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Original article

Computational and experimental strategies for combating MBL *P. aeruginosa* (MBLPA) biofilms using phytochemicals: Targeting the quorum sensing network

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterium, ubiquitously found in nature and causative agent in many infections. Due to increased antibiotic resistance, there is a need to develop more robust anti-bacterial agents from natural sources. In this study, we worked on two metallo-β-lactamase (MBL) producing *Pseudomonas aeruginosa* strains and targeted the Quorum Sensing mechanism (QS) of these bacteria to combat antibiotic resistance. Our study aimed at using phytochemicals which have been used since centuries in herbal medicine. We used fifteen commercially available phytochemicals and check their effects on biofilm formation, quorum sensing and inter-related mechanisms. Sub-inhibitory concentration of isoliquiritin inhibited biofilm formation 55 % in P8 at day 6 and 48 % in P6 at day 6; quorum sensing 83 % in P6 and 61 % in P8 whereas sub-inhibitory concentration of 6-gingerol suppressed biofilm formation by 48 % in P8 at day 6 and 44 % in P6 at day 6; quorum sensing 69 % in P6 and 48 % in P8, respectively. The results indicated isoliquiritin, epigallocatechin gallate, eugenol, luteolin and chrysin to be the potential candidates in inhibiting QS and related mechanisms. Isoliquiritin which was never been used before against biofilm and QS related studies, showed remarkable results and found to be more efficient in inhibiting QS than 6-gingerol –a known QS inhibitor. For examining the molecular interaction between phytochemicals and QS, In-silico molecular docking was performed between phytoligands and four QS proteins (*Las I, Las R, RhlI and Rhl R*). *In-silico* docking analysis revealed that isoliquiritin showed strong bond with amino acids (Trp34, Asp35, Asp35, Tyr105, Arg104, Val138, Thr140) present at the active site of RhlI with binding energy value of −8.4 kcal/mol as compared to that of 6-gingerol with Rhl1 (−7.3 kcal/mol). In conclusion, our study may help in controlling nosocomial infections caused by carbapenem-resistant metallo beta-lactamase *P. aeruginosa* (MBL-PA) by utilizing these phytochemicals in biofilms disruption and quorum sensing inhibition. Moreover their synergism with antibiotics may help in lowering the MIC of carbapenem antibiotics against such Multi-drug resistant strains.

1. Introduction

P. aeruginosa is a rod shaped, aerobic gram-negative opportunistic bacterium, ubiquitously found in nature. In various infections *P. aeruginosa* is found to be the causative agent. It has been transformed into more stringent metallo-beta-lactamase (MBL) producing bacteria by developing resistance against last class of antibiotics *i.e.* carbapenems (Husain et al., 2021). One of the antibiotic resistance mechanisms employed by bacterial pathogens is through Quorum sensing. QS controls the virulence and pathogenicity in bacterial pathogens and contribute in biofilm production, which is a vital source of antibiotic resistance in many infections (Sırıken et al., 2021). Quorum sensing is a cell-to-cell communication between microbes which occur through various signaling molecules known as autoinducer (like AHL in Gram negative bacteria) secreted by the bacteria. These signaling molecules then bind to their respective receptors and activate various virulence related mechanisms such as virulence factor secretion, motility, bioluminescence, sporulation, antibiotic production, competence and biofilm formation (Yi et al., 2021). In *P. aeruginosa*, QS is also responsible for the expression of various genes involved in pathogenicity. *P. aeruginosa* produces acyl homoserine lactones (AHLs) as an autoinducer in QS signaling systems. When the concentration of AHLs touches a threshold,

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it activates many different transcriptional regulators. Through this cellular communication, bacterial cells develop a biofilm which is a thick layer of bacterial cells embedded in an extracellular matrix, found attached to the living or nonliving surfaces (Duddy and Bassler, 2021). Therefore, QS has been suggested as an effective target in antimicrobial resistance therapy. Quorum sensing can be blocked by many ways, 1) by inhibiting the biosynthesis of AHL molecules, 2) by degrading the already synthesized AHL molecules 3) inactivating the AHL receptor protein (Sabat et al., 2021).

P. aeruginosa has devised two distinct acyl homoserine lactone (AHL) based pathways of quorum sensing: 1) lasI/lasR pathway: lasI gene codes for protein Las I synthase that forms 3-oxo-dodecanoyl homoserine lactone (3-oxo C12-HSL) which binds to its receptor LasR (which is a transcriptional activator), encoded by lasR gene. 2) the rhlI/rhlR pathway: rhlI gene encodes RhlI protein that forms butyryl acyl homoserine lactone (C4-HSL) which binds to its receptor RhlR (Zhao et al., 2020).

Over the past years, the antibiotic resistance in bacteria has transformed them into a more stringent and tougher strains and the development of new antibiotics has been severely dropped. Hence, there is a need to develop alternative approaches on urgent basis. These approaches need to target multi-drug resistant strains in both planktonic states and or in biofilm state. Phytotherapy could act as an efficient treatment to tackle multi-drug resistant microbes and their infections. Several phytochemicals have been reported as potential candidates in inhibiting *Pseudomonas* and other bacterial infections through different mechanisms of action (Akram et al., 2020).

Phytochemicals have captured the scientific community's attention since they are regarded as a sustainable and environmental friendly source of new molecules that have been shown to be excellent biofilm and beta lactamase inhibitors (Javid and Ahmed, 2023). Notably, phytochemicals have a wide range of structural features that differ greatly from standard antibiotics, which can be attributed to their multi-target mode of action. These distinguishing characteristics may make it simpler to overcome resistance. Because fruits and edible flora make up a major fraction of human and animal intakes and are generally safe and readily available, discovering QSI among these natural nutrients is of special significance (Alaoui Mdarhri et al., 2022).

The development of new antibiotics has not reduced the spread of bacterial infections which continue to threaten the global healthcare system. As the post-antibiotic era begins, the scientific community has developed an interest in anti-virulence drugs (QSIs). These "wonder medicines" contain bioactive phytochemicals that inhibit the expression of virulence genes while not eliminating or impeding the growth of dangerous bacteria. Compared to antibacterial drugs, this technique produces substantially less selection pressure (Subramanian et al., 2022).

Our study aimed at using compounds from plants which have been used for centuries in herbal medicine. We targeted the Quorum sensing mechanism (QS) of these bacteria to combat antibiotic resistance. We used fifteen commercially available phytochemicals to check their effects on biofilm formation, quorum sensing and inter-related mechanisms like motility, adhesion, hydrophobicity, and aggregation. So, the present study targets to validate the interaction of phytochemicals with the QS signaling in *P. aeruginosa* using an in-vitro and in silico approach.

2. Materials and Methods

A flow chart of the *in-vitro* and *in-silico* tests performed during this study are given in Fig. 1.

2.1. Bacterial strains and culture conditions

Two strains of *P. aeruginosa* P8 (Accession no. MT277358) and P6 (Accession no. MT277092) isolated from pus and urine samples respectively were kindly provided by Citi lab Lahore. The cultures were grown and maintained on Mac Conkey agar.

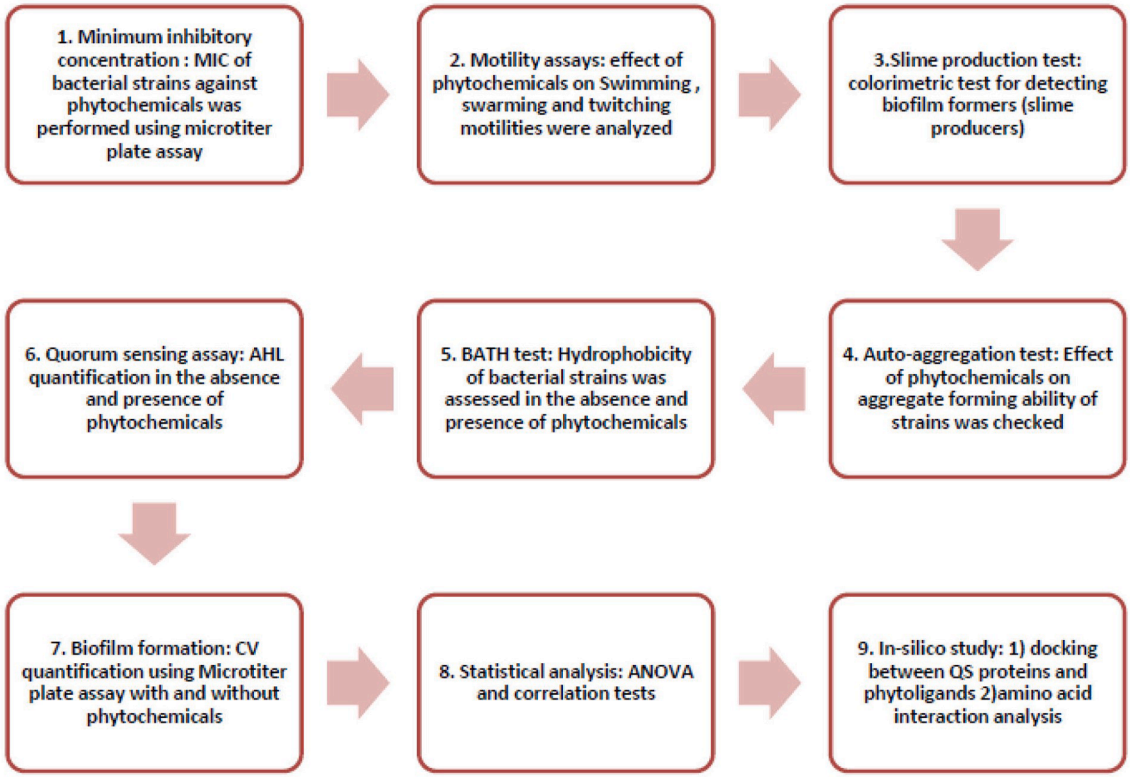

Fig. 1. Flow chart of in-vitro and in-silico tests performed during this study.

2.2. Phytochemicals and stock solutions

Fifteen phytochemicals (powdered form) namely Diosgenein (Chemimpex –USA), Artemisin (AK scientific-USA), Luteolin (AK scientific-USA), Chrysin (Chemimpex-USA), Anthraquinone (Ambeed-USA), Acetyl aleuritolic acid (Ambeed- USA), Piperine (Chemimpex-USA), Isoliquiritin (Adooq-USA), Caryophylline (Chemimpex-USA), allicin (carbosynth limited,UK), spermidine (Himedia), Emodin (himedia), 6-gingerol (carbosynth limited UK), Eugenol (carbosynth limited, UK), Epigallocatechin gallate (carbosynth limited,UK) were purchased and then dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) with the final DMSO concentration of the solution was 1 % (v/v).

2.3. Minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of all the phytochemicals against both strains were investigated by microtiter plate method (Kowalska-Krochmal and Dudek-Wicher, 2021). Briefly, the bacterial strains were grown in 0.1 μgml^{-1} to 1500 μgml^{-1} concentrations of phytochemicals and incubated at 37 °C for 24 h. After incubation, the absorbance was measured at 600 nm by using negative control (DMSO) as blank. All the subsequent tests were performed in triplicate with sub-inhibitory concentrations (½ × MIC) of phytochemicals.

2.4. Swimming, swarming and twitching Motilities

Motility assays was performed according to the method of (Saeki et al., 2021) with some modifications. Swimming, (a flagella directed movement in aqueous medium) and swarming (a flagella directed motility onto solid surface) were performed by transferring individual colonies from an agar growth to the surface of swimming and swarming agar by using a sterile inoculating needle and plates were incubated at 37 °C for 24 h. After incubation, diameter (mm) of the halo of growth formed around the point of inoculation was measured. Twitching motility (pilus-directed movement onto solid surfaces) was observed by inoculating the bottom of the twitching agar plate with sterile needle. After incubation, the agar was removed, plates were stained with 0.1 % crystal violet and zones of motility were recorded in mm.

2.5. Slime production assay

Effect of phytochemicals on slime production ability of strains was tested using Congo red medium according to (Rajkumar et al., 2016). The colonies from the agar growth were inoculated in the Congo red broth containing 1/2 × MIC concentrations of phytochemicals. The Eppendorf were incubated at 37 °C for 24 h. The bacteria producing slime layer showed black color with Congo red, whereas non-& weak slime producers gave brown, reddish pink and cream colors with Congo red.

2.6. Autoaggregation assay

$$Percentage\ \ Inhibition = \frac{O.D(Maximum\ \ Quorum\ \ Sensing) - O.D.(Phytochemical\ \ Treated)}{O.D.(Maximum\ \ Quorum\ \ Sensing)} \times 100$$

Auto-aggregation assay was performed to check the aggregate forming ability of different cells of the same specific strain (Ciandrini et al., 2017). The strains P6 and P8 were grown in L- broth at 37 °C for 24 h. After incubation, the bacterial cells were harvested by centrifugation at 6,000 × g for 20 min. The pellets of both strains were separately re-suspended in distilled water and OD time zero was measured at 600 nm (OD at t_0). After incubation of 90 min, OD_{600nm} was measured for each strain (OD at t_{90}). The %age auto-aggregation was calculated with the help of the following formula:

$$\%\mathbf{auto-aggregation} = \frac{[(\boldsymbol{OD\ \ at\ \ t_0}) - (\boldsymbol{OD\ \ at\ \ t_{90}})]}{(\boldsymbol{OD\ \ at\ \ t_0})} \times 100$$

2.7. Bacterial adherence to hydrocarbons (BATH) test

BATH test was performed for investigating the hydrophobicity of the strains in the presence and absence of phytochemicals (Subbiahdoss and Reimhult, 2020). The strains P6 and P8 were grown in L-broth at 37 °C for 24 h. After incubation, the cultures were centrifuged for ten minutes (6000 × g), supernatant was discarded, the pellets were washed (3 ×) with phosphate buffer saline (PBS) and finally resuspended in the same buffer and OD was adjusted to 0.5 (A_0) at 600 nm. Xylene (400 µL) was added to 4 ml of standardized bacterial suspension and was placed in water bath at 25 °C for 10 min. The tubes were left at room temperature for 15–20 min for phase separation and absorbance of the lower aqueous phase was recorded (A_1). The percentage of hydrophobicity was measured with the help of following formula:

$$\%\mathbf{adherence} = \frac{[(\boldsymbol{A_0}) - (\boldsymbol{A_1})]}{(\boldsymbol{A_0})} \times 100$$

The strains were assessed as follows:

Strongly hydrophobic > 50 %; Moderately hydrophobic 20–50 %; Hydrophilic < 20 %.

2.8. Quorum sensing inhibition assay

Quorum sensing inhibition assay was performed for both strains (Thakur et al., 2016). The bacterial isolates were grown with/without 100 µl of phytochemicals (1/2 × MIC) overnight in sterile Muller-Hinton broth (5 ml) at 37 °C. The culture (1.5 ml) was centrifuged at 10,000 × g for 15 min, pellets were discarded, and the procedure was repeated thrice. The supernatants were filtered, and filtrate was mixed with ethyl acetate and vortex for 10 min. The organic (upper) and aqueous (lower) immiscible phases are separated in a separating funnel. Organic layers were pooled and dried in an oven at 40 °C for 15 min and aliquot (40 µl) was inoculated into wells of 96-well polystyrene microtiter plate. Then 50 µl [1:1 mixture of hydroxyl amine (2 M) and NaOH (3.5 M)] were added in the well. Next, 50 µl of 1:1 mixture of ferric chloride (10 % in 4 M HCl) and 95 % ethanol was also supplemented to the well and O.D. was taken at 520 nm. A dark brown color was indicative of lactone compounds, while yellow or no color indicated that no or weak lactones were found in the samples. The culture supernatant having no phytochemical was considered as the maximum level of quorum sensing (Negative Control). Percentage quorum sensing inhibition was calculated using following equation:

2.9. Microtiter plate assay for biofilm formation

Biofilm formation on microtiter plate was performed by inoculating the microtiter wells with bacterial strains with and without phytochemicals (1/2 × MIC) (Haney et al., 2021). The strains were grown in LB broth at 37 °C for 24 h. After incubation, the OD of the cultures was

adjusted to 0.5 at 600 nm. In 96 well −microtiter plates, 200 µl of phytochemicals and cell suspension was added. LB broth, LB + phytochemical and LB + strains were used as controls. The plates were incubated at 37 °C for 2, 4 and 6 days. After incubation, the growth medium was discarded, wells were washed twice with 200 µl of 0.85 % NaCl and air dried for 30 min. After that, 200 µl of 100 % methanol per well was added as fixative. Methanol was discarded after 15 min and plates were air dried for 10 min. The bound cells were stained with 200 µl of 0.1 % (v/v) crystal violet (CV) solution for 10 min at room temperature. Excess dye was removed by washing each well (3 ×) with 200 µl of 0.85 % NaCl. The quantification of attached cells was performed by adding 200 µl of 33 % (v/v) glacial acetic acid as a CV solvent. O.D at 578 nm of dissolved CV was measured in a microtiter plate reader.

2.10. Statistical analysis

All the tests were performed in triplicate and results were presented as mean ± S.D. All the tests were analyzed using One Way ANOVA. P values ≤ 0.05 were considered significant. Correlation analysis of all the parameters with biofilm formation was also checked. R values between 0.51 and 0.64 were considered significant.

2.11. In-silico study

Three-dimensional structure of quorum sensing proteins of *P. aeruginosa* LasI (PDB ID: 1RO5) synthase and LasR (PDB ID: 2UV0) were retrieved from Protein Data Bank online database (https://www.rcsb.org) and downloaded in PDB format.

However, the 3D structure of the protein RhlI synthase and RhlR was not available on PDB, so the homology modeling of the protein structures was performed. The amino acid sequences of RhlI synthase and RhlR were obtained from NCBI and used for modeling. The online tool SWISS-MODEL (https://swissmodel.expasy.org) was used for homology modeling.

2.12. Visualization of 3D protein structures

3D structures of proteins were visualized using PyMOL. It is used for graphical representation of proteins structures and to determine the active site involved in protein ligand binding.

2.13. Retrieval of phytochemicals

All the phytochemicals were retrieved in 3D form from the online database NCBI Pubchem. The structures obtained from Pubchem were in SDF or JSON format which were converted into PDB format using PyMOL.

2.14. Molecular Docking

The phytochemicals (natural ligands) in PDB format were converted into PDBQT format using Autodock tools. For proteins, first already bounded ligand was removed from protein in PyMOL and then saved in PDB format. Then using Autodock tools, the water molecules were removed and polar hydrogens were added to proteins. These modified protein structures were then saved in PDBQT format. After the conversion of proteins and ligands into PDBQT format, the active sites were located in protein molecules using Autodock tools. Proteins with attached synthetic inhibitor were selected and opened in Autodock tools and Grid box was created around the inhibitor and PDBQT file was saved. The docking between protein active site and natural ligands was performed using Autodock vina. The configuration files of natural ligands were generated in which the receptor protein and ligand were mentioned as well as the location and size of active sites in xyz plane. The results of their docking were obtained using command prompt and recorded as log.txt files. The results of protein and ligand docking were visualized using PyMOL. The docking results of commonly used inhibitor 6-gingerol was also recorded and their affinities were compared to that of phyto-ligands. The amino acid residues of phyto-ligands involved in interaction and binding with protein active site were determined and visualized using discovery studio (DS). The amino acid interactions were presented in the form of 2-D diagrams.

3. Results

3.1. Minimum inhibitory concentration

Almost similar MIC values of all the phytochemicals against two strains were observed as shown in Table 1. The MIC values were in the range of 95 µg/ml to 1050 µg/ml. The lowest MIC value was of isoliquiritin being 95 µg/ml against P6 and 120 µg/ml against the strain P8. Piperine showed the highest MIC value of 900 and 1050 µg/ml against the strains P6 and P8, respectively. 6-gingerol which is a known quorum sensing inhibitor showed the MIC values of 170 and 180 µg/ml against the strains P6 and P8, respectively.

3.2. Slime production assay

Slime production from the strains P8 and P6 was assayed, and both the strains were found to be good slime producers as they gave black color with Congo red. While in the presence of phytochemicals, different colors like brown, reddish pink and cream were observed. In both strains, an almost similar trend was observed. All the phytochemicals were shown to affect the slime production ability of strains except acetyl aleuritic acid and spermidine (Sup. Table 1).

With Isoliquirtin, eugenol, epigallocatechin gallate and luteolin, both strains showed reddish pink color with Congo red. In the presence of emodin, anthraquinone, chrysin and allicin, reddish pink color was observed with both strains. Both strains showed light brown color in the presence of Piperine, diosgenin, caryophylline and artemisin (Sup. Table 1).

3.3. Motility assay

Both the *Pseudomonas* strains P8 and P6 showed significant swimming, swarming and twitching motilities in the absence of phytochemicals. The zones of swimming, swarming and twitching motilities in P8 were bigger as compared to that of the strain P6.

In swimming motility assay, control plates without phytochemicals, the strain P8 showed 20 mm zone of motility. 6 gingerol, a known QS inhibitor was used as a positive control. In its presence, motility of the

Table 1
Minimum inhibitory concentrations of phytochemicals against strains P8 and P6.

No. of obs.	Phytochemicals	Minimum inhibitory concentration (MIC) µg/ml	
		P6	P8
1	Diosgenin	70	80
2	Piperine	900	1050
3	Emodin	130	150
4	Epigallocatechin gallate	495	500
5	Anthraquinone	150	170
6	Caryophylline	200	220
7	Luteolin	80	90
8	Eugenol	160	180
9	Chrysin	90	100
10	Artemesin	390	410
11	Allicin	180	195
12	Acetyl aleuritic acid	500	550
13	Isoliquirtin	95	120
14	Spermidine	650	680
15	6-gingerol	170	180

strain P8 was impaired as indicated by the zone (15 mm). Three phytochemicals *i.e.,* emodin. diosgenin and anthraquinone reduced the swimming motility and showed zones like 6-gingerol (15 mm). Isoliquirtin inhibited up to 65 % motility (3 mm) which is greater as compared to gingerol (25 %), followed by Epigallocatechin gallate EGCG (60 % reduced motility), Luteolin and Eugenol (both showed 50 % reduction) and chrysin (45 %). Allicin (15 %), piperine (10 %) and artemisin (10 %), also inhibited swimming motility in the strain P8 but not more than gingerol. In the case of caryophylline, acetylaleuritic acid and spermidine, no change in motility zone was observed *i.e.,* they didn't affect the swimming motility. The strain P6 showed the 18 mm zone in the absence of phytochemicals. Isoliquirtin and EGCG both showed 61 % inhibition of swimming motility in the strain P6, followed by Eugenol (55.5 %), luteolin (55.5 %), Chrysin (50 %) and Allicin (17 %). Spermidine, caryophylline and acetyl aleuritic acid showed no change in motility (Fig. 2).

In the Swarming motility assay, the strain P8 showed 40 mm zone on swarming agar plate without phytochemicals. An almost similar trend of motility reduction was observed as in swimming motility assay. Caryophylline, acetyl aleuritic acid and spermidine didn't show any

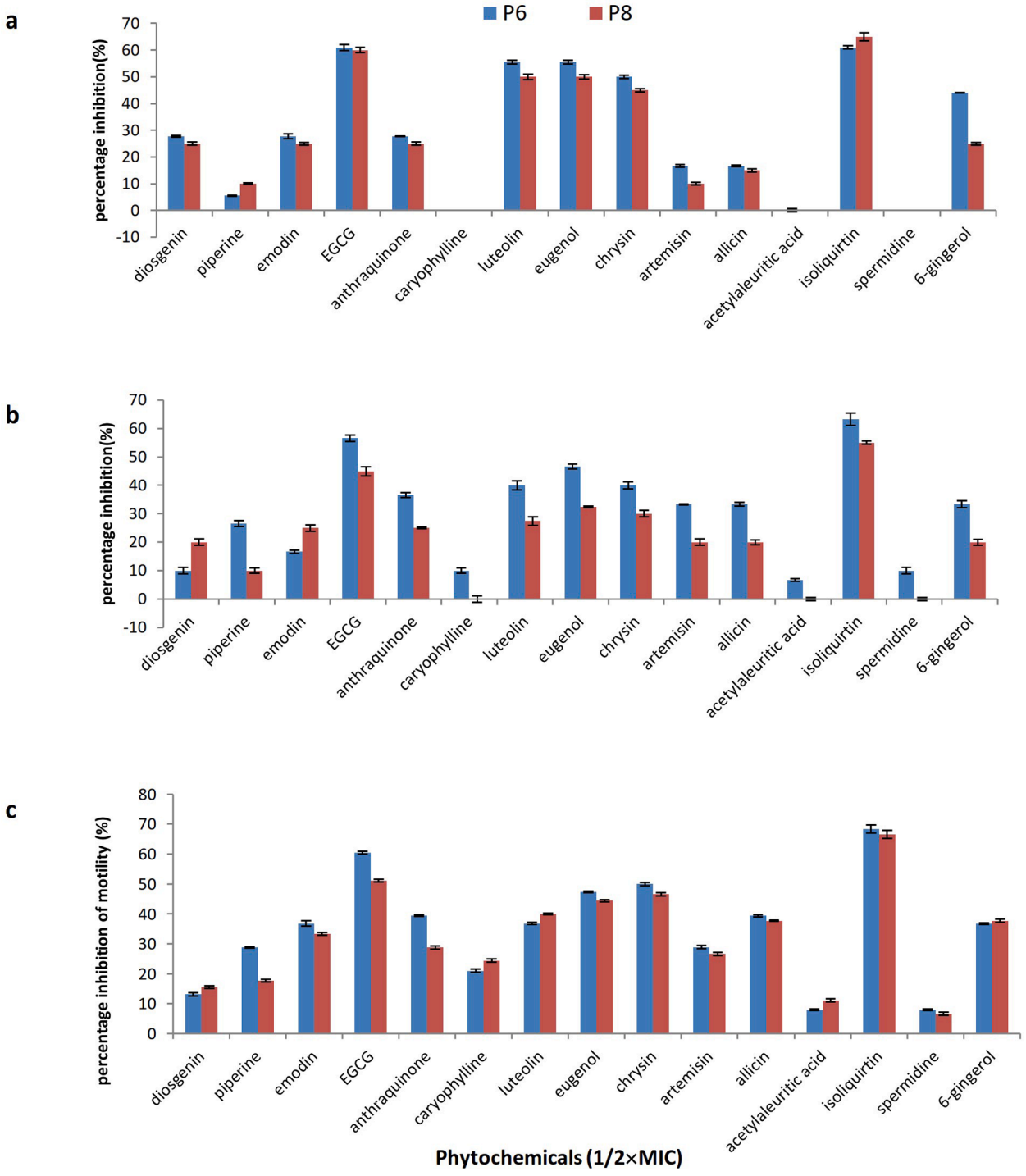

Fig. 2. Percentage inhibition of (a) swimming, (b) swarming and (c) twitching motilities in strains P8 and P6. Results are representative of three independent experiments. Error bars indicate standard deviations (mean ± SD).

reduction in swarming motility. In the presence of 6- gingerol, P8 showed 20 % reduced motility. Isoliquirtin reduced the swarming motility by 55 %, followed by EGCG (45 %) and Eugenol (32.5 %). Chrysin inhibited motility by 30 % followed by luteolin (27.5 %), emodin (25 %) and anthraquinone (25 %). In the case of artemisin, diosgenin and allicin, similar zones were observed as that of gingerol. The strain P6 showed 30 mm zone of swarming motility without any phytochemical. With gingerol, the strain P6 showed 33 % reduced swarming motility. Isoliquiritin showed 63 % reduction in swarming motility of P6 followed by EGCG (56.5 %), eugenol (46.6 %), luteolin (40 %) and chrysin (40 %) (Fig. 2b).

The strain P8 showed 45 mm zone of twitching motility without any phytochemical which is bigger than that of P6 (38 mm). With Gingerol, the strain P8 showed 28 mm zone of twitching motility. All the phytochemicals, except acetylaleuritic acid, caryophylline and spermidine, showed inhibition of twitching motility of the strain P8. Isoliquirtin was shown to inhibit twitching motility by 66.6 % which is far greater inhibition as compared to gingerol (38 %). EGCG also showed significant reduction (51 %), followed by chrysin (46.6 %), eugenol (44.4 %) and luteolin (40 %). In the case of the strain P6, all the phytochemicals were shown to inhibit the twitching motility. Isoliquirtin showed 68.4 % reduction, followed by EGCG (60.5 %), chrysin (50 %), eugenol (47.3 %) and anthraquinone (39 %). Luteolin and emodin showed similar reduction in twitching motility as that of gingerol (36.8 %) (Fig. 2c).

3.4. Bacterial adherence to hydrocarbons (BATH) test

In order to investigate the hydrophobicity of strains with and without phytochemicals, BATH test was performed by using xylene as hydrocarbon (Subbiahdoss and Reimhult, 2020). Both the strains were strongly hydrophobic in nature as indicated in Table 2.

In the presence of gingerol, strain P6 showed 34 % hydrophobicity. In the presence of Isoliquirtin, strain P6 showed 12 % hydrophobicity, followed by EGCG (16 %). With all other phytochemicals, the strain P6 falls in the range of 20–50 % hydrophobicity. Diosgenin (62 %) and spermidine (54 %) slightly change the hydrophobicity of the strain P6 (Table 2).

The strain P8 showed 36 % hydrophobicity with 6-gingerol. Six phytochemicals displayed affecting the hydrophobicity of the strain P8 more as compared to gingerol. With Isoliquirtin and Eugenol, the strain P8 showed 16 % and 18 % hydrophobicity which converted the strain from strongly hydrophobic to hydrophilic. With EGCG, chrysin, allicin and luteolin, the strain P8 showed 20 %, 22 %, 22 % and 26 % hydrophobicity respectively. However, spermidine didn't affect the hydrophobicity of the strain P8 (Table 2).

Table 2
Percentage Hydrophobicity and autoaggregation of P8 and P6 in the absence and presence of phytochemicals (1/2 × MIC).

No. of obs.	Phytochemicals	Hydrophobicity (%)		Autoaggregation (%)	
		P6	P8	P8	P6
1	Control (without phytochemical)	68 ± 0.58	60 ± 0.66	21 ± 0.59	19.8 ± 0.65
2	Diosgenin	62 ± 0.11	54 ± 0.67	13.67 ± 0.20	15.5 ± 0.17
3	Piperine	40 ± 0.42	36 ± 0.5	14.2 ± 0.15	15.5 ± 0.75
4	Emodin	44 ± 0.27	50 ± 0.77	14.4 ± 0.25	14.7 ± 0.40
5	Epigallocatechin gallate	16 ± 0.56	20 ± 1.51	7.69 ± 0.70	6.79 ± 0.36
6	Anthraquinone	50 ± 0.55	42 ± 0.5	12.8 ± 0.55	12.7 ± 0.59
7	Caryophylline	46 ± 0.40	46 ± 0.25	15.5 ± 0.35	13.7 ± 0.11
8	Luteolin	30 ± 1.21	26 ± 0.41	8.47 ± 0.92	8.65 ± 0.65
9	Eugenol	20 ± 0.92	18 ± 0.61	8.6 ± 0.40	9.7Z ± 0.50
10	Chrysin	20 ± 0.77	22 ± 0.60	7.76 ± 1.00	8.65 ± 0.65
11	Artemesin	46 ± 0.35	56 ± 0.17	14.4 ± 0.3	14.2 ± 0.44
12	Allicin	24 ± 0.90	22 ± 0.26	9.32 ± 0.11	11.42 ± 0.11
13	Acetyl aleuritic acid	50 ± 0.72	48 ± 1.52	20.3 ± 0.37	16.5 ± 0.45
14	Isoliquirtin	12 ± 1.08	16 ± 0.60	5.88 ± 0.75	4.8 ± 0.90
15	Spermidine	54 ± 0.36	60 ± 0	15.3 ± 0.45	19.04 ± 0.15
16	6-gingerol	34 ± 0.86	36 ± 0.5	12.17 ± 0.12	10.8 ± 0.73

All the values represents means of triplicates (n = 3) ± standard deviations.

3.5. Auto-aggregation assay

Auto-aggregation, the ability of forming aggregates by different cells of the same strain was analyzed in the absence and presence of all the phytochemicals. The results indicated that percentage aggregation was 21 % in control tubes having no phytochemical. In tubes having gingerol, the strain P8 showed an aggregation of 12.7 %. With the eight phytochemicals, the strain P8 formed less aggregates as compared to that with gingerol. In the presence of isoliquirtin, the strain P8 showed 6 % aggregates, followed by EGCG (7.6 %). Autoaggregation of the strain P8 in chrysin was 8 %, followed by luteolin (8.5 %), eugenol (8.6 %), allicin 9 %, caryophylline 10 %, anthraquinone 12.8 %, and diosgenin 13.6 %. In the presence of Acetyl aleuritic acid, 22.03 % autoaggregates were formed by the strain P8, which was least efficient in inhibiting aggregates formation (Table 2).

In the case of the strain P6, 19.8 % auto aggregates were formed in control tubes having no phytochemicals. With Gingerol, the strain P6 showed 10.8 % aggregates. In Isoliquiritin, 4.8 % autoaggregation was observed followed by 6.79 % in EGCG, 8.65 % each in chrysin and luteolin. Spermidine (19 %) showed least effect on aggregates forming ability of the strain P6 (Table 2).

Table 3
AHL quantification of P6 and P8 in the absence and presence of Phytochemicals (1/2 × MIC).

	Phytochemicals	Absorbance at 520 nm (P8)	Color	Absorbance at 520 nm (P6)	Color
1	Control (without phytochemical)	1.33 ± 0.04	Brown	1.91 ± 0.02	Brown
2	Diosgenin	1.04 ± 0.41	Brown	1.01 ± 0.58	Brown
3	Piperine	1.05 ± 0.43	Brown	1.04 ± 0.60	Brown
4	Emodin	0.99 ± 0.38	Brown	1.01 ± 0.25	Brown
5	Epigallocatechin gallate	0.52 ± 0.33	Yellow	0.37 ± 0.23	Yellow
6	Anthraquinone	1.01 ± 0.43	Brown	0.99 ± 0.67	Brown
7	Caryophylline	1.04 ± 0.15	Brown	1.03 ± 0.91	Brown
8	Luteolin	0.67 ± 0.58	Yellow	0.65 ± 0.37	Yellow
9	Eugenol	0.78 ± 0.36	Yellow	0.39 ± 0.92	Yellow
10	Chrysin	0.59 ± 0.34	Yellow	0.44 ± 0.21	Yellow
11	Artemesin	1.01 ± 0.21	Brown	1.02 ± 1.4	Brown
12	Allicin	0.99 ± 0.00	Brown	1.04 ± 0.21	Brown
13	Acetyl aleuritic acid	1.14 ± 0.5	Brown	1.74 ± 1.04	Brown
14	Isoliquirtin	0.49 ± 0.35	Yellow	0.32 ± 0.98	Yellow
15	Spermidine	1.17 ± 0.09	Brown	1.32 ± 0.36	Brown
16	6-gingerol	0.69 ± 0.51	Yellow	0.59 ± 0.4	Yellow

All the values represents means of triplicates (n = 3) ± standard deviations.

3.6. Quorum sensing inhibition

For assessment of quorum sensing inhibition by phytochemicals, AHL from both strains was quantified in the absence and presence of ½ × MIC values of phytochemicals. It is a colorimetric test in which dark brown color indicated the presence of AHL, and yellow or no color indicated the absence of AHL. The O.D $\leq$ 0.98 was considered as a threshold value. Above this threshold value, AHL concentration was considered significant. The value below or equal to this threshold was indicative of no or weak AHL.

In both strains, AHL was produced in high quantity in the absence of phytochemicals as indicated by dark brown coloration. In 6-gingerol, P8 showed O.D of 0.69 and yellow color which represented no or weak AHL. With isoliquiritin, P8 showed O.D value of 0.49, followed by EGCG (0.52), chrysin (0.59) and luteolin (0.67) and eugenol (0.78) (Table 3; Suppl. Fig. 1). In strain P6, a similar trend of AHL inhibition was observed (Table 3).

Table 4
Microtiter assay for biofilm formation of P8 and P6 at 2, 4 and 6 days in the absence and presence of Phytochemicals.

	Phytochemicals	Absorbance at 546 nm					
		P6			P8		
		2 days	4 days	6 days	2 days	4 days	6 days
1	Control (without phytochemical)	± 0.02	± 0.01	± 0.09	± 0.02	± 0.03	± 0.20
2	Diosgenin	1.04 ± 0.02	1.08 ± 0.30	1.01 ± 0.08	1.13 ± 0.01	1.08 ± 0.005	1.01 ± 0.06
3	Piperine	1.01 ± 0.29	1.21 ± 0.49	1.00 ± 0.09	1.00 ± 0.005	0.98 ± 0.01	0.96 ± 0.01
4	Emodin	1.03 ± 0.08	1.09 ± 0.11	1.03 ± 0.01	1.13 ± 0.005	1.09 ± 0.04	1.04 ± 0.005
5	Epigallocatechin gallate	0.62 ± 0.36	0.67 ± 0.17	0.51 ± 0.08	0.79 ± 0.01	0.70 ± 0.01	0.59 ± 0.20
6	Anthraquinone	0.73 ± 0.04	0.75 ± 0.02	0.71 ± 0.24	1.12 ± 0.02	1.11 ± 0.005	1.06 ± 0.03
7	Caryophylline	1.12 ± 0.12	1.21 ± 0.32	1.01 ± 0.19	1.19 ± 0.01	1.15 ± 0.01	1.03 ± 0.01
8	Luteolin	0.64 ± 0.43	0.68 ± 0.08	0.59 ± 0.19	0.82 ± 0.05	0.74 ± 0.005	0.64 ± 0.03
9	Eugenol	0.62 ± 0.02	0.69 ± 0.70	0.57 ± 0.11	0.80 ± 0.01	0.77 ± 0.01	0.61 ± 0.03
10	Chrysin	0.67 ± 0.06	0.69 ± 0.09	0.59 ± 0.19	0.84 ± 0.01	0.74 ± 0.05	0.67 ± 0.27
11	Artemesin	1.03 ± 0.03	1.14 ± 0.49	0.97 ± 0.16	1.21 ± 0.01	1.17 ± 0.11	1.02 ± 0.01
12	Allicin	0.91 ± 0.43	1.01 ± 0.16	0.84 ± 0.06	0.96 ± 0.01	0.92 ± 0.05	0.87 ± 0.13
13	Acetyl aleuritic acid	1.00 ± 0.04	1.18 ± 0.02	1.06 ± 0.09	1.20 ± 0.005	1.17 ± 0.05	1.14 ± 0.04
14	Isoliquirtin	0.58 ± 0.03	0.61 ± 0.06	0.56 ± 0.07	0.75 ± 0.03	0.69 ± 0.05	0.58 ± 0.03
15	Spermidine	1.03 ± 0.44	1.10 ± 0.37	0.99 ± 0.09	1.01 ± 0.03	0.99 ± 0.01	0.96 ± 0.01
16	6-gingerol	0.69 ± 0.49	0.71 ± 0.47	0.60 ± 0.33	0.83 ± 0.005	0.71 ± 0.03	0.68 ± 0.53

All the values represents means of triplicates (n = 3) ± standard deviations.

3.7. Biofilm formation on microtiter plate

Biofilm forming ability of the strains P8 and P6 was observed on microtiter plate for 2, 4 and 6 days in the absence and presence of phytochemicals. In strain P6, thick biofilm was observed after 2 days and it got more thickened after 4 days. After 6 days, a decrease in biofilm was detected. The same pattern of increased biofilm till 4 days and decrease afterwards was observed with all the phytochemicals. With isoliquiritin, biofilm formed after 2 days was thinner as compared to that of control and known inhibitor 6-gingerol. The biofilm was increased a little after 4 days but again started deteriorating after 6 days. EGCG, Eugenol, Chrysin, Luteolin showed similar pattern as was detected in isoliquirtin. These 4 phytochemicals were shown to affect the biofilm forming ability of the strain P6 even more than that of 6-gingerol (Table 4).

In strain P8, thick biofilm was developed at 2, 4 and 6 days and no decline in pattern was observed at any day in the absence of phytochemicals. P8 formed a more profound biofilm as compared to that of the strain P6. All the phytochemicals inhibited biofilm forming ability of strains when observed after 2, 4 and 6 days. But their optical densities were more as compared to P6 even after 6th day which showed their resistance towards phytochemicals. Isoliquirtin, EGCG, eugenol and luteolin all inhibited biofilm formation of P8 at all days more efficiently as compared to gingerol (Table 3).

3.8. Molecular docking

The phytochemicals which showed efficient AHL inhibition in in-vitro testing were selected and docked with quorum sensing proteins LasI, Las R, Rhl I and RhlR as shown in table (Table 5). All the 5 phytoligands were shown to bind efficiently with all four QS proteins tested. EGCG binds efficiently with all the four QS proteins and showed higher binding energy values when docked with LasI (−7.0 kcal/mol) and RhlR (−6.9 kcal/mol) than that of gingerol. Luteolin showed higher binding energy values with all QS proteins when comparing with gingerol. It showed strong binding with Las R protein (−10.6 kcal/mol). In the case of eugenol, higher binding energy was observed with LasI (−4.3) and RhlR (−5.1) than that of gingerol. Chrysin showed the highest binding energy value of −11 kcal/mol when docked with LasR. Isoliquiritin showed higher energy values than that of gingerol in all the three proteins except when docked with LasR (Table 5). Their percentage binding energies were calculated by using 6-gingerol as control (Table 5).

4. Discussion

P. aeruginosa is a Gram-negative opportunistic pathogen that causes deadly infections in immunocompromised individuals and burn patients. Due to increasing antibiotic resistance of *Pseudomonas* infections, it has arisen as a threat to public health worldwide. Consequently, it is vital to develop a new line of attack for the treatment of MDR infection of P. aeruginosa. In P. aeruginosa, the quorum sensing (QS) mechanism plays a principal role in its infection by regulating the biofilm formation and the secretion of virulence factors (Sırıken et al., 2021).

In this study, we worked on two metallo-β-lactamase (MBL) producing *Pseudomonas aeruginosa* strains P6 (Accession no. MT277092) and P8 (Accession no.MT277358), previously isolated from urine and pus sample respectively. These strains were previously checked and confirmed to be resistant to the last class of antibiotics *ie,* Carbapenems. Considering the significant role of Quorum Sensing in bacterial resistance, we targeted the Quorum sensing mechanism (QS) of these bacteria to combat antibiotic resistance. Our study aimed at using compounds from plants which have been used since centuries in our herbal medicine to treat various infections and diseases so we selected fifteen commercially available phytochemicals including alkaloids, flavonoids, catechins, sapogenins, phenols and polyamines to check their effects on biofilm formation, quorum sensing and inter-related

Table 5
Binding energy values (kcal/mol) of phytoligands docked with QS proteins.

		lasI	% efficiency as compared to control (6-gingerol = −4.2)	LasR	% efficiency as compared to control (6-gingerol = −8.6)	rhlI	% efficiency as compared to control (6-gingerol = −7.3)	rhlR	% efficiency as compared to control (6-gingerol = −4.9)
1	Epigallocatechin gallate	−7.0	66.6 %	−7.4	−13.95	−6.9	−5.4	−6.9	40.81
2	Luteolin	−6.3	50 %	−10.6	23.2 %	−8.1	10.9 %	−5.9	20.40
3	Eugenol	−4.3	2.3 %	−6.7	−22.09 %	−6	−17.8	−5.1	4.08
4	Chrysin	−5.9	40.47 %	−11	27.9 %	−8	9.5 %	−6.2	26.53 %
5	Isoliquirtin	−4.9	16.6 %	−5.6	−34.88 %	−8.4	15.06	−5.4	10.20

mechanisms like motility, adhesion, hydrophobicity and aggregation of MBL *P.aeruginosa* (Jubair et al., 2021).

MIC is the lowest concentration of any phytochemical and or antimicrobial agent which inhibits the growth of bacterial strain. In our study we have found a diverse range of MIC of phytochemicals, isoliquiritin having the lowest and piperine having the highest MIC values against both strains P8 and P6. P8 strain found out to be more resilient than strain P6 as indicated by their higher MIC values. Moreover, the strain P8 was resistant to both meropenem and imipenem. But the strain P6 was resistant to imipenem only. Resistance of P8 towards antibiotic and phytochemicals might be due to its origin/source of isolation *i.e.,* pus. In previous studies, it has been reported that strains isolated from pus samples are more resistant to antimicrobial agents than that from other sources (Paudel et al., 2021).

The results of swimming, swarming and twitching motilities indicated that isoliquiritin, EGCG, luteolin, eugenol and chrysin inhibited motility in both strains of *P. aeruginosa* more efficiently than that of 6-gingerol (ANOVA; p value = <0.05). Bacterial motility facilitates bacterial adhesion and biofilm formation. Swimming is flagellated movement which is required in initial attachment of bacterial cells to develop biofilms. Swarming influences biofilm structural architecture. Twitching, a pilus-mediated motility is also involved in biofilm development and facilitates the active expansion of biofilms across surfaces. Phytochemical Inhibition of swimming, swarming and twitching motilities inhibited different stages of biofilm formation. Our results are in accordance with previously reported studies that phytochemicals have the ability to inhibit motility and thus affecting biofilm attachment, maturation and dispersal (Hao et al., 2021).

Slime production test indicated the two strains as good slime producers as they give black color with Congo red. In the presence of phytochemicals, different colors like brown, reddish pink and cream were observed. Only spermidine and acetyl aleuritic acid didn't affect the slime production ability of both strains. Slime production is a key factor in bacterial adhesion to solid surfaces. The slime layer is polysaccharide in nature which gives black color with Congo red (CR). The presence of mucoid red, pink, or cream colors indicates weak slime producers. This color change is pH & temperature dependent. The phytochemicals alter the pH of the medium and thus affect the structure of proteins. Hence, by altering the structure, the binding affinity of proteins with Congo red got impaired, responsible for color change in Congo red assay (Lee et al., 2016).

The results of autoaggregation and hydrophobicity revealed that five phytochemicals *i.e.,* isoliquiritin, eugenol, epigallocatechin gallate, luteolin and chrysin significantly affected aggregates formation and hydrophobicity in both P8 and P6 as compared to that of 6-gingerol as shown in Table 2 (ANOVA; p value=<0.05). Allicin mimics 6-gingerol in inhibiting aggregate formation in both strains but affected hydrophobicity more than that of gingerol (Table 2).

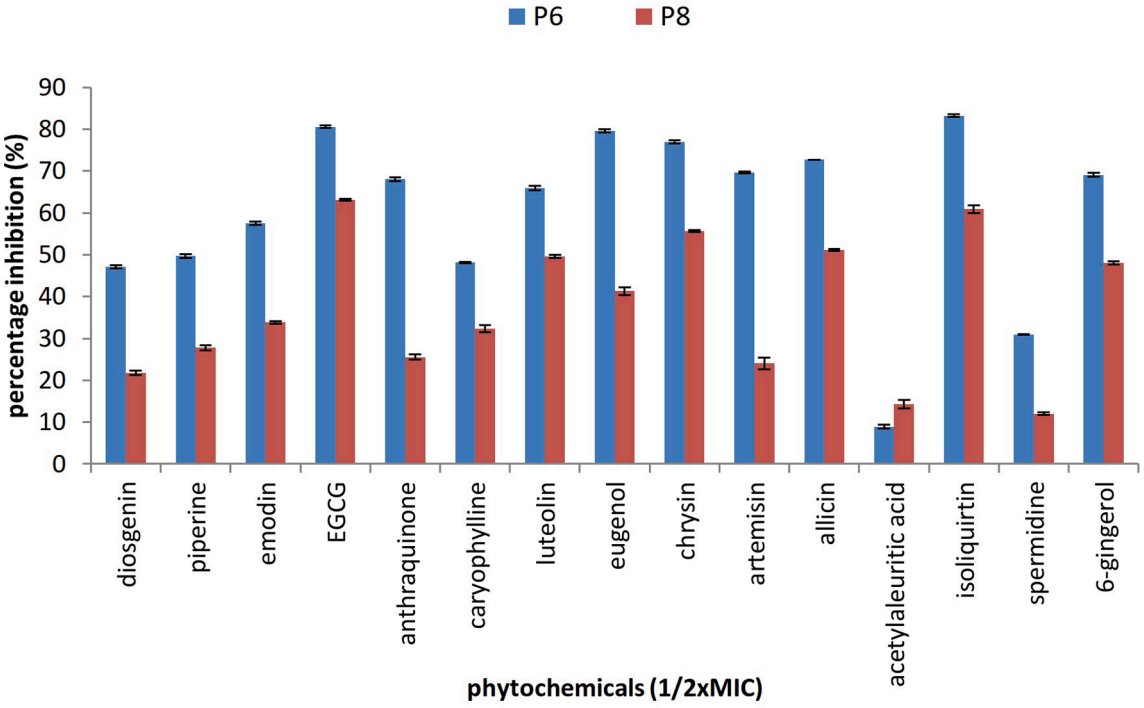

Fig. 3. Percentage inhibition of quorum sensing (QS) in P8 and P6 in the presence of phytochemicals (1/2 × MIC). Results are representative of three independent experiments. Error bars indicate standard deviation (±SD).

The effect of phytochemicals on quorum sensing in both strains was investigated by quantification of AHL. In both the strains P8 and P6 AHL was produced in high quantity in the absence of phytochemicals as indicated by dark brown coloration and O.D above 0.98. Isoliquiritin, EGCG, chrysin, luteolin and eugenol affected the AHL production in both the strains P8 and P6 as indicated by the yellow color wells in microtiter plate and O.D $\leq$ 0.98. Fig. 3 shows that AHL production in P8 was affected more severely than that of the strain P6 but the pattern of inhibition by phytochemicals is same in both strains. Gingerol-a known QS inhibitor inhibited AHL production up to 48 % in the strain P6 and 69 % in the strain P8. Isoliquiritin showed inhibition of 63 % in the strain P6 and 83 % in P8 followed by EGCG 61 % in the strain P6 and 81 % in the strain P8, chrysin 55 % in the strain P6 and 76 % in the strain P8, luteolin 50 % in the strain P6 and 65 % in the strain P8, eugenol 41 % in the strain P6 and 79 % in the strain P8.

Most Quorum Quenching phytochemicals have a heterocyclic ring structure resembling AHL molecules. This structural confirmation may allow for more stable interactions with QS receptors, as well as their potential to degrade signal receptors. Ajoene, allicin, and curcumin have similar structures to AHL side chains, but with differing oxygenation levels. In 6-gingerol, zingerone, eugenol, carvacrol, and cinnamaldehyde, the lactone ring is substituted by an aromatic moiety, making it difficult to open. A similar pattern may be found with naringin, quercetin, naringenin, vitexin, and baicalein, which all have a complex polycyclic structure. Furthermore, studies have showed that these phytochemicals efficiently interact with the various QS receptors of *P. aeruginosa* (Chadha et al., 2022).

However, interactions with bioactive phytochemicals have not been proven due to their non-proteinaceous nature. Nonetheless, multiple studies, both in vitro and in vivo, have found a significant correlation between molecular docking results and downregulation of key QS genes. Plant-based bioactives can now be used as anti-virulence medicines due to their versatility (Bose et al., 2021, Chadha et al., 2022).

Both the strains P6 and P8 were good biofilm formers but both showed different pattern of biofilm formation and deterioration when observed at 2, 4 and 6 days. The strain P6 formed thick biofilm and their biofilm increased with time. On the 6th day, a decrease in biofilm thickness was observed which indicated their dispersal. On the contrary, the strain P8 showed no detachment even after 6 days. With phytochemicals, the strain P6 showed a similar pattern of biofilm formation till day 4 and decrease afterwards, as shown in control. However, the strain P8 showed a decline pattern at all days when treated with phytochemicals. There are four main stages of biofilm formation: attachment, micro colony formation, maturation, and detachment/dispersal. Every microbe has its own rate of biofilm formation and detachment under different stresses (Coyte et al., 2017).

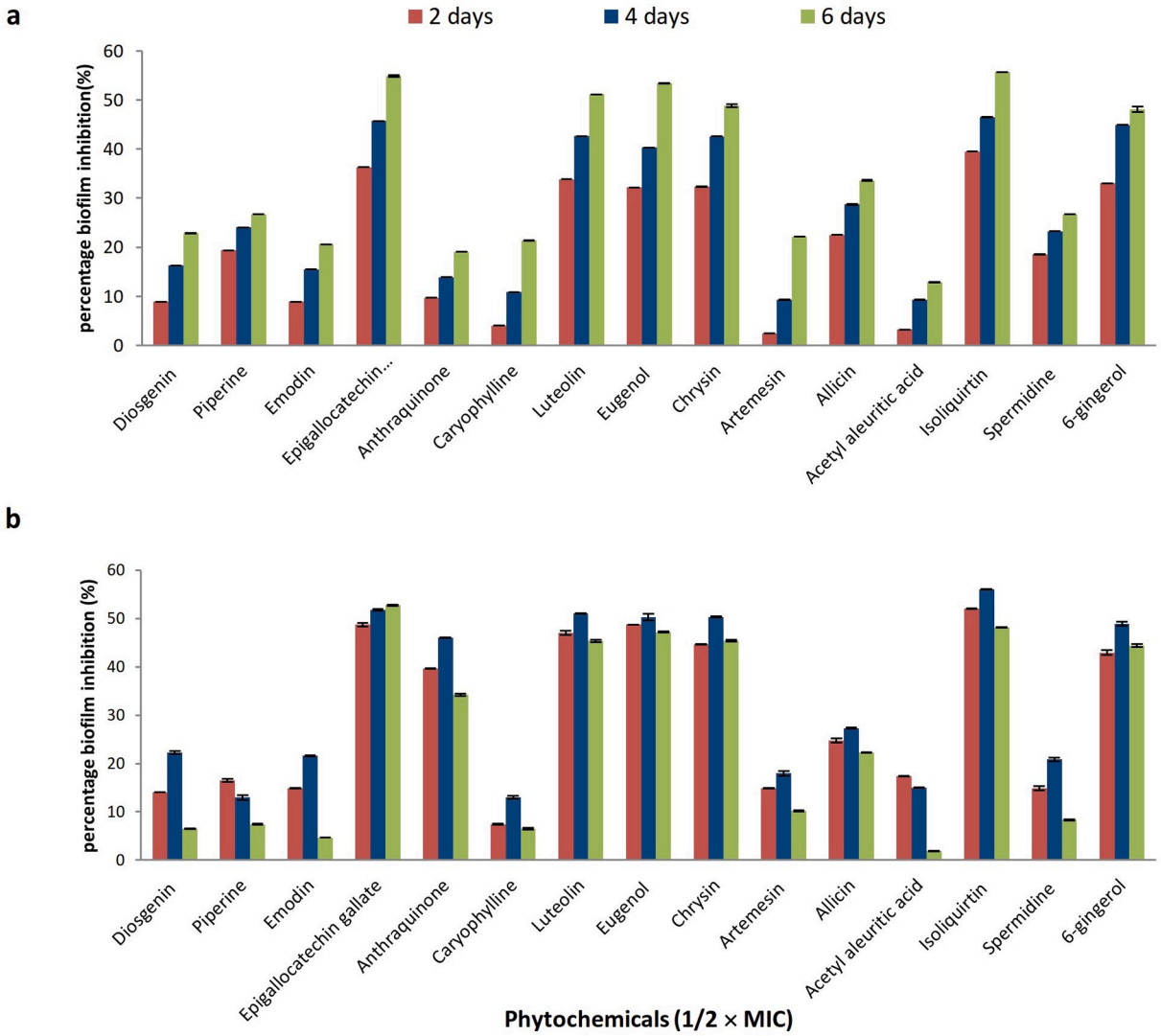

Fig. 4. Percentage inhibition of biofilm formation in P6 and P8 in the presence of phytochemicals (1/2 × MIC). Results are representative of three independent experiments. Error bars indicate standard deviations (±SD).

On day 2, percentage inhibition of biofilm of P8 in the presence of isoliquiritin was 39 % when comparing with gingerol (33 %), followed by EGCG (36 %), luteolin (34 %) and chrysin and eugenol (32 %). Isoliquiritin, EGCG and Luteolin were found to be more efficient inhibitors than gingerol. On day 4, isoliquiritin inhibited biofilm of the strain P8 about 46 %, EGCG 46 %, gingerol 44 %, luteolin and chrysin 43 % and eugenol 40 %. At day 6, percentage inhibition of biofilm of P8 in the presence of isoliquiritin was 56 %, followed by EGCG 55 %, eugenol 53 %, luteolin 51 % and chrysin 49 % which indicated these five phytochemicals to be more efficient inhibitors than gingerol (48 %) (Fig. 4).

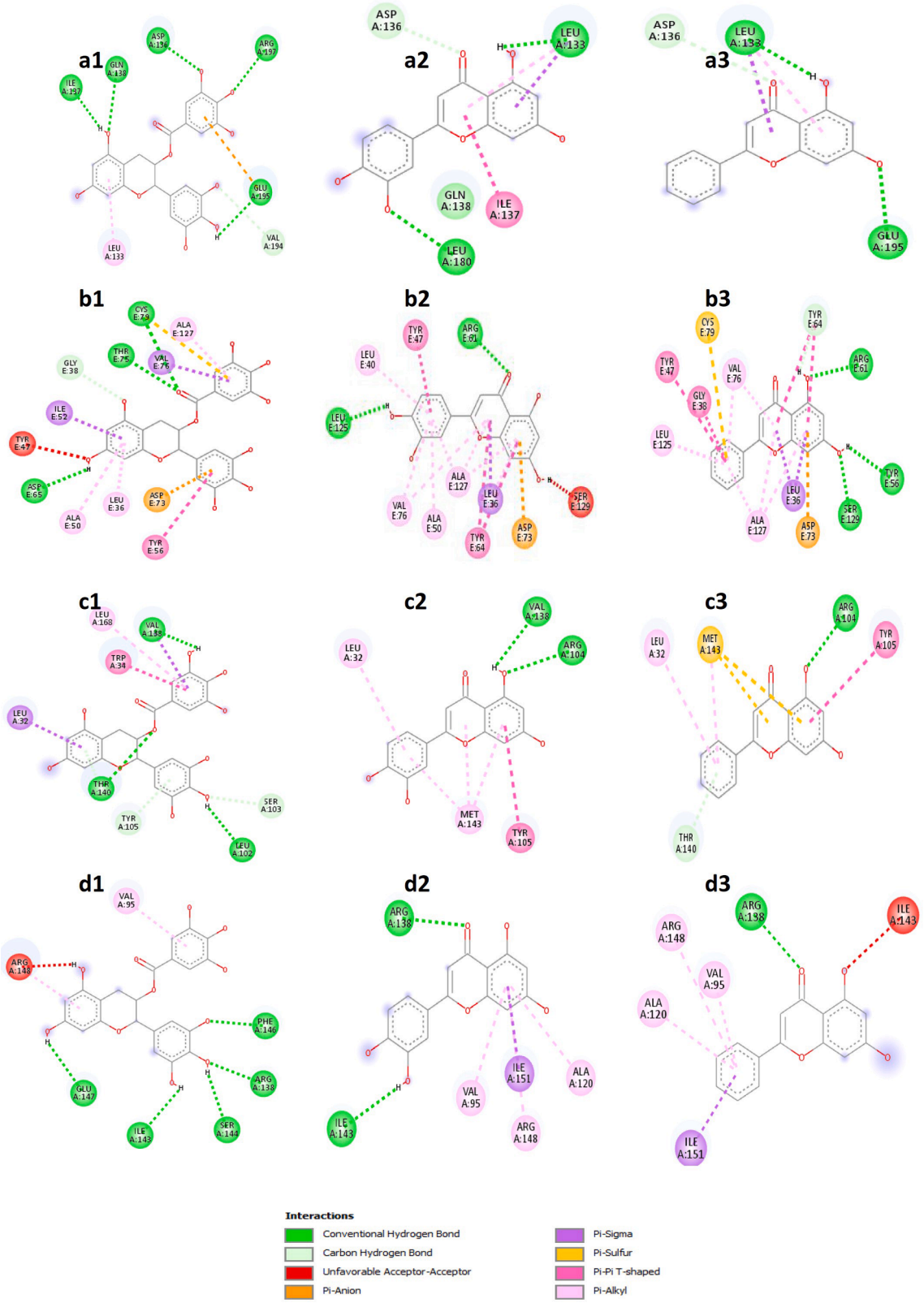

Fig. 5. Interaction of Phytoligands with amino acids of a) LasI, b) LasR, c) Rhl1, d) RhlR proteins with 1) Epigallocatechin gallate, 2) luteolin, 3) chrysin.

On day 2, the strain P6 showed 42 % biofilm inhibition when treated with gingerol as compared to control, 52 % inhibition with isoliquiritin, 49 % each with EGCG and eugenol, 47 % with Luteolin and 44 % with Chrysin. Anthraquinone (40 %) also inhibited biofilm of the strain P6. At day 4, gingerol inhibited biofilm of the strain P6 about 49 % when compared with control, isoliquiritin 56 %, EGCG 52 %, luteolin 51 %, Eugenol and Chrysin 50 % each, anthraquinone 46 %. At day 6, biofilm inhibition of the strain P6 with gingerol was 44 % when compared with control, EGCG 53 %, isoliquiritin 48 %, Eugenol 47 %, chrysin and luteolin 45 % each and anthraquinone 34 % (Fig. 4).

The correlation analysis indicated that all the parameters *ie,* motility (swimming, swarming, twitching), aggregation, hydrophobicity and quorum sensing are positively correlated with biofilm formation in both the strains P8 and P6. In the strain P8 swimming motility showed highly significant correlation with biofilm formation ($r = 0.81$, $N = 15$). Similarly, swarming motility ($r = 0.7$, $N = 15$), twitching motility ($r = 0.77$, $N = 15$), aggregation ($r = 0.86$, $N = 15$), hydrophobicity ($r = 0.85$, $N = 15$) and quorum sensing ($r = 0.83$, $N = 15$) also showed highly significant correlation with biofilm formation. Likewise, In the strain P6 all the parameters showed highly significant correlation with biofilm formation ($p \leq 0.05$).

Isoliquiritin is a flavonoid originating from liquorice. It is known for its variety of pharmacological effects such as inhibitory action against angiogenesis, depression and lipopolysaccharide induced inflammation. Its antifungal activities have also been reported (Luo et al., 2016). However, there is limited research on its antibacterial effects. In this study, we have found that sub-inhibitory concentrations of isoliquiritin have antibiofilm and anti-quorum sensing activity and inhibitory action on QS- regulated mechanisms like motility, adhesion, aggregation, and slime production. Moreover, the sub-inhibitory concentrations of Epigallocatechin gallate also proved to be promising candidate in inhibiting biofilm and quorum sensing activities of MBL producing *P. aeruginosa (*Serra et al., 2016*)*. Earlier research showed antimicrobial and antibiofilm activities of EGCG against *P. aeruginosa* isolated from skin wounds. One study reported that EGCG cause disruption of the QS system by amyloid remodeling and inhibits EPS production (Stenvang et al., 2016). Eugenol also reported to affect the synthesis of QS proteins which are involved in biofilm formation. These phytochemicals also affect the protein and carbohydrate content of EPS which is essential in forming biofilms (Lahiri et al., 2021). Chrysin is also a known flavone present in many plants and used in Chinese herbal medicine. Many previous studies had reported its antimicrobial, anti-inflammatory and anti-cancer activities (Bhowmik et al., 2022).

These five phytochemicals *ie,* Isoliquiritin, EGCG, eugenol, chrysin and luteolin which inhibited AHL production and biofilm formation more efficiently than 6-gingerol were selected and docked with QS proteins LasI, LasR, RhlI, RhlR. Molecular docking results indicated that Epigallocatechin gallate interacted with Las I more strongly as compared to gingerol as indicated by their percentage binding energy value *i.e.*, 66.6 % of EGCG. Luteolin also formed a more stable complex with LasI (50 %) as compared to that of gingerol, followed by chrysin (40.47 %), isoliquiritin (16.6 %) and eugenol (2.3 %). With Las R, only chrysin (27.9 %) and luteolin (23.2 %) showed more stable binding than gingerol. EGCG (−13.95 %), eugenol (–22.09). isoliquiritin (−34.88 %) showed negative percentage binding energy values with LasR which directed that gingerol made more stable complex with Las R. With RhlI, isoliquiritin (15.06 %) is shown to interact and bind more firmly as compared to gingerol, followed by luteolin (10.9 %) and chrysin (9.5 %). However, EGCG and eugenol showed less stable complex when compared with gingerol. With RhlR, all the phytoligands *ie,* EGCG (40.81 %), chrysin (26.53 %), luteolin (20.4 %), isoliquiritin (10.2 %) and eugenol (4.08 %) have shown to interact and form a more stable complex as compared to that of gingerol. The list of interacting amino acids of proteins LasI, LasR, RhlI and RhlR with phyto-ligands was given in Sup. Table 2 and their interaction is shown in Fig. 5.

In Las I protein, Leu133 was found to interact with all the phytoligands. Two unique residues Val 194 and Arg 197 were found to interact with EGCG which might be responsible for strong bonding between LasI and EGCG. Similarly, Leu 180 and Glu 179 were found interacting solely with Luteolin and isoliquiritin respectively. In Las R protein, Asp 73 was shown to interact with all phytoligands. Asp65 and Cys 78 were the unique residues found interacting with EGCG only. Similarly, Leu 40 in luteolin, Ala105 in isoliquiritin, Leu 125 and Leu110 in gingerol are the unique amino acids which were not present in other interactions. Long stretches of amino acids were seen interacting with chrysin which might be the reason of their strong and stable bonding (Sup. Table 2). In RhlI, no common amino acid was found in RhlI-phytoligand interactions. However, unique residues were found which might be responsible for more or less bonding *eg* Asp35 is unique to RhlI-isoliquiritin interaction, Phe173, Phe 147 and Leu80 were solely present in RhlI- gingerol bond, Glu166 in RhlI-Eugenol and Ser103 in RhlI-EGCG interactions. In RhlR, Val95 and Arg138 were common amino acids present in all RhlR-phytoligand interactions. Ser 144 is unique to RhlR-EGCG complex and might be responsible for their strong bonding. Phe 40 and Ser145 are solely present in RhlR- eugenol and RhlR-Isoliquiritin interactions respectively (Fig. 5). The amino acids which were found interacting with only single phytoligand help in maintaining the active site and may provide extra strength and stability to that specific protein–ligand complex (Bhat et al., 2021).

5. Conclusion

Phytotherapy is an alternative approach to conventional antibiotics in combating antimicrobial resistant microbes. Our study aimed at targeting quorum sensing and its related mechanisms to control antibiotic resistant infections. Isoliquiritin, a phytochemical, never been used before as an antibacterial agent in research, was proved to be a potent inhibitor of Quorum sensing as well as its related mechanisms like biofilm formation, motility, slime production and aggregation. Besides, epigallocatechin gallate (EGCG), eugenol, chrysin and luteolin were also shown to be promising candidates in inhibiting biofilm and quorum sensing systems in Metallo beta lactamase producing *Pseudomonas aeruginosa* in both *in-silico* and *in-vitro* studies.

CRediT authorship contribution statement

Maryum Fakhar: Data curation, Formal analysis, Investigation, Writing – original draft. **Mehboob Ahmed:** Conceptualization, Project administration, Resources, Writing – original draft, Writing – review & editing, Software, Supervision. **Anjum Nasim Sabri:** .

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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Appendix A. Supplementary data

Supplementary Figure 1: AHL production in the presence of phytochemicals (1/2xMIC). Brown color indicates AHL production. Yellow color indicates weak or no AHL production. Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2024.104001.

References

Akram, M., Riaz, M., Munir, N., Rasul, A., Daniyal, M., Ali Shah, S.M., Shariati, M.A., Shaheen, G., Akhtar, N., Parveen, F., 2020. Progress and prospects in the management of bacterial infections and developments in phytotherapeutic modalities. Clin. Exp. Pharmacol. Physiol.

Alaoui Mdarhri, H., Benmessaoud, R., Yacoubi, H., Seffar, L., Guennouni Assimi, H., Hamam, M., Boussettine, R., Filali-Ansari, N., Lahlou, F.A., Diawara, I., 2022. Alternatives therapeutic approaches to conventional antibiotics: Advantages, limitations and potential application in medicine. Antibiotics.

Bhat, M.A., Kumar, V., Ahmed, M.Z., Alqahtani, A.S., Alqahtani, M.S., Jan, A.T., Rahman, S., Tiwari, A., 2021. Screening of natural compounds for identification of novel inhibitors against β-lactamase ctx-m-152 reported among *Kluyvera georgiana* isolates: An in vitro and in silico study. Microbial Pathogenesis.

Bhowmik, S., Anand, P., Das, R., Sen, T., Akhter, Y., Das, M.C., De, U.C., 2022. Synthesis of new chrysin derivatives with substantial antibiofilm activity. Mol. Divers.

Bose, S.K., Sharma, K., Chhibber, S., Harjai, K., 2021. Therapeutic potential of nanolipoidal α-terpineol in combating keratitis induced by *Pseudomonas aeruginosa* in the murine model. Int. J. Pharm.

Chadha, J., Harjai, K., Chhibber, S., 2022. Repurposing phytochemicals as anti-virulent agents to attenuate quorum sensing-regulated virulence factors and biofilm formation in Pseudomonas aeruginosa. Microbial. Biotechnol.

Ciandrini, E., Campana, R., Baffone, W., 2017. Live and heat-killed Lactobacillus spp. Interfere with streptococcus mutans and Streptococcus oralis during biofilm development on titanium surface. Arch. Oral Biol.

Coyte, K.Z., Tabuteau, H., Gaffney, E.A., Foster, K.R., Durham, W.M., 2017. Microbial competition in porous environments can select against rapid biofilm growth. Proc. National Acad. Sci.

Duddy, O.P., Bassler, B.L., 2021. Quorum sensing across bacterial and viral domains. PLoS Pathog.

Haney, E.F., Trimble, M.J., Hancock, R.E., 2021. Microtiter plate assays to assess antibiofilm activity against bacteria. Nat. Protoc.

Hao, S., Yang, D., Zhao, L., Shi, F., Ye, G., Fu, H., Lin, J., Guo, H., He, R., Li, J., 2021. Egcg-mediated potential inhibition of biofilm development and quorum sensing in *Pseudomonas aeruginosa*. Int. J. Mol. Sci.

Husain, F.M., Perveen, K., Qais, F.A., Ahmad, I., Alfarhan, A.H., El-Sheikh, M.A., 2021. Naringin inhibits the biofilms of metallo-β-lactamases (mβls) producing Pseudomonas species isolated from camel meat. Saudi J. Biol. Sci.

Javid, A., Ahmed, M., 2023. A computational odyssey: Uncovering classical β-lactamase inhibitors in dry fruits. J. Biomol. Struct. Dyn.

Jubair, N., Rajagopal, M., Chinnappan, S., Abdullah, N.B., Fatima, A., 2021. Review on the antibacterial mechanism of plant-derived compounds against multidrug-resistant bacteria (mdr). Evid. Based Complement. Alternat. Med.

Kowalska-Krochmal, B., Dudek-Wicher, R., 2021. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. Pathogens.

Lahiri, D., Nag, M., Dutta, B., Dey, S., Mukherjee, D., Joshi, S.J., Ray, R.R., 2021. Antibiofilm and anti-quorum sensing activities of eugenol and linalool from *Ocimum tenuiflorum* against Pseudomonas aeruginosa biofilm. J. Appl. Microbiol.

Lee, J.-S., Bae, Y.-M., Han, A., Lee, S.-Y., 2016. Development of congo red broth method for the detection of biofilm-forming or slime-producing *Staphylococcus* sp. LWT.

Luo, J., Li, Z., Wang, J., Weng, Q., Chen, S., Hu, M., 2016. Antifungal activity of isoliquiritin and its inhibitory effect against *Peronophythora litchi*chen through a membrane damage mechanism. Molecules.

Paudel, P., Shrestha, S., Poudel, S., Tiwari, B.R., 2021. Multi-drug resistant bacteria isolated from pus and phenotypic detection of metallo β-lactamase activity of *Pseudomonas aeruginosa*. Nepal J. Health Sci.

Rajkumar, H., Devaki, R., Kandi, V., 2016. Evaluation of different phenotypic techniques for the detection of slime produced by bacteria isolated from clinical specimens. Cureus.

Sabat, A.J., Pantano, D., Akkerboom, V., Bathoorn, E., Friedrich, A.W., 2021. Pseudomonas aeruginosa and *Staphylococcus aureus* virulence factors as biomarkers of infection. Biol. Chem.

Saeki, E.K., Yamada, A.Y., De Araujo, L.A., Anversa, L., de Oliveira Garcia, D., Barros De Souza, R.L., Martins, H.M., Kobayashi, R.K.T., Nakazato, G., 2021. Subinhibitory concentrations of biogenic silver nanoparticles affect motility and biofilm formation in Pseudomonas aeruginosa. Front. Cell. Infect. Microbiol.

Serra, D.O., Mika, F., Richter, A.M., Hengge, R., 2016. The green tea polyphenol egcg inhibits e. Coli biofilm formation by impairing amyloid curli fibre assembly and downregulating the biofilm regulator csgd via the σe-dependent srna rybb. Mol. Microbiol.

Sırıken, B., Öz, V., Erol, İ., 2021. Quorum sensing systems, related virulence factors, and biofilm formation in *Pseudomonas aeruginosa* isolated from fish. Arch. Microbiol.

Stenvang, M., Dueholm, M.S., Vad, B.S., Seviour, T., Zeng, G., Geifman-Shochat, S., Søndergaard, M.T., Christiansen, G., Meyer, R.L., Kjelleberg, S., 2016. Epigallocatechin gallate remodels overexpressed functional amyloids in *Pseudomonas aeruginosa* and increases biofilm susceptibility to antibiotic treatment. J. Biol. Chem.

Subbiahdoss, G., Reimhult, E., 2020. Biofilm formation at oil-water interfaces is not a simple function of bacterial hydrophobicity. Biointerfaces Colloids Surf. B.

Subramanian, K., Selvaraj, H., Sampath Renuga, P., Aruni, W., 2022. Anti-quorum sensing in pathogenic microbes using plant-based bioactive phytochemicals. Adv. Mater. Sci. Eng.

Thakur, P., Chawla, R., Tanwar, A., Chakotiya, A.S., Narula, A., Goel, R., Arora, R., Sharma, R.K., 2016. Attenuation of adhesion, quorum sensing and biofilm mediated virulence of carbapenem resistant *Escherichia coli* by selected natural plant products. Microbial. Pathogenesis.

Yi, L., Dong, X., Grenier, D., Wang, K., Wang, Y., 2021. Research progress of bacterial quorum sensing receptors: classification, structure, function and characteristics. Sci. Total Environ.

Zhao, X., Yu, Z., Ding, T., 2020. Quorum-sensing regulation of antimicrobial resistance in bacteria. Microorganisms.