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Research article

Quorum quenching mediated biofilm impediment in *Chromobacterium violaceum* and *Staphylococcus aureus* by leaf extracts of *Delonix elata*

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ABSTRACT

Biofilms are complex communities of microorganisms that cause systemic infections, resistance development and delay in healing wounds. Biofilms can form in various parts of the human body, such as the teeth, lungs, urinary tract, and wounds. Biofilm complicates the effects of antibiotics in treating infections. In search of a cure, a plant-based phyto component was selected for this investigation as an anti-quorum-mediated biofilm restricting agent in Gram-negative *Chromobacterium violaceum* and Gram-positive *Staphylococcus aureus*. The bioactive components in *Delonix elata* (DE) ethyl acetate extract were identified using Gas chromatography and mass spectrometry. The extract was examined for toxicity using 3T3 cell lines and brine shrimp and ascertained to be non-toxic. Violacein was inhibited up to 68.81 % in *C. violaceum* at 0.6 mg/ml concentration. Hemolysin synthesis impediments in *C. violaceum* and *S. aureus* were 80 % and 51.35 %, respectively, at 0.6 mg/ml of DE extract. At 0.6 mg/ml, EPS was abated by up to 49 % in *C. violaceum* and 35.26 % in *S. aureus*. DE extract prevented biofilm formation in *C. violaceum* and *S. aureus* up to 76.45 % and 58.15 %, respectively, while associated eDNA was suppressed up to 67.50 % and 53.47 % at the respective sub-MIC concentrations. Expression of genes such as *cviI, cviR, vioA, vioB*, and *vioE* were dramatically reduced in *C. violaceum*, while genes such as *agrA, sarA, fnbA*, and *fnbB* were significantly reduced in *S. aureus*. Docking demonstrates that two or more DE molecules bind efficiently to the QS receptors of *C. violaceum* and *S. aureus*. Thus, DE extract can be investigated for therapeutic purposes against pathogenic microorganisms by rendering them less virulent through quorum quenching mediated action.

1. Introduction

Biofilm formation is a process by which microorganisms attach to a surface and produce a matrix of extracellular polymeric substances (EPS) which protect bacteria from environmental stresses [1]. Biofilms have a significant impact on human health, as they are involved in many chronic and device-related infections [2]. Moreover, biofilms can evade the host immune system, resist antimicrobial agents, and facilitate the spread of disease [3]. Biofilms occur on various medical devices, such as catheters, prosthetic heart valves, pacemakers, breast implants, contact lenses and cerebrospinal fluid shunts, as well as on dead and living tissues [4]. Infections stimulated by biofilms include endocarditis, urinary tract infections, wound infections, dental caries and periodontitis [5]. Biofilms

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affect the respiratory system of patients with cystic fibrosis, leading to lung damage and impaired lung function [6]. Quorum quenching renders an alternative way to diminish biofilm by preventing its formation.

Chromobacterium violaceum is a Gram-negative rod-shaped bacteria found abundantly in freshwater and soil microenvironments [7]. There were only a smattering few hundred human infective cases in the last century but currently, *C. violaceum* cases are expanding and grabbing research attention [8,9]. The characteristics of infection include skin lesions, internal organ abscesses and septicemia [10]. This bacterium was reported to cause fatal infections in hosts with debilitating immunity but later, reported with serious clinical manifestations in non-immune compromised hosts [11]. The Quorum Sensing (QS) system plays a predominant role in protecting pathogenic bacteria during adverse environments and harsh conditions. Eradication of preformed bacterial biofilm or preventing biofilm formation would help us to overcome severe infectious diseases caused by the bacteria [12]. CviI is the signal synthase employed by *C. violaceum* whereas CviR is the receptor protein; the signal molecule produced through this system is C6-HSL [13]. The biofilms formed by *C. violaceum* are resistant to disinfection, antibiotics, and phagocytosis. Genes that are controlled by QS system majorly include vioABCDE [14]. *Vio* operon produces the voguish QS-regulated purple pigment violacein, as a result of enzymatic coupling and oxidation of two molecules of tryptophan [15]. The prospect of creating biosensors is also made possible by the effortless way through which this QS-regulated pigment can be visualized and quantified [13].

Staphylococcus aureus is a pathogenic Gram-positive coccus, capable of constituting a biofilm matrix and was predominantly found on the human mucous environment and skin monolayer. Production of an antiphagocytic capsule, sequestering of host antibodies, protein A masking of antigens, biofilm formation, intracellular survival, and preventing leukocyte chemotaxis are a few examples of its virulence mechanisms to evade the host immune response which causes stringent infections [16,17]. Among these, biofilm formation would be considered pivotal because it can lead to plentiful supplemental complications [18]. The capacity of *S. aureus* to form biofilms curtails the efficacy of antimicrobial drugs, which could aggravate the infections and disease severity [19]. Thus, eradication of biofilms is of extensive concern in the context of disease suppression. Antibiotics are initially used to inhibit pathogen invasion and pathogenesis, but antibiotic resistance is revamped in bacteria that are capable of forming biofilms. Therefore, it is proven that antibiotics and chemical agents do not have a similar effect on bacterial biofilm as they do on planktonic cells [20,21]. *S. aureus* biofilm is primarily composed of EPS which possesses various proteins including surface binding protein A, fibrinogen binding protein, accumulation associated proteins, amyloid fibres, and extracellular matrix binding proteins [22]. Genes encode biofilm formation in *S. aureus*, for instance, fibrinogen-binding protein gene *fib*, fibronectin-binding proteins *fnbA* and *fnbB* genes, collagen binding protein *cna* genes, clumping factor *clfA* and *clfB* elastin binding protein *ebps,* intercellular adhesion genes *icaA, icaB, icaC and icaD,* elastin binding protein *ebps gene* and laminin binding protein *eno* gene [23–25]. *fnbA* and *fnbB* facilitate host cell penetration and aid in biofilm development. Under unfavourable conditions, biofilm dispersal takes place which is regulated by agr genes. Agr-associated genes are responsible for enzymes such as nucleases, proteases and lipase which are involved in metastatic infections and tissue destruction [22].

Currently, rather than killing the bacteria as such, eradicating bacterial biofilms by targeting quorum sensing will pave a novel strategy to overshade antimicrobial resistance [26]. Exploring natural bio-actives, especially those derived from plants, peaked during the past few years. Plants offer a wide range of therapeutics yet many remain unexplored. *Delonix elata* (DE) is a deciduous tree with numerous therapeutic properties such as antioxidant [27], anti-inflammatory [28,29], anti-arthritic [30], and immune modulations [31]. Here we explored the DE extract for anti-biofilm and associated virulence factor inhibition by targeting quorum sensing, which is one among the multiple ways of infection control. In view of these ethnomedical insist to discover the anti-quorum and anti-biofilm activity of DE was investigated in this study. To our knowledge, there are no supporting reports on the anti-quorum and anti-biofilm activity of DE. Hence this study deals with the anti-biofilm activity of DE extract against *C. violaceum* and *S. aureus* for the first time.

Table 1
Different compounds present in *D. elata* identified by GC-MS along with retention time.

S. No	Bioactives	Retention time	Chemical formula	Area%
1	O-METHYLISOUREA HYDROGEN SULFATE	3.07	$C_2H_8N_2O_5S$	9.841
2	MEGLUMINE	10.48	$C_7H_{17}NO_5$	2.154
3	MEGLUMINE	11.60	$C_7H_{17}NO_5$	3.173
4	1,3-PROPANEDIAMINE, N-METHYL-	12.30	$C_4H_{12}N_2$	4.206
5	5-AZIRIDINOPENTANOL	14.87	$C_7H_{15}NO$	3.724
6	1,3-PROPANEDIAMINE,N,N′-BIS(3-AMINOPROPYL)-	17.16	$C_9H_{24}N_4$	3.206
7	1-PENTATRIACONTANOL	17.62	$C_{35}H_{72}O$	16.797
8	2-BUTENE-1,4-DIAMINE, N,N′-DIMETHYL-	18.08	$C_6H_{14}N_2$	6.718
9	N-[3-METHYLAMINOPROPYL]AZIRIDINE	18.99	$C_6H_{14}N_2$	6.654
10	1,3-BUTANEDIAMINE	19.24	$C_4H_{12}N_2$	1.206
11	ARGININE	20.38	$C_6H_{14}N_4O_2$	6.006
12	1,10-DECANEDIOL	20.72	$C_{10}H_{22}O_2$	10.901
13	1,10-DECANEDIOL	21.31	$C_{10}H_{22}O_2$	2.516
14	[1-(DIETHYLAMINO) ETHYLIDENIMINO]SULFUR PENTAFLUORIDE	24.03	$C_6H_{13}F_5N_2S$	1.032
15	N-[DIMETHYLAMINOMETHYL]AZIRIDINE	25.32	CH_2NHCH_2	1.074
16	THREO-4-HYDROXY-L-LYSINE LACTONE	26.17	$C_6H_{12}N_2O_2$	2.999
17	BENZENEETHANAMINE, 3,4-DIMETHOXY-N-METHYL-	28.07	$C_{11}H_{17}NO_2$	1.203
18	PHENETHYLAMINE, 3-METHOXY-.ALPHA.-METHYL-4,5-(METHYLENEDIOXY)-	28.83	$C_{11}H_{15}NO$	6.242
19	SPERMINE	29.70	$C_{10}H_{26}N_4$	3.518
20	2-BUTENE-1,4-DIAMINE, N,N′-DIETHYL-	31.15	$C_8H_{18}N_2$	6.830

2. Results

2.1. Bioactive profiling by gas chromatography - mass spectroscopy analysis

The GCMS utilizing NIST and Wiley libraries was employed to profile the possible bioactive in the ethyl acetate (EA) extract. We confirmed the presence of several bioactive components in the DE extract by the total ion chromatogram. Various compounds eluted at different periods (Retention time) were characterized using mass spectroscopy. Table 1 illustrates the identified 20 bioactive compounds, their retention time, area percentage, and molecular formula. Among the 20 bio-actives, Pentatriacontanol had the most significant area percentage of 16.797, followed by 1,10-decanediol at 13.417 and O-Methylisourea hydrogen sulfate at 9.841. The total chromatogram is shown in Fig. 1.

2.2. Toxicity of D. elata extract against 3T3 cells and brine shrimps

The toxicity of DE extract was assessed using mouse fibroblast 3T3 cell lines and shown in Fig. 2. At the selected sub-MIC concentrations of 0.6 and 0.15 mg/ml, DE extracts were rather proliferative up to 108 % and 103 % respectively than toxic. To further affirm the nontoxic attribute of the plant extracts brine shrimps were employed in which the extracts show no toxicity at the sub-MIC concentrations of 0.6 and 0.15 mg/ml and all the shrimps were alive.

2.3. Antibacterial activity of D. elata ethyl acetate extract

The EA extract of DE suppressed the growth of *C. violaceum* and *S. aureus* at the lowest concentrations of 4.1 mg/ml and 3 mg/ml, respectively. Sub-MIC concentrations of 0.6 and 0.15 mg/ml were chosen to examine further anti-quorum and anti-biofilm action.

DE treated and untreated bacterial cells were analyzed by propidium iodide staining using flow cytometry. There is no significant difference between treated and untreated samples in the dead cell population confirming the non-bactericidal action at sub-MIC concentrations. The dead cell percentages were 6.70, 6.68 and 6.28 in *C. violaceum* control, 0.6 mg/ml DE treated and 0.15 mg/ml DE treated samples of *C. violaceum* respectively. In *S. aureus* the dead cells corresponded to 3.44, 4.41 and 4.10 % respectively for control, 0.6 and 0.15 mg/ml. The results are depicted in Fig. 3A–F.

2.4. QS regulated virulence factor inhibition in C. violaceum and S. aureus

In *C. violaceum*, a sub-MIC concentration of DE extract minimized QS-controlled virulence factor violacein production. When treated with 0.6 and 0.15 mg/ml of DE extract, the inhibition percentages were 68.81 % and 49.36 % respectively which were illustrated in Fig. 4A as a bar graph. The optical density (OD) of *C. violaceum* cells was simultaneously evaluated to ensure that growth was not impeded. Another virulence factor regulated through quorum sensing is hemolysin production. DE extract at sub-MIC doses of

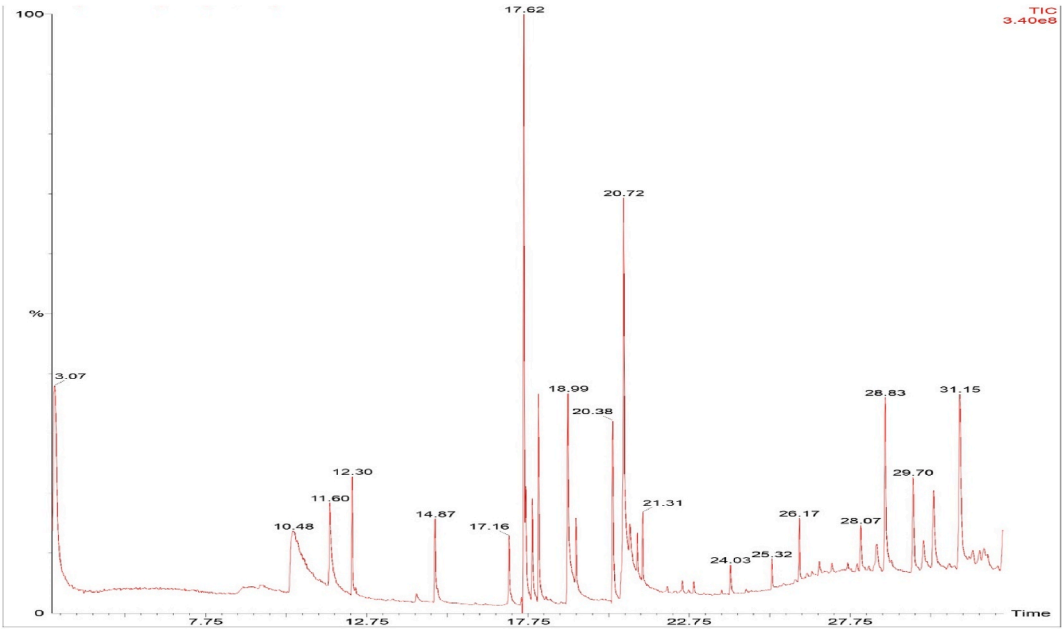

Fig. 1. GC-MS chromatogram depicts major peaks in ethyl acetate leaf extract of Delonix elata. The spectra of unknown were compared with NIST and Wiley library and matched fractions were listed in Table 1.

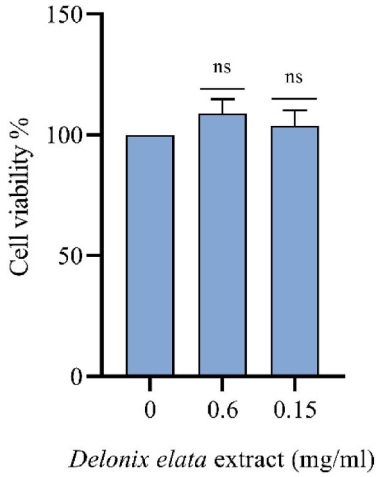

Fig. 2. 3T3 cytotoxicity effects of *D. elata* at 0.6 and 0.15 mg/ml concentration. At this sub-MIC concentration, the *D. elata* extract shows proliferation rather cytotoxicity.

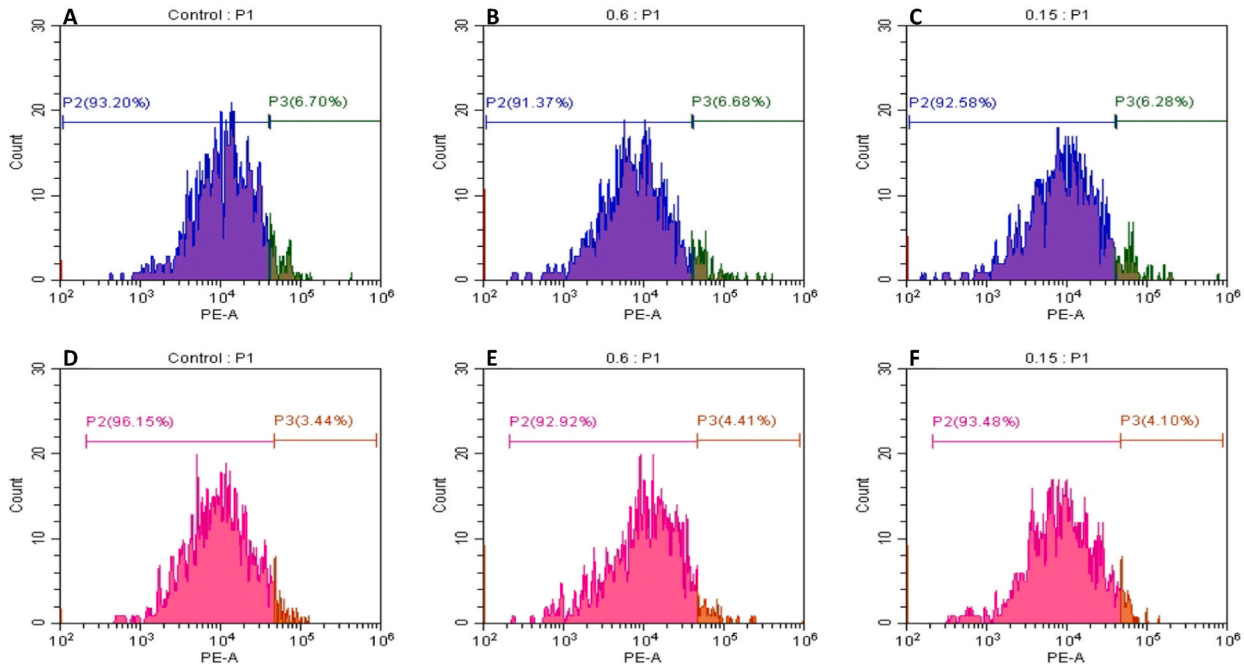

Fig. 3. Antimicrobial effects of *D. elata* were confirmed using flow-cytometry analysis. A, B and C represents untreated, 0.6 and 0.15 mg/mL of *D. elata* treated *C. violaceum* whereas, D,E and F shows the untreated, 0.6 and 0.15 mg/mL of *D. elata* treated *S. aureus* respectively. There is no significant inhibition in the treated groups in comparison with the untreated control. This signifies that *D. elata* leaf extract might exhibit a reduction in virulence factor mediated by quorum quenching activity.

0.6 and 0.15 mg/ml hindered hemolysin production in *C. violaceum* up to 80.05 % and 44.02 % as depicted in Fig. 4B and in *S. aureus* up to 51.35 % and 34.94 % respectively (Fig. 5A). QS regulates EPS production in these microorganisms and plays an essential role in biofilm formation and pathogenicity. Sub-MIC concentrations of DE extract 0.6 and 0.5 mg/ml suppressed EPS synthesis in *C. violaceum* by 49 % and 37.79 % (Fig. 4C) and in *S. aureus* by 35.26 % and 24.23 % (Fig. 5B).

2.5. Antiadhesion activity of DE extract

To assay the adhesion inhibition potential of DE extract against *C. violaceum* and *S. aureus*, a cell infection model was utilized. The extract at concentrations of 0.6 and 0.15 mg/ml was able to inhibit the adhesion of *C. violaceum* and *S. aureus* on mouse fibroblast cells L929. The log reduction in treated samples was depicted in the bar graph Figs. 4D and 5C.

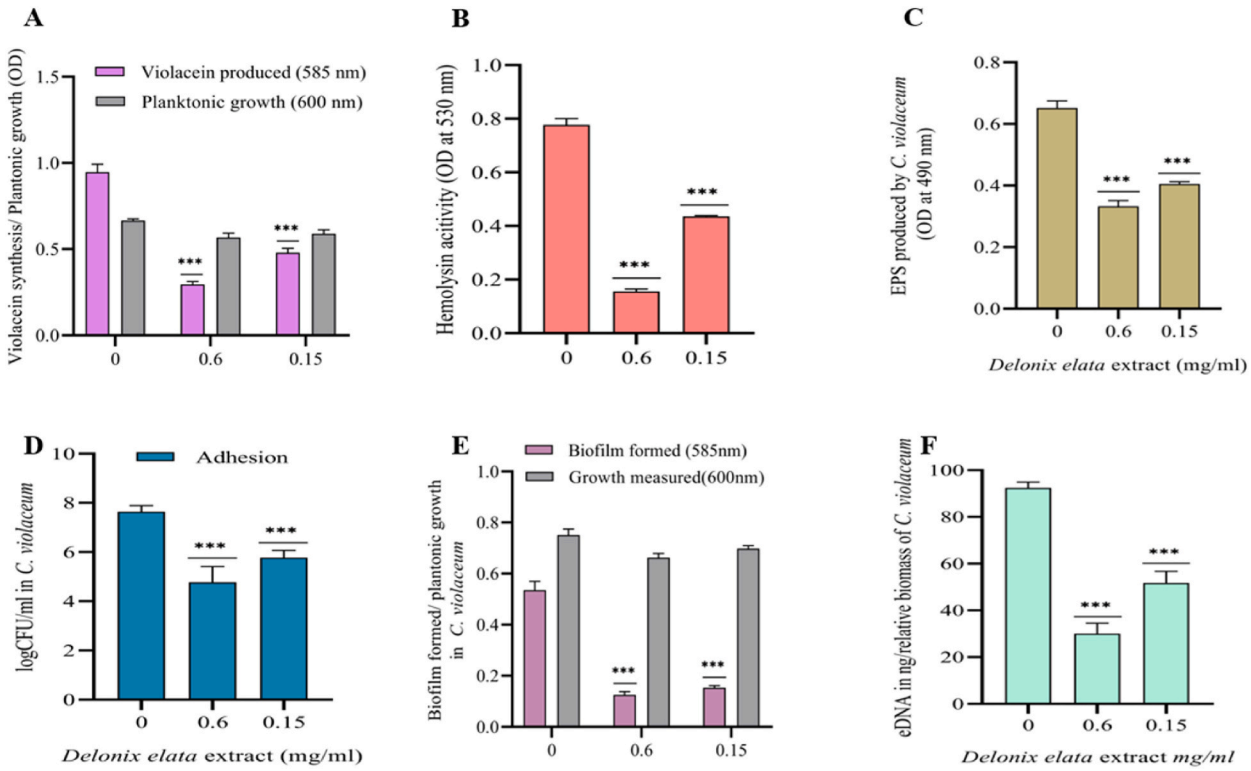

Fig. 4. *D. elata* extract effects on QS regulated virulence factors in *C. violaceum* such as Violacein, hemolysin, EPS, Biofilm, eDNA
A. From the *D. elata* treated *C. violaceum* the violacein was extracted to check whether the bacterial QS system was inhibited as there is no significant reduction in cell viability in accordance with the previous experiment. Furthermore, the violacein pigments were reduced in treated groups in comparison with the untreated control. Since violacein pigment production was mediated by QS system this affirms that *D. elata* has anti-quorum activity. The growth inhibition is also cross-verified by measuring the pellet OD at 600 nm
B. Hemolysis activity was checked against the control and *D. elata* treated *C. violaceum*. Compared to the control the treated groups resulted in a greater reduction of hemolysin production to the extent of 80.05 % at 0.6 mg/ml.
C. Production of EPS was considered to be a significant virulence factor for biofilm formation in pathogenic bacteria. The result shows a significant reduction in EPS production which further contributes to anti-quorum activity. The inhibition percentage in *C. violaceum* were 49 % and 37.79 % respectively at 0.6 and 0.15 mg/ml of *D. elata*.
D. Cell adhesion ability was performed as described by others. The bacteria were co-cultured in pregrown cells and the adhered biofilm cells were quantified by CFU determination which exhibited significant reduction in treated groups compared to the control group.
E. Biofilm formation was also inhibited efficiently by *D. elata* extract compared with control in *C. violaceum*. The inhibition percentage by crystal violet staining was recorded to be 76.15 and 71.40 % in *C. violaceum*
F. e-DNA quantification from biofilm cells depicts reduction compared with the untreated control which further supports to the anti-quorum activity of *D. elata*. The inhibition percentages were 67.50 and 44.05 % for *C. violaceum* when treated with *D. elata*. ($P < 0.05$, $P < 0.001$).

2.6. Antibiofilm quantification and morphology visualization

At sub-MIC concentrations of 0.6, and 0.15 mg/ml, DE extract inhibited biofilm formation in *C. violaceum* by up to 76.45 % and 71.40 % and in *S. aureus* by up to 58.15 % and 45.5 % respectively. The bacterial growth was not hindered (Figs. 4E and 5D). SEM was used to visualize the control and DE extract treated group at 0.6 and 0.15 mg/ml concentration on *C. violaceum* and *S. aureus*, which showed an apparent suppression in biofilm architecture (Fig. 6A–F).

Extracellular DNA associated with bacterial biofilms was quantified for control and treated samples of *C. violcaeum* and *S. aureus*. At the sub-MIC concentrations of 0.6 and 0.15 mg/ml DE restrained the amount of eDNA at the biofilm environment up to 67.50 % and 44.05 % and 53.47 % and 35.51 % for *C. violaceum* and *S. aureus* respectively (Figs. 4F and 5E).

2.7. QS gene expression evaluation using qRT PCR

RT-qPCR was used to assess the influence of DE extract at sub-MIC doses of 0.6 and 0.15 mg/ml on the quorum-sensing genes of *C. violaceum* and *S. aureus*. The influence of quorum sensing regulators *cviI* and *cviR* and the *vio* operon genes *vioA, vioB and vioE* was investigated. The DE extract downregulated the expression of the genes *cviI, cviR, vioA, vioB* and *vioE* genes in *C. violaceum* (Fig. 7A). Similarly, the impacts of *S. aureus* QS system regulators *sarA* and *agrA*, as well as biofilm-related adhesion genes *fnbA* and f*nbB*, have

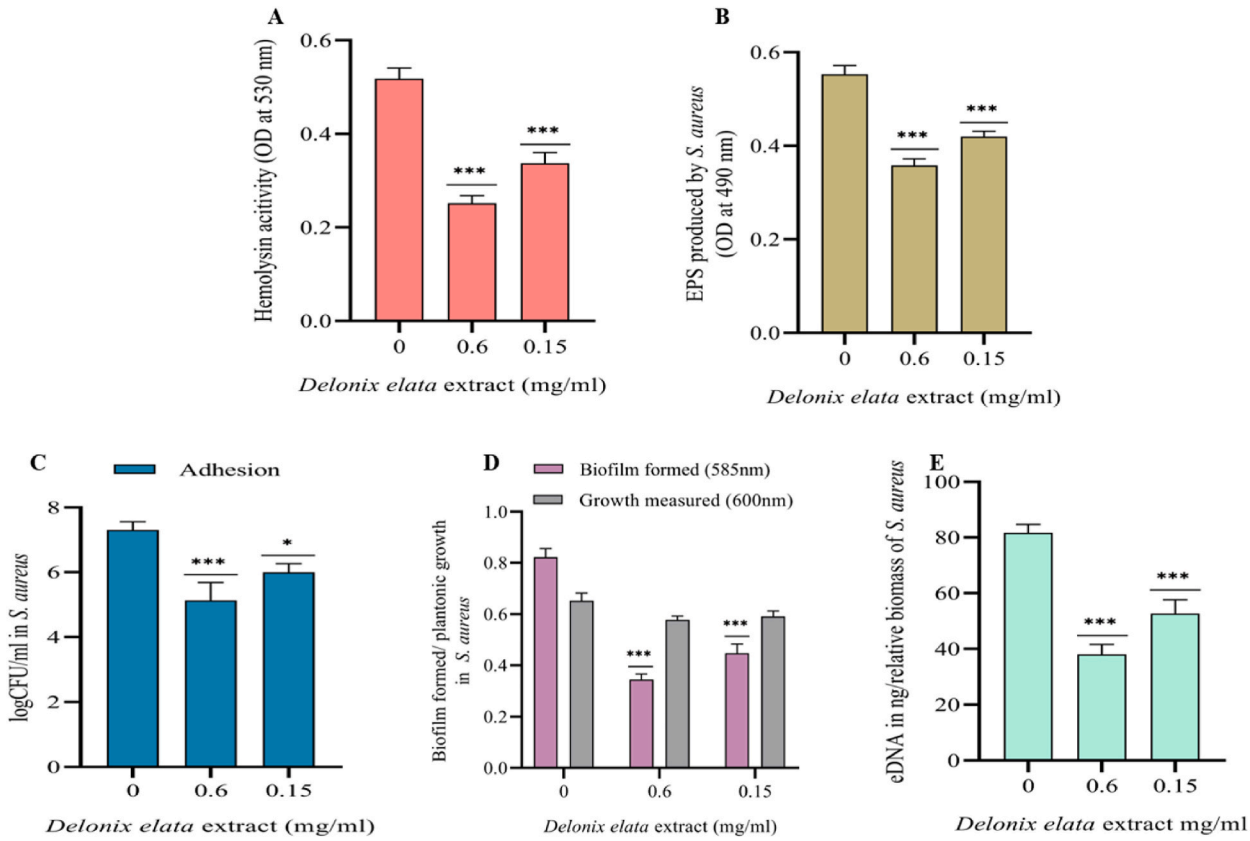

Fig. 5. Effects of *D. elata* extract on QS-regulated virulence components in *S. aureus*, including hemolysin, EPS, cell adhesion, Biofilms, and eDNA
A. Hemolysis activity was measured in comparison to the control and *D. elata*-treated *S. aureus*. In comparison to the control, the treated groups reduced hemolysin production by 51.35 % and 34.94 % at 0.6 mg/ml and 0.15 mg/ml *D. elata* respectively.
B. The data demonstrate a considerable decrease in EPS generation, which adds to anti-quorum action. At 0.6 and 0.15 mg/ml of *D. elata*, the inhibition percentages in *S. aureus* were 35.26 % and 24.23 %, respectively.
C. *S. aureus* was cocultured in pregrown 3T3 cells, and the adherent biofilm cells were measured by CFU determination, which shows a substantial reduction in treated groups compared to controls.
D. When compared to the control in S.aureus, *D. elata* extract effectively prevented biofilm formation. The inhibition percentage by crystal violet staining was reported to be 58.15 and 45.5 % in *S. aureus* respectively.
E. e-DNA quantification from biofilm cells, indicates the anti-quorum action of *D. elata*. When *S. aureus* was treated with *D. elata*, the inhibition percentages in eDNA were 53.47 % and 35.51 %, respectively. ($P < 0.01$, $P < 0.001$).

been examined. The DE extracts were able to suppress the expression of the genes *sarA, agrA, fnbA* and *fnbB* compared with the control to a more considerable extent (Fig. 7B).

2.8. Docking DE bioactive with QS proteins

Docking results displayed favorable interactions of a few bioactives of DE extract against CviR and AgrA quorum sensing responsible receptors of *C. violaceum* and *S. aureus* respectively. Benzeneethanamine, 3,4-Dimethoxy-N-Methyl- and Phenethylamine, 3-Methoxy- Alpha-Methyl-4,5-(Methylenedioxy) interacted in the active site of CviR with the binding energy of −6.33 kcal/mol and −6.27 kcal/mol respectively. 3 hydrogen bonds were formed in the active site of CviR by Benzeneethanamine, 3,4-Dimethoxy-N-Methyl- at atoms TRP 84, TYR 88, and ASP 97 with the hydrogen bond distance of 1.91, 2.85 and 1.90 Å respectively. Phenethylamine, 3-Methoxy- Alpha-Methyl-4,5-(Methylenedioxy) bound in the active site of CviR by forming a hydrogen bond with atom TRP84 with a hydrogen bond distance of 1.74 Å. Threo-4-Hydroxy-L-Lysine Lactone bound in the active site of AgrA with four hydrogen bonds with the atoms ASN185 and GLU 188 with the bond distance of 2.91, 3.15, 3.58 and 2.54 Å respectively. The binding energy is −6.69 kcal/mol 1,3-Propanediamine, N,N′-Bis(3-Aminopropyl)- binds with an energy of −5.42 kcal/mol by four hydrogen bonds with GLU144, TYR183, GLU188 with the bond distance of 2.01, 2.02, 2.06 and 1.78 Å. All the binding energies of ligands with CviR and AgrA receptors were represented in Tables 2 and 3 and the interactions were depicted in Fig. 8A–Q and 9A-P.

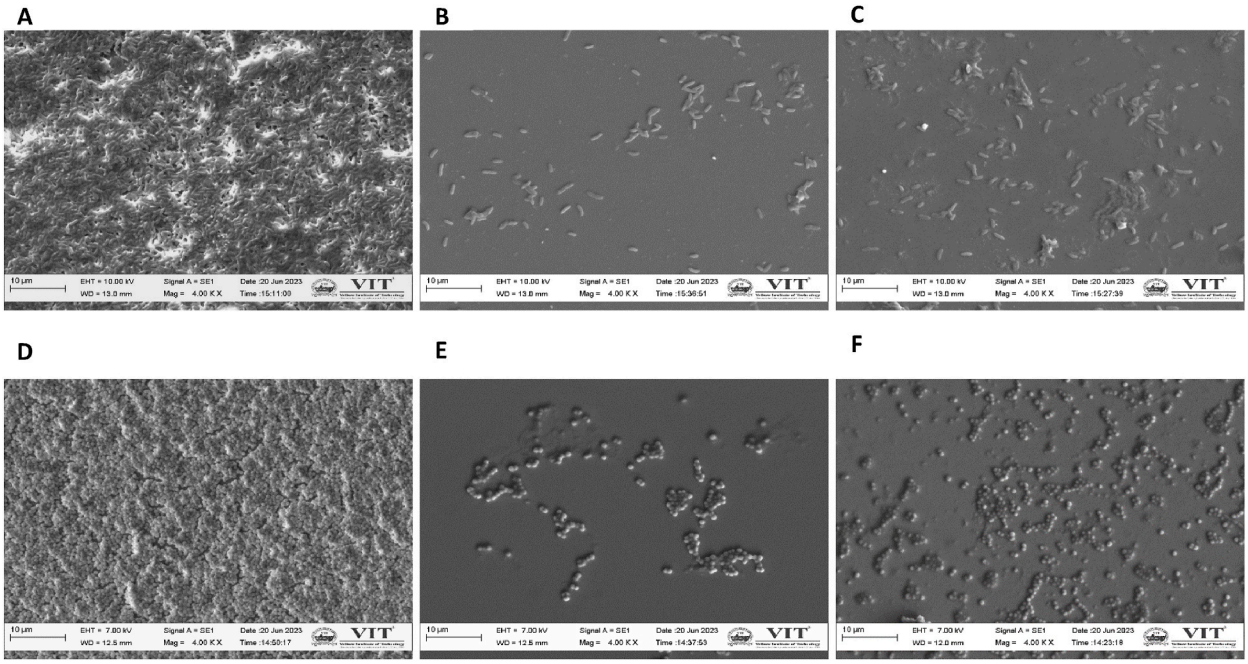

Fig. 6. Inhibition of biofilm formation by *D. elata* against *C. violaceum* and *S. aureus*
A, B and C depicts the biofilm morphology of *C. violaceum* without and with treatment of *D. elata* at 0.6 and 0.15 mg/mL concentrations respectively. The SEM micrographs confirms the anti-biofilm activity of *D. elata* and shows efficient reduction of *C. violaceum* biofilm at 0.6 mg/ml. While, D, E and F are the micrographs of *S. aureus* biofilms without and with treatment of *D. elata* at 0.6 and 0.15 mg/mL concentrations respectively. The *S. aureus* biofilms were significantly reduced at 0.6 mg/mL concentration.

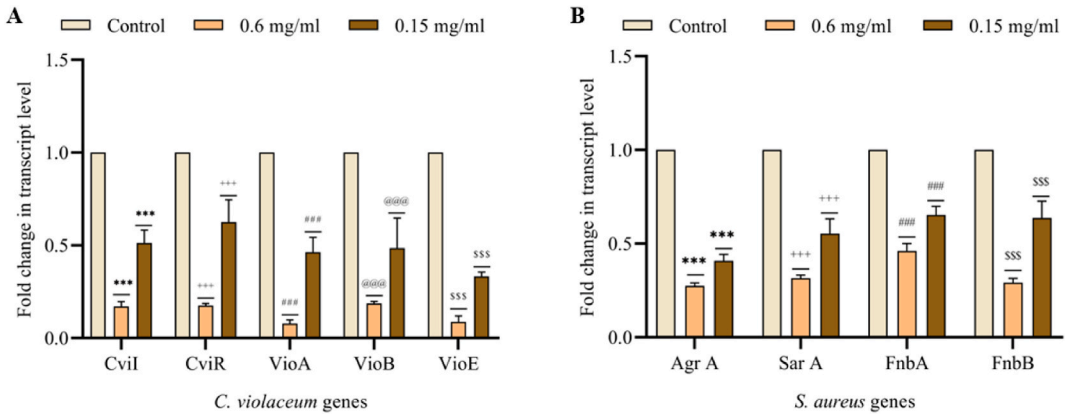

Fig. 7. Gene expression analysis of the major Quorum sensing regulated virulent genes of both *C. violaceum* and *S. aureus* were examined. Where in both cases there is a significant reduction in the virulence gene expressions in all the treated groups. Further, efficient reduction was seen in the groups treated with *D. elata* at 0.6 mg/mL concentration than 0.15 mg/ml concentration ($P < 0.01$, $P < 0.001$).

3. Discussion

Biofilm is one of the crucial virulence factors regulated by QS in bacteria such as *C. violaceum* and *S. aureus*. They are a vital causative agent of resistance and persistence in those bacteria [32]. Here we impede bacterial biofilm by targeting quorum sensing systems Cvi and Agr in *C. violaceum* and *S. aureus* respectively using the plant DE.DE has heretofore reported biological significance such as antimicrobial, anti-inflammatory, and antioxidant benefits [33]. DE leaves were extracted using ethyl acetate and the bio-actives were identified using the GC-MS platform. GC-MS revealed 20 major bio-actives with different retention times. Many medicinal plants have various phytochemical constituents with potential toxicity to humans. For instance, Datura and *Atropa belladonna* are well-known medicinal plants utilized in traditional medicine but unfortunately associated with toxic effects on human beings [34,35]. Further, *Momordica charantia* was reported to have anti-malarial and anti-diabetic properties along with negative effects such as deadly

Table 2
Docking score of bioactive present in *D. elata* against *C. violaceum* QS receptor cviR.

S. No	Bioactives	Docking score
1	O-METHYLISOUREA HYDROGEN SULFATE	−2.44
2	MEGLUMINE	−4.2
3	1,3-PROPANEDIAMINE, N-METHYL-	−4.85
4	5-AZIRIDINOPENTANOL	−3.59
5	1,3-PROPANEDIAMINE, N,N′-BIS(3-AMINOPROPYL)-	−6.19
6	1-PENTATRIACONTANOL	–
7	2-BUTENE-1,4-DIAMINE, N,N′-DIMETHYL-	−4.68
8	N-[3-METHYLAMINOPROPYL]AZIRIDINE	−4.06
9	1,3-BUTANEDIAMINE	−5.65
10	ARGININE	−5.18
11	1,10-DECANEDIOL	−4.66
12	[1-(DIETHYLAMINO)ETHYLIDENIMINO]SULFUR PENTAFLUORIDE	−5.22
13	N-[DIMETHYLAMINOMETHYL]AZIRIDINE	−3.06
14	THREO-4-HYDROXY-L-LYSINE LACTONE	−6.12
15	BENZENEETHANAMINE, 3,4-DIMETHOXY-N-METHYL-	−6.33
16	PHENETHYLAMINE, 3-METHOXY-.ALPHA.-METHYL-4,5-(METHYLENEDIOXY)-	−6.27
17	SPERMINE	−5.86
18	2-BUTENE-1,4-DIAMINE, N,N′-DIETHYL-	−5.33

Table 3
Docking score of bioactive present in *D. elata* against *S. aureus* QS receptor agrA.

S. No	Bioactives	Docking score
1	O-METHYLISOUREA HYDROGEN SULFATE	−2.74
2	MEGLUMINE	−3.71
3	1,3-PROPANEDIAMINE, N-METHYL-	−4.4
4	5-AZIRIDINOPENTANOL	−3.46
5	1,3-PROPANEDIAMINE, N,N′-BIS(3-AMINOPROPYL)-	−5.42
6	1-PENTATRIACONTANOL	–
7	2-BUTENE-1,4-DIAMINE, N,N′-DIMETHYL-	−4.26
8	N-[3-METHYLAMINOPROPYL]AZIRIDINE	−3.09
9	1,3-BUTANEDIAMINE	−4.88
10	ARGININE	−5.29
11	1,10-DECANEDIOL	−3.2
12	[1-(DIETHYLAMINO)ETHYLIDENIMINO]SULFUR PENTAFLUORIDE	−3.23
13	N-[DIMETHYLAMINOMETHYL]AZIRIDINE	−2.26
14	THREO-4-HYDROXY-L-LYSINE LACTONE	−6.69
15	BENZENEETHANAMINE, 3,4-DIMETHOXY-N-METHYL-	−4.92
16	PHENETHYLAMINE, 3-METHOXY-.ALPHA.-METHYL-4,5-(METHYLENEDIOXY)-	−4.38
17	SPERMINE	−5.04
18	2-BUTENE-1,4-DIAMINE, N,N′-DIETHYL-	–

hypoglycemic side effects in children [36]. Thus, it is very essential to investigate the toxic effects of the selected plant species. The sub-MIC concentration of DEshowed nontoxic effects in mice T36 cells. Similarly, previous research had reported the nontoxicity of *M. calabura* leaf extract against Vero cell lines which is an effective quorum quenching plant extract [37]. DE extracts were nontoxic to brine shrimps at the selected concentration which is following a previous report where several Pacific Northwest Forest plants have appeared to be nontoxic to brine shrimps [38].

Multiple aspects influence the ability of bacteria to cause infection. Current research is exploring those factors including i) violacein biosynthesis, ii) hemolysin production, iii) exopolysaccharide excretion, iv) biofilm formation, v) eDNA and vi) virulence gene expressions. To confirm the nonmicrobicidal nature of DE extract at sub-MIC concentration flow cytometry was employed and found sub-MIC concentrations of 0.6 and 0.15 mg/ml were not detrimental to the bacterial cells. Subhasree et al., research results predicted that *V. cholerae* was not killed by treatment with anti quorum compound naringenin, but the virulence factors were reduced by quorum quenching [39].

Furthermore, the previously mentioned six mechanisms that play a major role in bacterial virulence became the focus of the research trajectory. Violacein synthesis is regulated by the QS system of *C. violaceum* in a population density-dependent manner. DE extract prevented the violacein biosynthesis at sub-MIC concentration to the extent of 68.81 % and 49.36 % at 0.6 and 0.15 mg/ml of DE respectively. These results were in correspondence with the previous research in which dichloromethane extract of *C. praecox* inhibited the violacein synthesis up to 80 % [40]. Another research depicts extracts of *Syzygium aromaticum* and *Dionysia revolute* inhibit violacein biosynthesis up to 77 and 83 % respectively [41,42]. Systemic infections of pathogenic microbes cause deadly sepsis by hemolysis. There were numerous sepsis cases caused by *C. violaceum* and *S. aureus* which ruptured RBCs by production of hemolysin. DE EA extract impeded hemolysin production by up to 80 % in *C. violaceum* and 51.3 % in *S. aureus* in our experiments. Previous reports exhibited that food additive 2-tert-Butyl-1,4-benzoquinone inhibited hemolysin synthesis in *C. violaceum* up to 70 % and

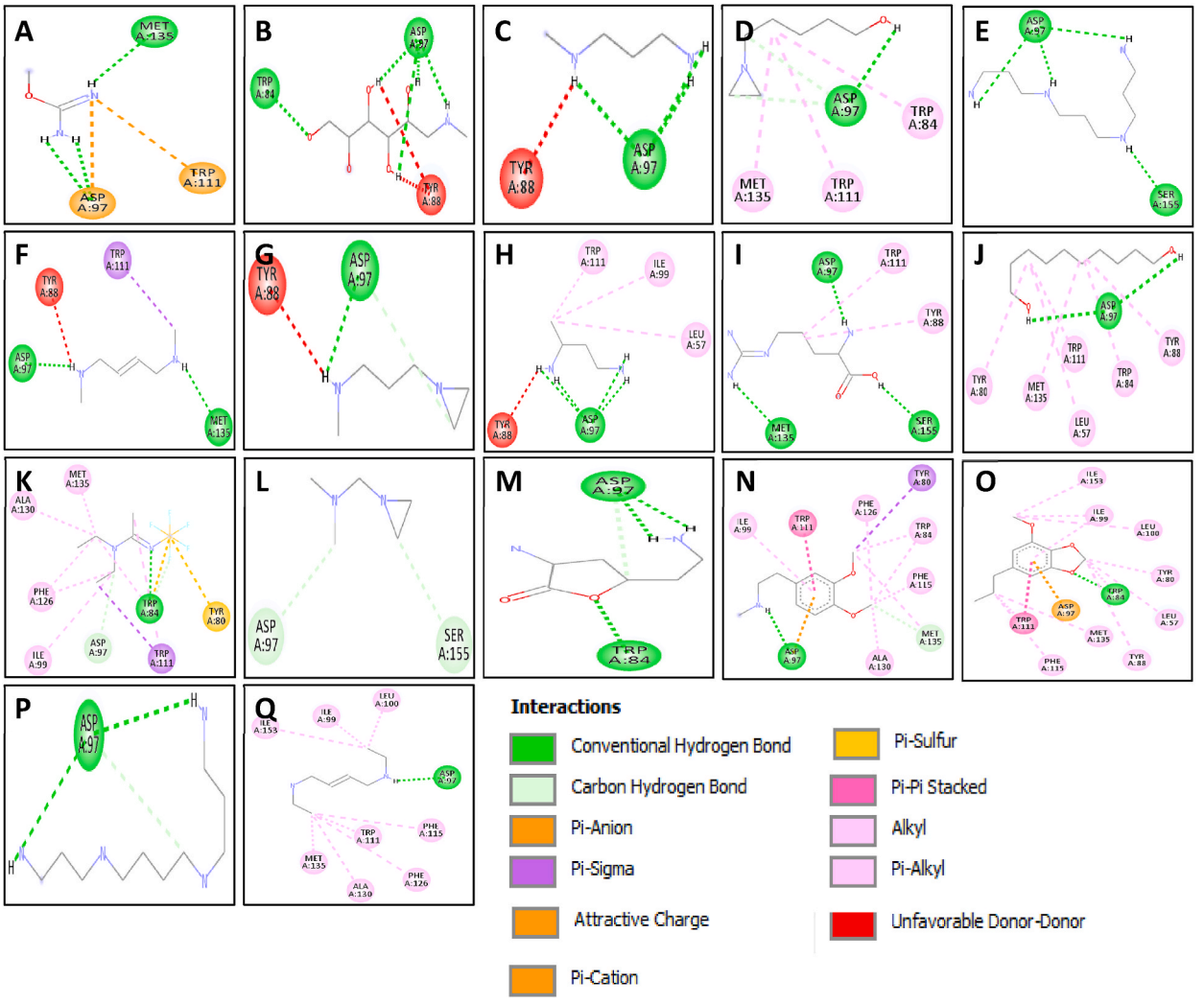

Fig. 8. Docking 2D schematic diagram of Bioactives binding with cviR receptor of *C. violaceum* were depicted. A) O-METHYLISOUREA HYDROGEN SULFATE B) MEGLUMINE c) 1,3-PROPANEDIAMINE, N-METHYL- D) 5-AZIRIDINOPENTANOL E) 1,3-PROPANEDIAMINE, N,N′-BIS(3-AMINOPROPYL)- F) 2-BUTENE-1,4-DIAMINE, N,N′-DIMETHYL- G) N-[3-METHYLAMINOPROPYL]AZIRIDINE H) 1,3-BUTANEDIAMINE I) ARGININE J) 1,10-DECANEDIOL K) [1-(DIETHYLAMINO)ETHYLIDENIMINO]SULFUR PENTAFLUORIDE L) N-[DIMETHYLAMINOMETHYL]AZIRIDINE M) THREO-4-HYDROXY-L-LYSINE LACTONE N) BENZENEETHANAMINE, 3,4-DIMETHOXY-N-METHYL- O) PHENETHYLAMINE, 3-METHOXY-. ALPHA.-METHYL-4,5-(METHYLENEDIOXY)- P) SPERMINE Q)2-BUTENE-1,4-DIAMINE, N,N′-DIETHYL-.

bioactive resveratrol at 32 μg/ml inhibited hemolysin biosynthesis in *S. aureus* without growth inhibition [43,44]. Exopolysaccharide aids in the initial attachment which results in intercellular and surface adhesion and triggers the compact multilayered biofilm formations. EPS hindrance would limit biofilm formation and reduce virulence of any pathogenic microorganisms. Our DE extract diminished the EPS production by up to 49 % in *C. violaceum* and 35 % in *S. aureus* commensurate with published results. Previous reports present that lysine inhibited EPS production in *C. violaceum* by 12.5 % at 0.684 mM concentration [45]. A fruit extract of *Passiflora edulis* attenuated EPS production up to 72.8 % in *C. violaceum* as reported earlier [46]. Biofilm formation impedes antibiotic actions by protecting microbes in an enclosed environment. Thus, eradicating biofilms will be a leap step toward alternative therapeutic strategies. Adhesion in cells of humans or animals benefit microbes to form complex biofilms which cause heightened pathogenesis. Mouse fibroblast cells L929 were utilized for the antiadhesion potential of DE extract against *C. violaceum* and *S. aureus*. The DE extract unveiled an efficient reduction in the CFU count that formed biofilms which complied with the previous research report which projects the reduction in biofilm formation in *Proteus mirabilis* by extract of *Alhagi maurorum* [47]. Our DE extract hindered biofilm formation up to 76.45 % in *C. violaceum* and 58.15 % in *S. aureus* at a concentration of 0.6 mg/ml as revealed by crystal violet staining. The results were similar to previous research in which seed extracts of *Psoralea corylifolia* reduced biofilm formation in *C. violaceum* up to 64 % and 2.4 mg/ml of *Illicium verum* inhibited the biofilm formation in *S. aureus* up to 74 % [48,49]. Biofilm architecture was visualized utilizing SEM, which compared control and treated biofilms of *C. violaceum* and *S. aureus*. The morphology showed reduction in DE-treated samples compared to control biofilms of *C. violaceum* and *S. aureus*. This is in synonym with previous

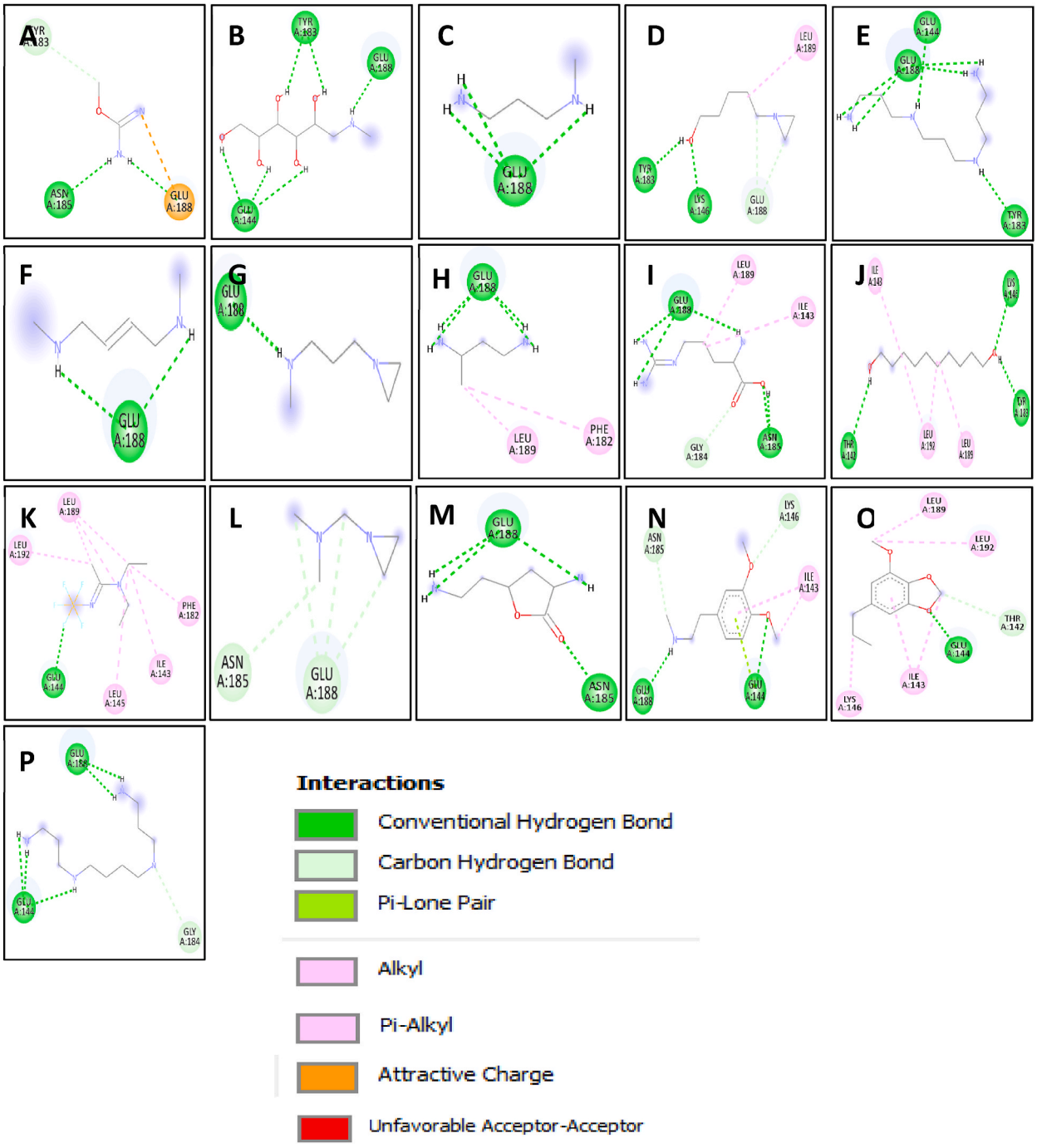

Fig. 9. Docking 2D schematic diagram of Bioactives binding with agrA receptor of *S. aureus* were depicted. A) O-METHYLISOUREA HYDROGEN SULFATE B) MEGLUMINE c) 1,3-PROPANEDIAMINE, N-METHYL- D) 5-AZIRIDINOPENTANOL E) 1,3-PROPANEDIAMINE, N,N′-BIS(3-AMINO-PROPYL)- F) 2-BUTENE-1,4-DIAMINE, N,N′-DIMETHYL- G) N-[3-METHYLAMINOPROPYL]AZIRIDINE H) 1,3-BUTANEDIAMINE I) ARGININE J) 1,10-DECANEDIOL K) [1-(DIETHYLAMINO)ETHYLIDENIMINO]SULFUR PENTAFLUORIDE L) N-[DIMETHYLAMINOMETHYL]AZIRIDINE M) THREO-4-HYDROXY-L-LYSINE LACTONE N) BENZENEETHANAMINE, 3,4-DIMETHOXY-N-METHYL- O) PHENETHYLAMINE, 3-METHOXY-. ALPHA.-METHYL-4,5-(METHYLENEDIOXY)- P) SPERMINE.

results published where biofilms of *C. violaceum* were impeded when treated with *Mentha suaveolens* essential oil and *S. aureus* biofilms was inhibited by *Polygonun chinense* L. extract [50,51]. eDNA is an essential component of bacterial biofilms which protect bacteria from antibiotics and host immune systems. We quantified the eDNA in the biofilm environment of control and treated *C. violaceum* and *S. aureus*. The eDNA was reduced up to 67.5 and 53.47 % in *C. violaceum* and *S. aureus* respectively in treated conditions which

conformed with previous reports of which *Polygonum chinense* L. extract inhibited eDNA in *S. aureus* biofilm matrix [51]. Virulence genes such as *vioA, vioB* and *vioE* were regulated by master quorum sensing genes such as *cviI* and *cviR* in *C. violaceum*. In *S. aureus* adhesion responsible genes *fnbA* and *fnbB* were regulated by quorum sensing system *agrA* and *sarA*. RT qPCR gene expression study revealed hindrance in the expression level of the above-mentioned genes in *D. elata* treated samples compared with the control. These results were in accordance with the previously published results where butein and bavachin inhibited quorum-sensing genes of *C. violaceum* such as cviI and cviR [52]. 2-[(Methylamino)methyl] phenol attenuated QS genes in *S. aureus*.

Docking employing bioactive ligands against quorum sensing proteins of *C. violaceum* and *S. aureus* revealed better interactions which were in accordance with previous results where components of *Passiflora edulis* interacted with CviR receptor and attenuated quorum sensing mediated virulence production [46]. In another study 3 HBA interacted with *S. aureus* AgrA and impeded QS mediated virulence factor [49]. These interactions might be responsible for the DE extract-mediated quorum quenching in *C. violaceum* and *S. aureus* addition to the diminished expression of the receptor itself as depicted in the qPCR studies.

4. Conclusion

The emergence of antimicrobial resistance poses a serious threat to global health and necessitates the development of novel therapeutic strategies. One promising approach is to target the quorum-sensing system of pathogenic bacteria, which regulates their virulence and biofilm formation. However, there are currently no approved drugs that modulate quorum sensing in clinical settings. Future research should focus on identifying and optimizing quorum-sensing inhibitors such as *Delonix elata* extract and their bioactive that can effectively attenuate bacterial infections without inducing resistance or toxicity. This would have significant implications for the advancement of science and the improvement of human health.

5. Materials and methods

5.1. Bacterial culture conditions

Chromobacterium violaceum wild-type strain (MTCC2656) and *Staphylococcus aureus* (MTCC 737) were obtained from MTCC, India. *C. violaceum* was routinely grown in the Luria Bertani (LB) media purchased from Hi Media, India, at the temperature of 30 °C at an rpm of 120 for 24 h. *S. aureus* was aerobically cultivated at 37 °C with 100 rpm, and sub-cultured before every experiment for 16–24 h [53,54].

5.2. Delonix elata extract (DE) preparation

Delonix elata leaves were collected from Valangaiman, India (10°53′26″N 79°23′33″ E) in a sterile polythene bag. The leaves were dried under shade and pulverized finely employing a mechanical mixer. Then 25 g of course powder were soaked in 100 ml of ethyl acetate and agitated at 150 rpm for 48 h. The solvent was separated from the crude using Whatman filter paper number 1 and evaporated at 50 °C using a rotary flash evaporator. Then the samples were stored at 4 °C until further use, and a sterile environment was maintained [33,55,56].

5.3. GCMS for identifying bioactive compounds in the DE extract

The Clarus 680 PerkinElmer Gas Chromatography and Clarus 600 (EI) mass spectrometry. VIT, Vellore was employed for GCMS analysis, which utilizes a fused silica column packed with Elite-5MS (5 % biphenyl 95 % dimethylpolysiloxane, 30 m 0.25 mm ID 250 m df). Helium, with a flow rate of 1 mg/ml, was used as a carrier gas. During the chromatographic run, the injector temperature was established to 260 °C. About 1 μL sample of *Delonix elata* ethyl acetate leaf extract was injected into the instrument, and the oven temperature was maintained at 60 °C for 2 min, followed by 300 °C at a rate of 10 °C min-1, then 300 °C for 6 min. The mass detector settings were as follows: transfer line temperature 240 °C, ion source temperature 240 °C, ionization mode electron impact at 70 eV, scan time 0.2 s, and scan interval 0.1 s. The range of fragments varies in size from 40 to 600 Da. The component spectrums were correlated to a database of known spectrums recorded in the GC-MS NIST library [57,58].

5.4. MTT toxicity assay of Delonix elata extract

The toxicity of ethyl acetate extracts of *Delonix elata* was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. Mouse fibroblast cells (3T6) were preattached and treated with sub-MIC concentrations of *Delonix elata* at 0.6 and 0.15 mg/ml. After 24 h, 100 μL of MTT was added to the wells after removing DE extract and incubated for 4 h. The formed formazan crystals were dissolved by 100 μL of DMSO, and absorbance was measured at 570 nm spectrophotometrically [59,60].

5.5. Brine shrimp lethality assay

Artemia salina eggs were hatched in artificially made seawater (38 g/ml of salt). Matured nauplii shrimps were ready for assay after 24 h. The plant extract was added to attain the final concentration of 0.6 and 0.15 mg/ml and a control without any extract. Ten brine shrimps were added to each concentration and observed for any death at 6,12 and 24 h [38,61,62]. The death percentage was

calculated using the formula given below.

Percentage of Death (%): (Total nauplii – Alive nauplii)/ Total nauplii x 100%

5.6. MIC determination using Triphenyl tetrazolium chloride (TTC)

TTC, a modified CLSI broth microdilution method, is used to determine the MIC of the ethyl acetate extract of *Delonix elata* against *C. violaceum* and *S. aureus*. 10 μL of bacterial cultures with cell mass equivalent to (0.5) McFarland unit is added to the control tubes with LB broth and with DE extract with varying concentration from 10 mg/ml to 0.016 mg/ml by two-fold serial dilution method. *C. violaceum* tubes were maintained at 30 °C at 12 rpm, and *S. aureus* tubes were kept at 37 °C at 100 rpm for 24 h. Then the cell density was quantified at 600 nm, and to confirm the obtained results, 10 μL TTC was added and inspected for a cherry red colour to be developed. The concentration at which no visible bacterium growth was monitored is taken as MIC. The DE extract at sub-MIC concentration was utilized for further anti-quorum and biofilm experiments downstream [63].

5.7. Bacterial viability assay using flow cytometer

The cell viability was determined using flow cytometry analysis utilizing propidium iodide.

C. violaceum and *S. aureus* were grown in the presence and absence of DE extract for overnight. Then they were diluted for 1 OD and incubated with 15 μg/ml of propidium iodide for 15 min in the dark. Then the cells were centrifuged and PBS-washed to remove unbound dye. Then PI positive events were recorded and distinguished from the SSC/FSC bacterial population. The population of live cells was calculated by subtracting the dead cell population. The cytoFLEX Beckman Coulter, USA was employed for flow cytometry analysis [39].

5.8. Violacein quantification

Ethyl acetate (EA) extract of DE was engaged in quantifying violacein synthesized in a quorum-sensing regulated manner in *C. violaceum*. An overnight culture of *C. violaceum* corresponding to approximately (1 X 10^8 CFU/ml) was added to the control and DE-treated tube at the concentrations of 0.6 and 0.15 mg/ml. After incubation for 24 h, the bacterial cells were pelleted, and violacein was extracted with 200 μL of DMSO. Then the remaining cell pellets were measured at 600 nm after centrifugation at 10000 rpm to determine growth. The absorbance at 585 nm was quantified using a spectrophotometer for soluble violacein [64].

5.9. Hemolysin quantification

The load of hemolysin synthesized by *C. violaceum* and *S. aureus* was quantified indirectly, as described by others. *C. violaceum* and *S. aureus* were added to the control and 0.6 mg and 0.15 mg/ml of extract-treated tubes and incubated for 24 h. Then the supernatant encompassing hemolysin was separated using centrifugation. The supernatants were purified and added with sheep blood (2 % in PBS) in a ratio of 1:9 and incubated for 1 h at room temperature (28 °C). Then the cells were pelleted and liberated red colour by lysis of blood was measured at 530 nm using a spectrophotometer [44].

5.10. Quantification of EPS

Extraction and quantification of EPS were performed using protocols described by others. Briefly, 10 μL of overnight cultures of *C. violaceum* and *S. aureus* were added to control without plant extract and sub-MIC concentrations of 0.6 and 0.15 mg/ml of EA DE extract. The tubes were incubated for 24 h at 30 °C for *C. violaceum* and 37 °C for *S. aureus*. The bacterial cells were pelleted by spinning at 10000 rpm and resuspended in a high salt buffer. Then the mixture was centrifuged, and supernatants were collected. An equal volume of ethanol was added to the supernatant and centrifuged at 10000 rpm for 30 min. The precipitated EPS was added with 1 ml of 5 % cold phenol and 5 ml of conc H_2SO_4. The developed red colour was measured using the spectrophotometer at 490 nm [65].

5.11. Antiadhesion assay

Mouse fibroblast cells (L929) were seeded with 0.6 and 0.15 mg/ml of *D. elata* and without plant extract in 24 well plates. Then they were infected with *C. violaceum* and *S. aureus* for 4 h and incubated at 37 °C at 5 % CO_2. Then the wells were washed carefully with PBS to remove nonadherent bacteria. Subsequently, 500 μL of 0.025 % Triton X 100 was applied to the plate to lyse and detach the cells. Later the lysates were serial diluted and plated to count CFU to compare the control and treated samples [47].

5.12. Antibiofilm formation – quantitative assay using crystal violet

The ethyl acetate extract of *Delonix elata* is screened for inhibition of biofilm formation in *C. violaceum* and *S. aureus* using a static microtiter plate assay. Overnight cultures of 10 μL of *C. violaceum* and *S. aureus* were inoculated in LB broth with 0.6 and 0.15 mg/ml of

DE extract. The plates were incubated at 30 °C for *C. violaceum* and 37 °C for *S. aureus*. After 24 h, the supernatants were discarded, and the wells were washed with deionized distilled water three times, added 2 % crystal violet, and washed again. Then biofilm bounded crystal violets were dissolved in 95 % ethanol, and the absorbance was measured at 595 nm [66,67].

5.13. Antibiofilm – Qualitative assay utilizing scanning electron microscopy

The microscope was used to visualize the biofilm formation in control and 0.6 and 0.15 mg/ml DE extract-treated samples. The coverslips were carefully removed from the 6-well plates using sterile forceps and rinsed with PBS to remove unbound planktonic cells. Then the cells in the biofilm were air-dried for 15 min and fixed with 2.5 % glutaraldehyde for 5 min. After fixation, the foils were serially dehydrated with 70–100 % ethanol. The coverslips were sputter coated with gold/palladium and observed in Carl Zeiss Evo/18 scanning electron microscope at 10 μM magnification [68].

5.14. eDNA quantification from biofilm environment

eDNA quantification was performed as described earlier by others with some minor modifications. Overnight cultures of 10 μL C. *violaceum* and *S. aureus* were inoculated in LB media with 0.6 and 0.15 mg/ml of *D. elata* and without the extract as control and incubated at 30 °C and 37 °C for *C. violaceum* and *S. aureus* respectively for 24 h. Moreover, the biofilms were treated with 1 ml of Tris EDTA NaCl buffer and cooled at 4 °C for 1 h. Additionally, the supernatants were removed along with planktonic cells carefully. Furthermore, 700 μL of TE buffer was added to remove the adhered biofilm cells and they were pelleted by centrifugation at 15000 rpm for 5 min at 4 °C. Then eDNA in the supernatants was extracted using phenol-chloroform isoamyl alcohol and resuspended in TE buffer. Subsequently the quality of the DNA was accessed using gel and quantified in the spectrophotometer [51].

5.15. Relative gene expression using qRT-PCR

Total RNA was extracted from *C. violaceum* and *S. aureus* cells using the Qiagen AllPrep RNA isolation kit, Germany according to the manufacturer's instructions. The bacterial cultures were grown at appropriate conditions with 0.6 and 0.15 mg/ml and without DE plant extract. Total RNA was extracted and converted into cDNA using Prime Script RT reagent kit (Takara, Japan) at 37 °C for 15 min (reverse transcription) and 85 °C for 5 min reverse (transcriptase inactivation) for a total volume of 30 μL. Integrated DNA Technology, India, synthesized the primers.

Real-time quantitative PCR was performed according to the manufacturer's protocol of the kit TB green Premix ex taq II by Takara, Japan. cDNA converted from total RNA is used as a template for RT-qPCR. BIO-RAD CFX96 Real-time PCR system was used to perform the experiments. The reaction conditions were set as follows: 95 °C for 30S, pre-denaturation, followed by 40 cycles of 95 °C for 5S denaturation and 60 °C for 30S extension. 16srRNA was used as the housekeeping gene, and all the genes' relative mRNA expression was calculated using the 2 -ΔΔCt method. The experiments were repeated three times independently [52,69].

5.16. Docking DE bioactive with QS proteins

The bioactive phytochemicals structures were retrieved from NCBI-PubChem and unavailable structures were drawn in Chemdraw and converted into a suitable format using the open Babel online tool. The ligands were optimized by minimizing energies. X-ray crystal structures of *C. violaceum* CviR (PDB ID:3QP5) and *S. aureus* AgrA (PDB ID:4G4K) were obtained from Protein Data Bank (PDB). The structures were optimized using a Swiss PDB viewer prior to processing for docking. Water was removed and polar hydrogens were added for assigning bond order. Then grids were made in the active sites of the protein CviR and AgrA in chain A. Then the proteins were subjected to docking using the application Autodock with the ligands using genetic algorithms and the energy required for binding was recorded and the interaction was saved as a complex file which was viewed in the Discovery studio visualizer [49].

5.17. Statistical analysis

All experimental works were done in triplicates and statistical significance was determined for toxicity assay, violacein biosynthesis, hemolysin production, EPS production, adhesion, biofilm formation, and eDNA quantification, using one-way ANOVA followed by Tukey's test using graph pad prism 8 software.

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Ethical approval

This article does not contain any studies with human participants or animals by any of the authors.

Consent for publication

"Informed consent was obtained from all the authors who have contributed to the study."

Data availability statement

No data was used for the research described in the article.

CRediT authorship contribution statement

Venkatramanan Mahendrarajan: Writing – original draft, Investigation, Data curation. **Huldah Lazarus:** Writing – original draft, Methodology, Investigation. **Nalini Easwaran:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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