of analytical grade. Luria Bertani (LB) medium, *Pseudomonas* broth, TTC (2,3,5-TTC - Triphenyltetrazolium chloride), crystal violet dye, and glutaraldehyde solution was procured from Hi-media, Mumbai. Azocasein and Elastin Congo red was procured from Sigma-Aldrich Pvt Ltd. All the solvents Methanol, Chloroform, n-hexane, Ethyl acetate, Ethanol, and HCL used in the study were of 99.9% purity obtained from Thermo Fisher Scientific India Pvt Ltd., Mumbai, India.

2.2. Bacterial strains and growth conditions

The test bacterial strains, *Chromobacterium violaceum* ATCC 12472 (ATCC, USA), wild type strain capable of producing purple pigment in response to its own C6 AHL (QS) molecules, *Serratia marcescens* MTCC 97 (procured from MTCC, India), and *Pseudomonas aeruginosa* PAO1 (gifted by Prof. R. J. C. McLean, USA) were taken in the study. The bacterial strains were maintained in Luria Bertani (LB) medium (0.5% yeast extract, 15.0 g tryptone, 0.5% NaCl, 20 g/L Agar). *C. violaceum*12472 was grown at 30 °C, and others were grown at 37 °C.

2.3. Plant material used

Various authenticated plant samples were taken as a gift from the Himalaya Drug Company (Dehradun, India) or procured/collected locally at Aligarh, India. The taxonomic identity of all the plants used was confirmed with the help of department of botany, AMU, Aligarh, India. The voucher specimens were deposited at the Department of Agricultural Microbiology, AMU, Aligarh, India. Details of plants used and their ethnopharmacological information is listed in Table S1.

2.4. Preparation of plant extracts and their fractionation

For the preparation of methanolic extract, shade dried plant material (leaves or fruit/pods) were pulverized to a fine powder with the help of an electric grinder and the extract was prepared as described previously [32]. For the preparation of crude extract, 100 g powdered plant material was suspended in 500 ml of methanol (Thermo Fisher Scientific India Pvt Ltd., Mumbai, India) for five days with intermittent shaking. The methanolic extracts were filtered using Whatman No.1 filter paper and then subjected to centrifugation (Sigma 2-16KL High-Speed Table Top Centrifuge) at 10000 rpm for 15 min. The filtrates were concentrated under reduced pressure using a rotator evaporator (RE-2000A, Associated Scientific Technologies, Delhi, India) at 40 °C. The concentrated crude extracts were then suspended in hot double distilled water and fractionated with solvents of increasing polarity such as n-hexane, chloroform, ethyl acetate and the remaining is aqueous. Each fraction was concentrated as described above using a rotatory evaporator (RE-2000A, Associated Scientific Technologies, Delhi, India) and stored at 4 °C. The dried fractions were reconstituted in $\leq$1% DMSO (v/v) to prepare stock solutions for the assessment of biological assays.

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

Methanolic extracts of all 18 medicinal plants were screened for their ability to inhibit pigment production in *C. violaceum* 12472 strain as adopted by the method described previously [32]. Briefly, the overnight grown culture of *C. violaceum* 12472 (100 μl) containing 10^6 CFU/ml of cells were taken with 3 ml of molten LB agar (0.5% w/v agar), subsequently poured over LB agar plate and allowed to solidify. The plant

extracts were screened at 1 mg/ml concentration for their anti-QS activity. The plates were incubated overnight at 30 °C. The inhibition of violacein production and/or growth was measured in terms of inhibition zone diameter of pigment and/or growth. The most active plant extract was selected for further studies.

2.6. Determination of minimum inhibitory concentration (MIC) of Acacia nilotica

MIC of most active plant extract i.e. *Acacia nilotica* ethyl acetate fraction (ANEa) was determined in order to select sub-inhibitory concentrations (Sub-MICs) for further experiments against bacterial test strains. MIC was determined using the broth microdilution method using TTC dye as described earlier [33]. Briefly, active fractions (ethyl acetate) of methanolic extract (from 8 mg/ml to 0.125 mg/ml) was diluted twofold in Luria Bertani broth to make a total volume of 200 µl in 96-well microtiter plate. The inoculum density of test pathogens was adjusted to 0.5 McFarland standard (10^8 CFU/mL) and diluted to 1:100 for MIC determination. The positive control was the medium with bacterial culture while the negative control (sterility) was medium without bacteria. The microtiter plate was incubated at 37 °C for 18 h. After incubation 20 µl of 2 mg/mL solution of TTC dye (Hi Media, India) was added in each well and incubated for 10 min at 37 °C. Reduction of TTC to its formazan product was apparent indication of growth/no growth. The development of pink colour was considered as growth. Antibiotic doxycycline was used as positive control for MIC determination. The concentrations at which no change of colour observed was regarded as MIC. Absence of growth or mottled growth was further confirmed by transferring 100 µl of treated bacterial culture on to the LB agar plates.

2.7. Analysis of bacterial growth curve

The effect of the highest sub-MIC of ANEa fraction on the growth of *Pseudomonas aeruginosa* PAO1, *Serratia marcescens* MTCC 97, and *Chromobacterium violaceum* 12472 was investigated as described previously [32]. Bacterial cultures were inoculated into 100 mL of LB broth with and without sub-MIC of ethyl acetate fraction. The flask was incubated for 24 h at 37 °C. The absorbance of the culture was measured at 600 nm every 2 h to plot the growth curve.

2.8. Quantitative estimation of violacein

The quantification of violacein was performed using *C. violaceum* 12472 according to previously described method with slight modification [34]. Briefly, *C. violaceum* 12472 was grown in absence and presence of sub-inhibitory concentrations (125, 62.5, and 31.25 µg/ml) of ANEa fraction in Luria Bertani (LB) broth and allowed to incubate for overnight at 30 °C with shaking (220 rpm) condition. After overnight incubation insoluble violacein was extracted through centrifugation (Sigma 2-16KL High-Speed Table Top Centrifuge) at 10000 rpm for 10 min. The collected pellet was suspended in 1 ml of DMSO, cyclo-mixed for 30 s and bacterial cells were removed by centrifugation at 10000 rpm for 10 min. Absorbance of soluble violacein in supernatant was recorded using LABMAN LMSP-V3205 spectrophotometer at 585 nm. The experiment was performed in triplicates and the percentage of violacein pigment inhibition as compared to untreated control was calculated using following eq. (1):

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

where, A_c and A_t are the absorbances of control and treated samples at 585 nm.

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

2.9.1. Assay for pyocyanin production

The quantitative estimation of pyocyanin was performed in *Pseudomonas* broth (PB) medium (1.4 g/l $MgCl_2$, 10 g/l K_2SO_4 and 20 g/l peptone) to quantify the production of pigment pyocyanin as described previously with slight modifications [35]. The *P. aeruginosa* PAO1 culture was grown in the presence or absence of sub-inhibitory concentrations (500, 250, 125 µg/ml) of ANEa at 37 °C with shaking for 18 h. After incubation, the treated or untreated bacterial culture was centrifuged (Sigma 2-16KL High-Speed Table Top Centrifuge) at 10000 rpm for 10 min and the resulting supernatant (5 ml) was mixed with 3 ml of chloroform, followed by the addition of 1 ml of 0.2 M HCl in the organic phase. After centrifugation (Sigma 2-16KL High-Speed Table Top Centrifuge) at 10000 rpm for 10 min the absorbance of the upper pink or deep red layer was recorded using LABMAN LMSP-V3205 spectrophotometer at 520 nm. The concentration of pyocyanin is retrieved in µg/ml by multiplying the OD520 by 17.072.

2.9.2. Assay for pyoverdine production

The assay for the evaluation of pyoverdine was performed by the method as described previously with slight modifications [36]. Briefly, cell-free supernatants of *P. aeruginosa* PAO1 was obtained in the presence or absence of sub-inhibitory concentrations of ANEa (500, 250, 125 µg/ml) were collected. Nine hundred microliters of 50 mM Tris-HCl (pH 7.4) was added in 100 µl of in culture supernatant. The fluorescence of the solution was recorded at excitation and emission wavelength of 400 and 460 nm using RF-5301PC spectrofluorometer (Shimadzu, Japan).

2.9.3. Assay for inhibition of proteolytic activity

The method as described by Kessler et al. [37] was employed to determine the exo protease activity of *P. aeruginosa* PAO1 by azo-casein degradation assay. Briefly, 100 µl of cell-free treated or untreated culture supernatants were added to 1 ml of 0.3% (w/v) azocasein (0.5 mM $CaCl_2$ in 0.05 M Tris- HCl, pH 7.5) and incubated for 15 min at 37 °C. The reaction was terminated by adding 500 µl of trichloroacetic acid (10% w/v) and then centrifuged (Sigma 2-16KL High-Speed Table Top Centrifuge) for 10 min at 12000 rpm. The absorbance of the supernatant was determined at 400 nm with a LABMAN LMSP-V3205 spectrophotometer.

2.9.4. Swarming motility assay

The QS-inhibitory effect of selected plant extract on swarming motility in *P. aeruginosa* PAO1 was performed as described previously [38]. Briefly, LB agar plates (0.5% agar) containing varying concentrations of 500, 250, 125 µg/ml of ANEa, were prepared on the same day and allowed to dry at room temperature for 1 h. An aliquot of 5 µl bacterial culture comprised of approximately 10^8 CFU/ml was point inoculated at the centre of the LB plates. The plate that received no treatment was used as a control. The plates were incubated at 37 °C in an upright position for 18 h and the diameter of swarm zones was measured from the point of inoculation.

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

2.10.1. Quantitative estimation of prodigiosin

QS regulated production of prodigiosin (2-methyl-3-phenyl-6-methoxyprodyginine) pigment is a virulence trait of *S. marcescens* MTCC 97. The inhibition assay of prodigiosin was performed as described previously [39]. Briefly, cells were grown in absence and presence (250, 125, and 62.5 µg/ml) of sub-MICs of ANEa fraction and then cells were harvested by centrifugation at 10,000 ×g for 15 min. From the harvested

cells, the pigment prodigiosin was extracted by using acidified ethanolic solution (4 ml 1 M HCl + 96 ml ethanol). The production of prodigiosin was quantified by measuring the absorbance at 534 nm using LABMAN LMSP-V3205 spectrophotometer. The percentage of pigment prodigiosin inhibition compared to untreated control was calculated using following eq. (2):

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

where, A_c and A_t are the absorbances of control and treated samples at 534 nm.

2.10.2. Swarming motility

The QS-inhibitory effect of ANEa fraction on swarming motility *S. marcescens* MTCC 97 was performed as described above [38]. Briefly, swarm agar plates (0.5% agar) supplemented with 250, 125, and 62.5 μg/ml were prepared on the same day, and left to dry at room temperature. Plates without ANEa fraction was taken as control. For *S. marcescens* MTCC 97 5 μl of culture was point inoculated on the centre of the plate. Then, the plates were incubated at 30 °C for 16 h and observed for inhibition of swarming motility.

2.10.3. Inhibition of exo-protease activity

The inhibition assay of exo-protease activity of *S. marcescens* MTCC 97 was performed as described above. Hundred microlitre of treated (250, 125, and 62.5 μg/ml) or untreated culture supernatant was combined with 1000 μl of 0.3 % azocasein. (0.05 M Tris-HCl with 0.5 mM CaCl2 at pH 7.5). The reaction mixture was incubated at 37 °C for 15 min before being stopped with 500 μl of trichloroacetic acid (10% w/v). The sample was centrifuged (Sigma 2-16KL High-Speed Table Top Centrifuge) at 12000 rpm for 10 min and the optical density of the supernatant was measured at 400 nm.

2.10.4. Quantitative assessment of biofilm formation

Quantitative estimation of biofilm formation was performed on 96-well microtiter plate as described previously [34]. Briefly, overnight grown bacterial cultures were seeded into wells containing LB medium. The treatment groups received sub-MIC concentrations of ANEa fraction for the respective bacterial culture. The control group consisted of wells that received no treatment. The plate was allowed to incubate at 37 °C for 24 h under static conditions. After incubation, excess broth and planktonic cells were removed by gently washing three times with sterile phosphate buffer (0.1 M, pH 7.4). The plate was allowed to dried at room temperature for 20 min. The adhered biofilms were stained with crystal violet (0.1 percent w/v) for 15 min and then gently washed to remove any unbound dye. The biofilm cells stained with crystal violet was solubilized in 90% ethanol. The absorbance of the solution was measured at 620 nm using microplate reader (Thermo Scientific, Multiscan Ex, India). The percent of biofilm inhibition was calculated using following eq. (3):

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

where, A_c and A_t are the absorbances of control and treated samples at 620 nm.

2.10.5. Microscopic evaluation

The overnight grown bacterial cultures were inoculated into 12-well tissue culture plates containing LB medium and surface sterile glass coverslips for light microscopic analysis, and plates were incubated for 24 h in the absence and presence of treatment groups. After overnight incubation glass coverslips were carefully rinsed with sterile phosphate buffer (0.1 M, pH 7.4) to remove unbound cells. Following staining with 0.1% w/v crystal violet solution. The stained coverslips were air-dried and visualized under the light microscope (Olympus BX60, Model BX60F5, Olympus Optical Co. Ltd. Japan) with an attached digital VGA camera (Sony, Model no. SSC-DC-58AP, Japan) at 40× magnification.

2.10.6. Detection of major phytocompounds in extract

The freshly prepared crude methanolic extract of *A. nilotica* and its fractions were subjected for qualitative determination of various bioactive phytoconstituents like flavonoids, alkaloids, saponins and glycosides etc by standard colour test procedures [40].

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

FTIR analysis was performed to identify the occurrence of various functional groups or chemical bonds in ANEa fraction. A small amount of plant material was mixed with potassium bromide to a fine powder and loaded in FTIR Spectroscope (Shimadzu, Japan). The characteristics peaks were recorded after loading the samples in the scan range of 4000-400cm[1] using PerkinElmer Spectrophotometer (Version 10.4.00). Using the interpretation of IR absorption spectra presence of various functional groups can be deduced.

2.10.8. Gas chromatography-mass spectrometry (GC-MS) analysis

The ethyl acetate fraction of *A. nilotica* was analyzed by system comprised of Gas chromatography-mass spectrometer (Shimadzu, Kyoto, Japan) with mass selective detector at Advanced Instrumentation Research Facility (AIRF), JNU, New Delhi, India. As a carrier gas helium was used with an injection temperature 260 °C with a split ratio 1:10 of helium with a constant flow rate of 1.21 ml/min. The separation was achieved on using the following temperature program: The initial temperature was 50–280 °C at a rate of 10 °C/min. Final oven temperature was 280 °C at a 10 °C/min and a holding time of 25 min. Scan 40 to 650 scan was performed the compound were found by comparing mass spectra from the library to a database (NIST Mass Spectral Library). It was stated in percentage in the relative area in the entire chromatogram, as well as percentage of time with a hold time of 2 min. The final oven temperature was set 280 °C at the rate of 10 °C/min with holding time of 25 min. Data was acquired in Scan mode (m/z range 40–650). Various compounds were identified by comparing mass spectra of obtained peaks with the standard database library (NIST Mass Spectral Library). The relative amounts of compounds detected was expressed in percentage, with respect to peak area in total chromatogram and retention time.

2.10.9. Liquid chromatography-mass spectrometry (LC-MS) analysis

The ethyl acetate fraction of *A nilotica* was also analyzed for the presence of polar compounds using LC-ESI-Q-TOF MS (Agilent Technologies -G6550A) at Sophisticated Analytical Instrument Facility (SAIF) IIT Bombay, Mumbai, India. The solvents system used were 0.1% Formic acid in water for channel A and 90% Acetonitrile +10% water+ 0.1% Formic acid for channel B using 5 μl injection volume. The gradient system started with 95.00 % of A and 5.00% of B, reaching 0 % of A: 100% of B at 25 min and then back to initial composition 95% of A and 5% of B in 1 min and which was maintained at the same composition for 5 min at a flow rate of 0.300 mL/min at 1200.00 bar pressure. The MS analysis was performed using ESI in both positive (+ve) and negative (−ve) modes to ionize the compounds between *m/z* 150 and 1000. The scan source parameters set in Q-TOF MS were as follows: capillary voltage 3500 V, nozzle voltage 1000 V, frag mentor 175 V, skimmer 65 V, and Octupole RF peak 750 V, gas flow 13 (l/min), gas temp 250 °C, sheath gas flow 11, sheath gas temp 300 and nebulizing gas 35 (psig). The Mass Hunter software was used to profile, classify, identify, and quantify the compounds present in fractions using high-resolution MS and MS/MS at SAIF IIT, Bombay, Mumbai, India.

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).

2.10.10. In silico *molecular docking studies*

The bioactive phytoconstituents of ANEa fraction identified in GC/MS and LC/MS analysis were subjected to *in silico* studies against the QS receptor proteins and biofilm specific proteins in *C. violaceum* 12472 and *P. aeruginosa* PAO1. The 3D crystal structures of proteins (CViR LasI, LasR, PilY1 and PilT) were downloaded in pdb format from Protein data bank except the RhlR which is retrieved from UniProt (https://www.uniprot.org/uniprot/P54292) because of its unavailability on RCSB protein data bank. The tertiary structures of phytocompounds i.e. methyl gallate (CID: 7428), Pyrogallol (CID: 1057), Betulin (CID: 72326), Phloroglucinol (CID: 359), Oleic acid (CID: 445639), 5-hydroxyvanillin (CID: 77535), 2,3-dihydro-benzofuran (CID: 10329), Dehydroabietic acid (CID: 94391), Pyrocatechol (CID: 289), Homomangiferin (CID: 5491388) and Retronecine (CID: 10198) were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov) in sdf format and converted into pdb file through Chimera 1.10.2. MGL Tools-1.5.6 was used to create the pdbqt files of both receptor proteins and ligand (Morris et al. 2009). The complete detail of grid size and centre of each receptor protein is mentioned in Table S2. The receptor protein and ligand residues were prepared, and docking analyses were run using virtual screening software, AutoDock Vina [41]. LigPlot^{+} was employed to observe hydrophobic interactions and the number of hydrogen bonds between the protein-ligand complexes [42]. Figures were created using the graphical visualizer Pymol and Biovia discovery studio 4.2.

2.11. *Statistical analysis*

All of the experiments were carried in triplicates, and the data was provided as mean ± SD (Standard Deviation). The results are statistically significant at $P \leq 0.05$. The significance level of the data was determined by calculating the P-value in Microsoft Excel 2019 using the t-test.

3. Results

3.1. *Screening of some plants for their anti-QS activity*

Interference in QS mediated purple pigment production in *C. violaceum*12472 is indicative of QS inhibition. Out of 18 medicinal plants screened for their anti-QS activity (pigment inhibition) against the bio monitor strain *C. violaceum* 12472, only 8 plants, namely *Acacia nilotica*, *Andrographis paniculate, Cinnamomum verum, Gymnema sylvestre, Murraiyya koenigii, Myristica fragrans, Punica granatum, Syzygium aromaticum* showed varied level of pigment inhibition (Table S1). Methanolic extract of *Andrographis paniculate, Gymnema sylvestre, Murraiyya koenigii* and *Myristica fragrance* solely have antibacterial activity but slight anti-QS activity at tested concentration. The plant *Acacia nilotica* showed maximum QS inhibition (14 mm) followed by *Cinnamomum verum* (8 mm), *Syzygium aromaticum* (5 mm) and *Punica granatum* (4 mm) (Table 1). *Acacia nilotica* demonstrated maximum anti-QS activity was further fractionated in solvents hexane, chloroform and ethyl acetate. The ethyl acetate fraction was found to be the most active fraction (in terms of pigment inhibition) as depicted in Figure S1. The ethyl acetate fraction was further tested for broad spectrum anti-QS activity against QS mediated virulence factors of *P. aeruginosa* PAO1 and *S. marcescens* MTCC 97.

3.1.1. *Minimum inhibitory concentration (MIC)*

MIC value of *A. nilotica* ethyl acetate fraction (ANEa) against *C. violaceum* 12472, *P. aeruginosa* PAO1 and *S. marcescens* MTCC 97 was first determined to select sub-MICs for their anti-infective effect. The MIC of ANEa fraction against *C. violaceum* 12472, *P. aeruginosa* PAO1 and *S. marcescens* MTCC 97 was 250, 1000 and 500 μg/ml respectively. The MIC of positive control doxycycline against *C. violaceum* 12472 and *S. marcescens* MTCC 97 was found to be 8 μg/ml. *P. aeruginosa* PAO1 had a MIC of 16 μg/ml.

3.2. *Quantitative estimation of violacein*

Quantitative estimation of QS-inhibitory activity ANEa fraction revealed concentration dependant manner inhibition in violacein pigment as compared to untreated control (Figure 1). In presence of sub-inhibitory concentration 125 μg/ml, 62.5 μg/ml and 31.2 μg/ml of ANEa

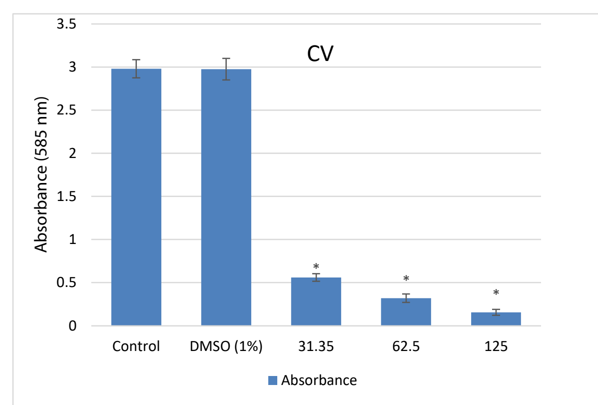

Figure 1. Inhibition of violacein in *C. violaceum* 12472 at varying sub-MICs (125 μg/ml, 62.5 μg/ml, and 31.25 μg/ml) of ethyl acetate fraction of *A. nilotica* (ANEa). Secondary y-axis shows percent inhibition. The data presented is average of three independent replicates with standard deviation. The statistical significance of the data was analyzed by calculating p-values with respect to control. * indicates p-value ≤ 0.05 with respect to control.

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.

fraction the reduction in pigment was found 94.7%, 89.2% and 81.2% respectively over control.

3.3. Analysis of growth curve

The growth curve analysis of ANEa fraction at its highest sub-MIC of *P. aeruginosa* PAO1 (500 μg/ml), *S. marcescens* MTCC97 (250 μg/ml), and *C. violaceum* 12472 (125 μg/ml) did not cause any significant change in growth of bacteria as depicted in Figure S2.

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

The ANEa fraction was tested against the major QS-associated virulence factors of *P. aeruginosa* PAO1 such as pyocyanin, pyoverdine, and swarming ability. The production of pyocyanin in broth cultures was significantly inhibited in the presence of varying concentrations of ANEa fraction. As evident from Table 2, the concentration dependant inhibition in production of pyocyanin by ANEa fraction was observed. The ANEa fraction at highest sub-MIC (500 μg/ml) resulted in maximum inhibition of pyocyanin (51.7%) and pyoverdine (36.5%). However at lower sub-MIC relatively low level of inhibition was recorded. The azocasein degrading protease assay was used to compare the development of proteases in the absence and presence of ANEa fraction. It was found that there was 49.3%, 43.8%, and 26.8% reduction in azocasein degrading protease as compared to untreated control (Table 2). In addition, swarming motility of *P. aeruginosa* tested in LB agar medium was also significantly reduced to 11.6 ± 0.5 mm at highest tested concentration of ANEa fraction (500 μg/ml) in comparison to untreated control (19.3 ± 1.1 mm) as shown in Table 2 and Figure S3.

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

In prodigiosin assay, the extract of ANEa (250–62.5 μg/ml) effectively interfered with the QS-linked factors of *S. marcescens* MTCC 97 and caused significant reduction in production of prodigiosin to a level of 10–79% as shown in Figure 2. Similarly in presence of ANEa swarming motility of *S. marcescens* MTCC 97 significantly reduced by 34.2 mm, 18.6 mm and 10.9 mm in concentration dependent manner (62.5–250 μg/ml) when compared to the untreated control (61 mm) as evident from Figure 2. Furthermore, there was a 69.9, 52.6, and 34.5% reduction in azocasein degrading exoprotease activity in the presence of 250, 125, and 62.5 μg/ml of ANEa fraction respectively.

3.6. Quantitative assessment of biofilm inhibition in gram-negative pathogens

Biofilm formation in *S. marcescens* MTCC 97 and *P. aeruginosa* PAO1 is also QS related attribute. As apparent from Figure 3, ANEa fraction reduced the formation of biofilm in concentration dependant fashion. The biofilm of *C. violaceum* 12472 was significantly reduced 74.1% and

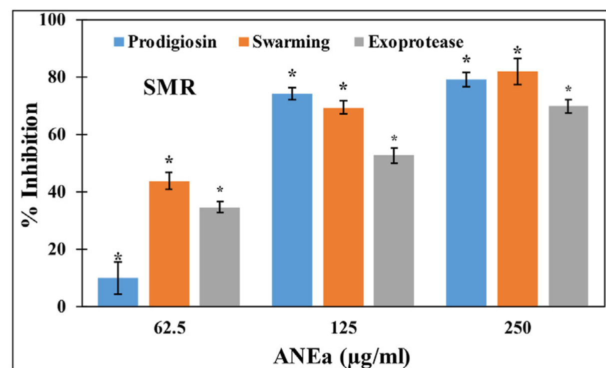

Figure 2. Inhibition of prodigiosin production, swarming motility and exoprotease activity of *S. marcescens* MTCC 97 at varying sub-MICs (250, 125, and 62.5 μg/ml) by ethyl acetate fraction of *A. nilotica* (ANEa). The data presented is average of three independent replicates with standard deviation. The statistical significance of the data was analyzed by calculating p-values with respect to control. * indicates p-value ≤ 0.05 with respect to control.

64.5% by ANEa at the highest tested concentrations (125 and 62.5 μg/ml). In this investigation, there were 57.07%, 38.9%, and 24.4% reduction in biofilm formation at 500 μg/ml, 250 μg/ml, and 125 μg/ml of ANEa fraction against *P. aeruginosa* PAO1 (Figure 3). Likewise in *S. marcescens* MTCC 97 biofilm formation was reduced to 61.1% at highest tested concentration *i.e.,* 125 μg/ml (Figure 3). The quantitative results obtained were further confirmed by light and scanning electron microscopy. In *P. aeruginosa* PAO1, there was dose dependent inhibition by ANEa fraction, where reduced cell density and microcolonies

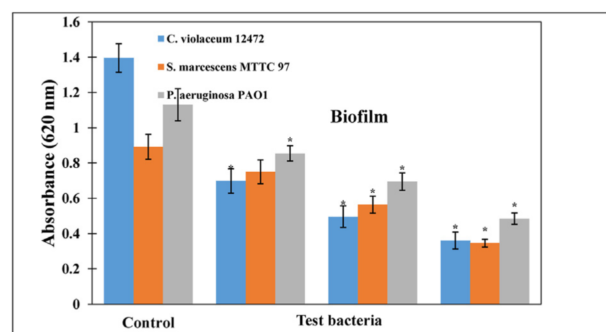

Figure 3. Inhibition of biofilm formation of *C. violaceum* 12472, *S. marcescens* MTCC 97, *P. aeruginosa* PAO1 and at varying sub-MICs of ethyl acetate fraction of *A. nilotica* (ANEa). α, β, and γ for *C. violaceum* 12472 are 31.25 μg/ml, 62.5 μg/ml, and 125 μg/ml; α, β, and γ for *S. marcescens* MTCC 97 are 62.5 μg/ml, 125 μg/ml, 250 μg/ml; α, β, and γ for *P. aeruginosa* PAO1 are 125 μg/ml, 250 μg/ml, and 500 μg/ml. Secondary y-axis shows percent inhibition. The data presented is average of three independent replicates with standard deviation. The statistical significance of the data was analyzed by calculating p-values with respect to control. * indicates p-value ≤ 0.05 with respect to control.

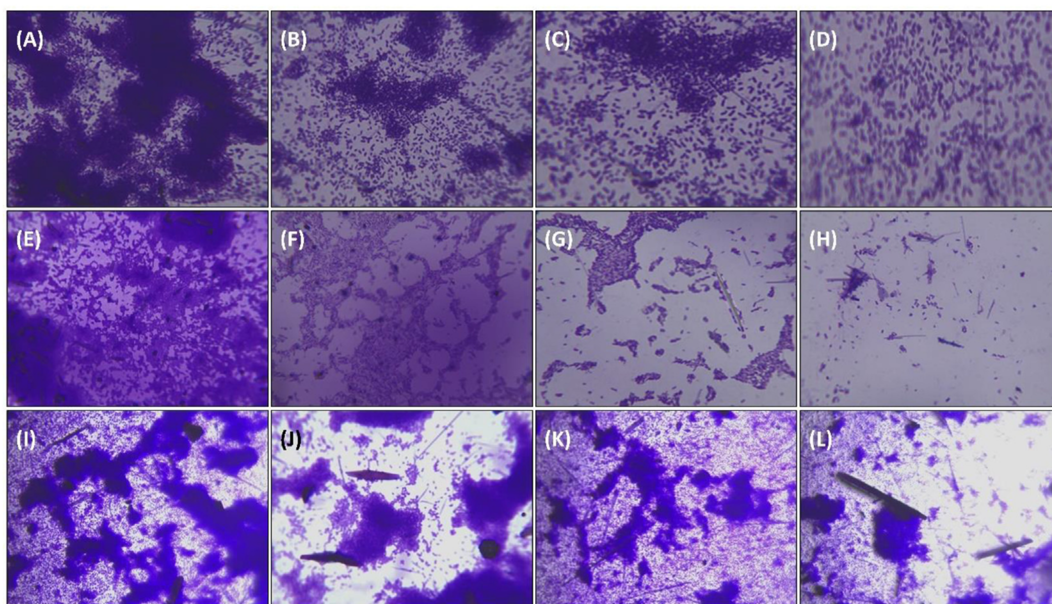

Figure 4. Representative light microscopic images showing the effect of *A. nilotica* ethyl acetate fraction (ANEa) against the biofilm formation of *C. violaceum* 12472, *S. marcescens* MTCC 97, and *P. aeruginosa* PAO1. The images were recorded at 40X magnification. (A) Untreated control *P. aeruginosa* PAO1; (B) *P. aeruginosa* PAO1 treated with 125 μg/ml ANEa; (C) *P. aeruginosa* PAO1 treated with 250 μg/ml ANEa; (D) *P. aeruginosa* PAO1 treated with 500 μg/ml ANEa; (E) Untreated control *S. marcescens* MTCC 97; (F) *S. marcescens* MTCC 97 treated with 62.5 μg/ml ANEa; (G) *S. marcescens* MTCC 97 treated with 125 μg/ml ANEa; (H) *S. marcescens* MTCC 97 treated with 250 μg/ml ANEa; (I) Untreated control *C. violaceum* 12472; (J) *C. violaceum* 12472 treated with 31.25 μg/ml ANEa; (K) *C. violaceum* 12472 treated with 62.5 μg/ml ANEa; (L) *C. violaceum* 12472 treated with 125 μg/ml ANEa.

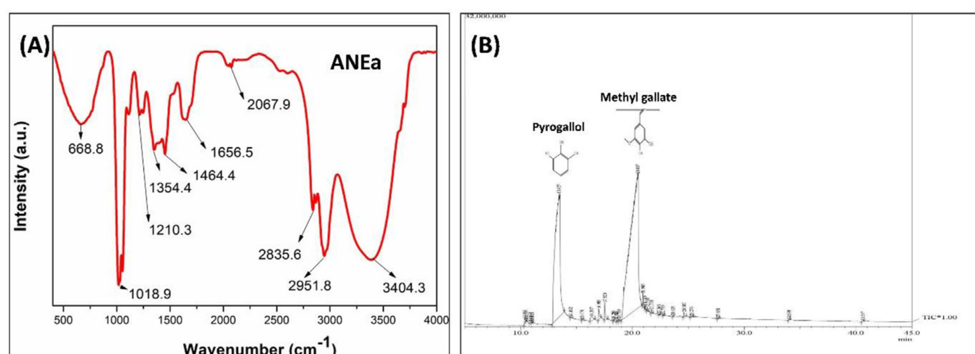

Figure 5. (A) Fourier-transform infrared spectroscopy (FTIR) spectrum of *Acacia nilotica* ethyl fraction (ANEa). (B) Gas chromatography–mass spectrometry (GC-MS) chromatogram of ethyl acetate fraction of *A. nilotica* (ANEa). Numbers above the peak refer to retention time.

observed as compared to dense biofilm matrix of untreated control (Figure 4A, B, C,D). The dose dependant effect on biofilm matrix was also seen *S. marcescens* MTCC 97 compared to untreated control (Figure 4E, F, G, H). In *C. violaceum* 12472 similar observations were found where in presence of ANEa fraction scattered and smaller clumps of bacterial cell were found compared to untreated control (Figure 4I, J, K, L).

3.7. Preliminary phytochemical screening of extracts

The result of preliminary investigation of major group of phytocompounds of crude as well ANEa fraction is depicted in Table S3. Alkaloids were present in both crude extract as well as their fractions. The bioactive fraction i.e. ethyl acetate fraction of *A. nilotica* gave the idea of tentative occurrence of phytocompounds such as tannin, phenolics, flavonoids and saponins except the glycosides.

3.8. Fourier transform of infrared spectroscopy (FTIR) analysis

Based on peak values in the infrared region, the FTIR spectrum was used to classify the functional groups corresponding to each of the active components present in the extract as depicted in Table S4 and Figure 5A. The FTIR spectra in high wave region correspond to O–H stretching in phenols (3000–3600 cm^{-1}), C–H stretching in alkanes (2850–3000 cm^{-1}), observed band at 1672 cm^{-1} can be assigned to C=C–C(O)–OH, C=O stretching of –COOH functional group, –OH in alcohols (1000–1300 cm^{-1}) and the other bands in the spectral range are assigned to C–O–C stretching in ethers in ANEa fraction. The FTIR spectral analysis provided the information of functional groups corresponding to phenols, alcohols, ethers, tannins, aromatic compounds, alkynes and alkyl halides in ANEa fraction.

3.9. GC-MS analysis of ANEa fraction

GC-MS analysis of ANEa fraction revealed the presence of various phytocomponents such as alkanes esters, fatty acids, aldehydes and ketones as listed in Table S5. The GC–MS chromatogram of ANEa fraction revealed a total of 23 peaks corresponding to bioactive components of different classes, which were identified by comparing their peak retention time, peak area (%), height (%), and mass spectral fragmentation patterns to those of known compounds in the National Institute of Standards and Technology (NIST) library. The GC-MS chromatogram of

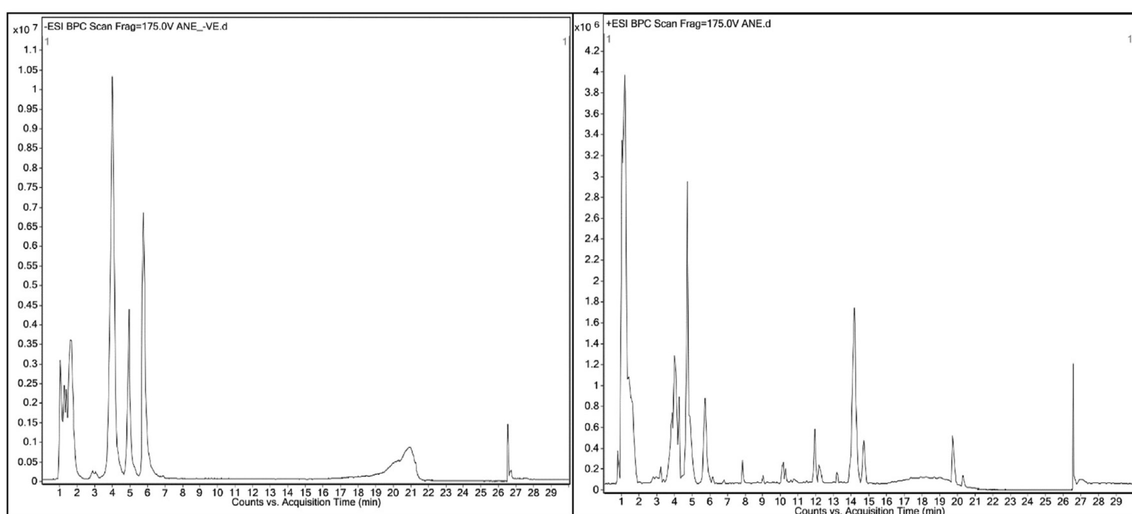

Figure 6. (A) LC-MS chromatogram obtained in a positive mode of ionization of *A. nilotica* ethyl acetate fraction (ANEa). (B) LC-MS chromatogram obtained in a negative mode of ionization of *A. nilotica* ethyl acetate fraction (ANEa).

ANEa fraction is shown in Figure 5B. The major compounds identified in ANEa fraction were Benzoic acid, 3,4,5-trihydroxy-, methyl ester or methyl gallate (61.24%), 1,2,3-benzenetriol, (35.05%), benzene methanol (0.73%), 3,4-dihydroxy-5-methoxybenzaldehyde (0.67%), oleic acid (0.49%) and 6 benzene-1,2-diol (0.29%), 2-cyclohexen-1-one (0.19%), 9, 12-Octadecanoic acid (0.12%), and Hexadecenoic acid (0.05%) etc.

3.10. LC-MS analysis of ANEa fraction

LC-ESI-Q-TOF MS system was used for the analysis of ANEa fraction which resulted in the identification of total 90 tentative compounds, of which 80 were detected in positive mode of ionization and 10 were identified in the negative mode of ionization. The details of some selected identified compounds with their theoretical and observed mass to charge ratio, molecular formula, and retention time (Rt) in positive and negative ionization mode in ESI are enlisted in Table S6, and the chromatograms generated is shown in Figures 6A and 6B. The detected compounds includes phenolics, flavonoid and various alkaloid compounds in the extract of *A. nilotica* such as ECG/Epicatechin gallate (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). The ESI-Q-TOF MS spectra of some of the identified compounds is depicted in Figure S4.

3.11. In silico *studies*

Molecular docking confirmations were analyzed to elucidate the potential binding affinity of selected phytocompounds detected in GC/MS analysis to the ligand-binding domain of QS and biofilm-associated proteins (PilY1 and PilT) as depicted in Table 3. The details of interacting H-bond with their bond length (in Å) and hydrophobic residues of protein-ligand complexes is mentioned in Table S7. The compound pyrogallol showed strong binding affinity in lasI, lasR, RhlR and CviR with the binding affinity of −7.6, −6.9, −6.7, −7.8 kcal mol^{-1}, respectively. Similar to pyrogallol, dehydroabietic acid showed a good binding affinity with LasI having all hydrophobic residues interactions. Thr145, Val148, Phe105, Trp33, Val143, Thr144, Phe27, Arg30, Val26 were the surrounding amino acids supported by hydrophobic interactions (Figure S5). Furthermore, pyrocatechol and 5-hydoxyvanilline also interact with Ser135 and Ala44 of RhlR that was found to play to be key interacting residues of its active sites in addition to other hydrophobic residues. The strong binding affinity of −9.7 kcal mol^{-1} of dehydroabietic acid with CviR suggest the binding of ligand on the catalytic site (Figure S6). The docking of betulin with PilY1 resulted in protein-ligand complexes

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

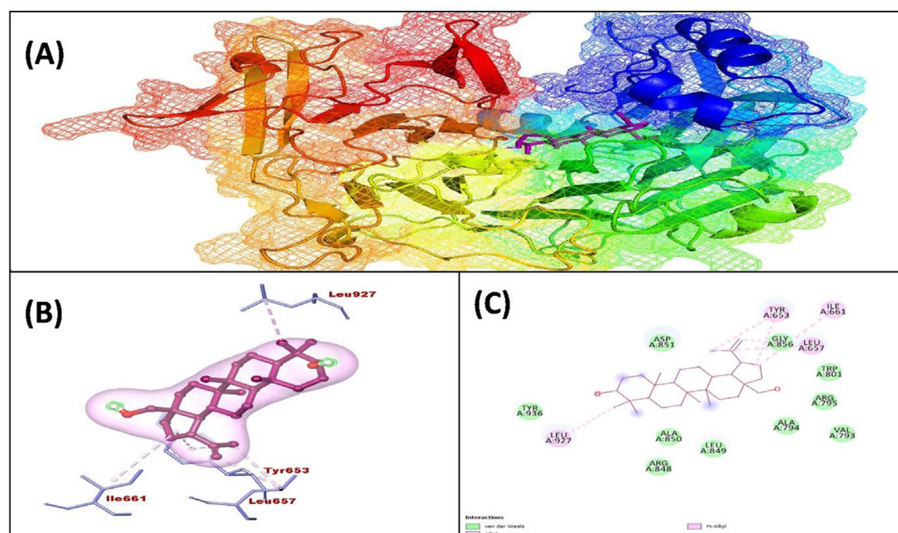

Figure 7. (A) Molecular docked conformation showing interaction of Betulin with PilY1 a surface view. (B) Ball and stick model of ligand in magenta colour and interacting residues of PilY1 in blue colour. (C) 2D diagram of Betulin and PilY1 interaction.

exhibiting a significant binding affinity score of -8.5 kcal mol^{-1} among all the tested ligands (Figure 7).

4. Discussion

The screening study revealed that many Indian medicinal plants have antibacterial activity with the promising anti-QS property. The extract of *A. nilotica* was found to be the most active plant extract in terms of maximum QS inhibition (14 mm). Fraction based study revealed that the ethyl acetate fraction of *A. nilotica* was most active. The effect of ANEa fraction on the growth kinetics of all the three tested bacteria showed that there was insignificant growth inhibition at their respective sub-MIC concentration listed. The potency of the suppression of QS-mediated violacein production in the biosensor organism *C. violaceum* 12472 in presence of ANEa fraction provided a preliminary indication of the extract's anti-QS property. In a similar study, a significant arrest in the presence of hexane extract of Amphipterygium adstringens was observed, inhibiting the release of violacein pigment by 91.6% and 94% at 55 and 166 μg/ml of concentration, respectively [43]. ANEa fraction significantly inhibited virulence factors of *P. aeruginosa* in a concentration dependant manner. The release of pyocyanin helps in the pathogenicity of *P. aeruginosa* that interferes with variety of cellular functions, particularly due to pyocyanin redox-active properties. It also has indirect role in the development of biofilm on solid surfaces [44]. Pyoverdine is also another major virulence factor in *P. aeruginosa*, which is escaped by the neutrophil gelatinase called as lipocalin and thus helps in the severity of infection particularly in the case of cystic fibrosis of the lungs [45]. Pyoverdine is a siderophore of *P. aeruginosa* which chelate iron from host-iron sequestering factors such as transferring, ferroproteins that are resident of host mitochondria in addition to sequestering iron from inorganic sources, and thereby trigger surveillance pathways and disrupting its biological functions [46, 47]. The findings of this study are comparable with those of previous reports where *Senna alexandriana* Mill acetone extract effectively inhibited QS- associated attributes pyocyanin and protease production by 58% at highest tested concentration [48]. The QS associated virulence factors of *S. marcescens* MTCC 97 was also significantly attenuated by ANEa fraction. Findings are also corelated with those reported at sub-MIC concentration of streptomycin i.e. 5 μg/ml which inhibited prodigiosin production up to 82.7% [49].

Interestingly, ANEa fraction showed profound activity against the biofilm formation of all the tested pathogens (>50% inhibition). Biofilm formation on solid surfaces is the one of the hallmark properties of *P. aeruginosa*, which is associated with AHL mediated QS system. Many of the QS interfering compounds have the ability to inhibit the formation of biofilm by the pathogenic strains [50, 51]. Molecules with QS inhibitory potential have prime importance against *P. aeruginosa* infection because of the undesirable consequences of inflammatory injuries and flourishing antimicrobial resistance [52]. Previously broad spectrum inhibition of quorum sensing and biofilm was also achieved by *Mangifera indica* and *Carum copticum* against MDR enteric bacteria [32, 33]. Furthermore, the activity guided fractionation of *A. nilotica* has resulted in profound anti-QS and anti-biofilm activity against drug-resistant Gram-negative pathogens that is comparable to other plants [34]. It indicates that the ANEa fraction potentially inhibited the production of virulence factors and biofilm formation in target bacterial pathogens.

Phytochemicals such as alkaloids, flavonoids, tannins and saponins detected through colour test procedures were also confirmed by FTIR analysis. The FTIR spectrum of *A. nilotica* ethyl acetate fraction showed similarity as reported earlier with those of FTIR spectra of leaves and bark [53]. The symmetric stretching of the C–O bond of the ester feature is assigned to the peak at 1354.4 cm^{-1} in FTIR spectrum is a characteristic peak of hydrolyzable tannins [54]. The occurrence of various phytocompounds identified through GC/MS analysis in ANEa fraction of pods is also confirmed by the literature. Presence of 9, 12-Octadecanoic acid (m/z 41, 55, 67), oleic acid (m/z 41, 55, 69), and 2-cyclohexen-1-one (m/z 107, 125, 140) has been previously detected [55, 56] in *Acacia* genus. Though the presence of methyl gallate and pyrogallol has been detected previously in *Nymphaea tetragona* (Water Lily), with the best of our knowledge this the first report showing the presence of methyl gallate and pyrogallol in *A. nilotica*. The compound pyrogallol inhibits quorum sensing in *Vibrio harveyi* by the fact that it promotes the generation of peroxide radicals, having a minor harmful effect that can be mitigated by adding catalase [57]. In addition, the methyl gallate component of *Galla rhois* also showed antimicrobial activity against drug-resistant intestinal bacteria [58, 59]. It is now obvious that pyrogallol and methyl gallate played a critical part in the activity of ANEa fraction, accounting for approximately 96.29% of the total. The combined activity of methyl gallate and pyrogallol and some hydrocarbons might be responsible for the anti-QS activity of ANEa fraction. The LC-MS technique has improved in terms of feasibility, making it a better alternative for the precise determination of secondary metabolites in plant material [60]. In a negative mode of ionization, the compound relative to *m/z* 441.0 showed one peak at RT = 5.931 corresponding ECG/Epicatechin gallate, compound relative to *m/z* 457.0 showed one peak at RT =

5.117 corresponding to 8- Demethyltetracenomycin C, and the compound relative to *m/z* 83.02 showed one peak at RT = 4.106 corresponds to Methyl 2,4,6- trihydroxybenzoate. Negative ionization mode evidenced the occurrence of phenolic compounds belonging to the catechin/gallactocatechin with their galloyl esters. The compounds identified in the negative mode were similar to the findings observed in the previous reports [61].

A. nilotica also holds the largest concentration of phenols and flavonoids various solvent extracts conferred various antioxidant capacities [56, 62]. Though the ANEa fraction is shown to be effective against the QS linked virulence factors, and biofilm indicate that inhibition may not be only due the nonpolar components, but there exist some other compounds such as polyphenols and flavonoids that also participate in the inhibition. Singh et al. (2009) reported that hydrolyzed ethyl acetate fraction (HEF) of green pods of *A. nilotica* have strong antioxidant and anti-QS activity in *C. violaceum* 12472 [63]. Various parts of the *A.* nilotica however, to the best of our knowledge, this is the first report of broad-spectrum anti-QS and antibiofilm activity of ethyl acetate fraction of *A. nilotica* pods. The results of molecular modelling studies suggest that phytoconstituents of ANEa fraction have antagonizing ability where these ligands tie with the active site and inhibit the response of its natural ligands. The identified phytocompounds showed a varied level of binding affinity to th QS and biofilm linked proteins. The highest binding energy i.e. -8.6 kcal mol^{-1} was obtained through *in silico* analysis against biofilm pilus assembly protein (PilY1). The results are comparable where derivatives of betulin showed good binding score (-9.2 kcal mol^{-1})with the proteins of Enterovirus E and Human alpha herpes virus 1 [64]. In a recent study it was found that betulin attenuates biofilm formation in *P. aeruginosa* and binding of natural ligands against QS receptors LasI and RhlR under *in vitro* and *in vivo* conditions [65]. Furthermore, betulin also showed higher affinity to AMF (Autocrine Motility Factor Receptor) -7.26 kcal/mol against multi drug resistant tumour cells [66]. In addition, Dehydroabietic acid also showed good binding score against the biofilm associated proteins PilY1 and PilT followed by betulin and pyrogallol. *In vitro* studies suggest dehydroabietic acid efficiently inhibit viability and biomass of established biofilms of *Staphylococcus aureus* [67]. From *in silico* based study it can be hypothesized that betulin may disable the pilus assembly or may the contributing factor in enhanced antibiofilm activity of ANEa fraction.

In conclusion, the screening study revealed many Indian medicinal plants with potential anti-QS property. The active extract of *A. nilotica* demonstrated broad-spectrum anti-QS and antibiofilm activity against *C. violaceum* 12472, *P. aeruginosa* PAO1, and *S. marcescens* MTCC 97. Attenuation of virulence factors of test strains highlights promising anti-QS potential. Major phytocompounds detected in GC/MS and LC/MS analysis showed fair binding affinity to QS and biofilm-associated proteins of *P. aeruginosa* PAO1 and *C. violaceum* 12472 has further strengthens our findings. *In vivo* based investigations are further required to uncover the therapeutic efficacy of plant extracts and phytocompounds. Active compounds may also be exploited as combination therapy with antibiotics to combat drug-resistant pathogens.

Declarations

Author contribution statement

Samreen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Faizan Abul Qais: Performed the experiments.

Iqbal Ahmad: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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