Bioorganic Chemistry 87 (2019) 291–301

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Inhibition of biofilm formation, quorum sensing activity and molecular docking study of isolated 3, 5, 7-Trihydroxyflavone from *Alstonia scholaris* leaf against *P.aeruginosa*

M. Abinaya, M. Gayathri*

Department of Biotechnology, School of Biosciences & Technology, Vellore Institute of Technology (VIT), Vellore 632014, Tamil Nadu, India

ARTICLE INFO

Keywords:
3, 5, 7-Trihydroxyflavone
Biofilm
Virulence factors
Quorum sensing
Molecular docking

ABSTRACT

The current study is to evaluate the inhibition of biofilm formation and quorum sensing activity of isolated 3, 5, 7-Trihydroxyflavone (TF) from *A.scholaris* leaf extract against *Pseudomonas aeruginosa.* The effects of isolated TF on quorum sensing-regulated virulence factors production such as swimming motility, pyocyanin production, proteolytic, EPS, metabolic assay and inhibition of biofilm formation against *P.aeruginosa* was evaluated by standard protocols. In addition, the interaction between the isolated TF and active sites of QS- gene (LasI/rhlI, LasR/rhlR, and AHLase) in *P.aeruginosa* was evaluated by molecular docking studies using AutoDock Tools version 1.5.6. Based on the structural elucidation of the isolated compound was identified as 3, 5, 7-Trihydroxyflavone. Consequently, the isolated TF shows a significant reduction of biofilm formation through the inhibition of QS-dependent phenotypes such as pyocyanin production, proteolytic, swimming motility, EPS activities against *P.aeruginosa* in a dose-dependent manner. Molecular docking analysis of isolated TF can interfere the signaling [N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL)] molecules in *P.aeruginosa* by QS genes (LasI, LasR, rhlI, and AHLase) regulation. The isolated TF compound from *A.scholaris* reveals a greater potential to inhibit biofilm and QS dependent virulence factor production in *P.aeruginosa.* Docking interaction studies of TF-LasR complex express higher binding affinity than the other QS gene in *P.aeruginosa.*

1. Introduction

Generally, the diseases that are caused by fungi, bacteria, parasites, and viruses play a vital role in the morbidity and mortality in all the sector of the world, mainly in developing countries [1]. Mostly, the bacteria and fungi are resistance to all type of antibiotic in the past decades, because of various issues in the environmental changes. Therefore, the rate of detection of novel antibiotic is gradually decreasing, due to the absence of particular antibiotic discoveries, encompass, without a novel mechanism, poor investment and fewer pharmaceutical companies [2]. However, the increased multi-drug resistance and biofilm formation are very difficult to reduce nowadays, so the effort of searching alternative for novel antibacterial drug therapy. There is a growing interest in the development of ceramic biomaterials. However, most of the implant failure was caused by biofilm formation. Hence it is necessary to control the biofilm formation in the biomaterials field also [3].

Biofilm is the bacterial populations in which the microbial cells are attached to each other on a living or non-living surfaces and these adherent cells are embedded within a slimy extracellular matrix that composed of Extracellular polysaccharides (ECP) [4]. The process of biofilm population occurs through the process of cell-to-cell interaction by the dispersion of signaling molecules are commonly known as quorum sensing (QS) system [5].

These signaling molecules can regulate certain behavior such as regulation and expression of virulence factor genes, bioluminescence, sporulation, motility, mating, and biofilm formation [6]. The inhibition of bacterial virulence without terminating the pathogen in an anti-pathogenic approach is increasing day by day. The chemical signaling molecules of QS were released during the production of bacterial accumulation, this type of signaling was also known as Autoinducer (AIs)/bacterial pheromones. The main function of AIs is to regulate the gene expression on another new cells group, which in turn will switch the number of bacterial responses. The autoinducers may fluctuate; mostly depend on Gram-negative and Gram-positive bacteria. Among those, the most common autoinducers molecules are initiated in Gram-negative bacteria by LasI homolog synthase of N-acyl homoserine lactones (AHLs) [7].

* Corresponding author.
E-mail addresses: gayathrigopinath@vit.ac.in, gayathrimahalingamelsevier@gmail.com (M. Gayathri).

https://doi.org/10.1016/j.bioorg.2019.03.050
Received 14 December 2018; Received in revised form 18 February 2019; Accepted 17 March 2019
Available online 18 March 2019

The Gram-negative bacteria can efficiently increase the cell population density and resistance to the antibiotic and they are frequently linked with virulent pathogens such as *Pseudomonas aeruginosa, V.fisheri, Pseudomonas fluorescens, Pseudomonas chlororaphis, Acinetobacter tumefaciens, Chromobacterium violaceum* and *Erwinia carotovora* [8]. It also has been well recognized by the cell-dependent phenomenon which has been expressed in 30 different species of Gram-negative bacteria. Recently, the quorum sensing mechanism of *Chromobacterium violaceum* and its biocomponents has been reported in detail and used to estimate the antiquorum activity. Consequently, the inhibition of the QS system is a recent approach to control biofilm production than targeting particular virulence gene factors for therapeutic infections.

P.aeruginosa is an opportunistic pathogen which was well explored in human, plant and animal pathogens that are responsible to induce several virulence factors. The bacterial infection which was caused by clinical pathogens are influenced by cystic fibrosis, cancer, burn victims, nosocomial infection, immunocompromised and AIDS patients [9]. The extracellular polysaccharide enzymes which produce virulence gene in *P.aeruginosa* like protease, elastase, biosurfactants, phospholipases, rhamnolipid, and hemolysins. The quorum sensing circuits and virulence gene of *P.aeruginosa* were linked by two Autoinducer (AIs) (signaling) molecules [10,11]. The Las and *rhl* gene are responsible for signaling network, the synthase of LasI gene generates the N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) i.e. acyl-homoserine lactone (AHL), this signal was recognized by LasR gene through the transcriptional regulator. The LasR-AHL complex was stimulating the expression of several genes, which contains the expression of virulence factors and quorum sensing [12]. The same process for the *rhlI* gene generates the second N-butanoyl-L-homoserine lactone (C_4HSL) signal, which was detected by the transcriptional regulator gene *rhlR* (Fig. 1). This *rhlR*- C_4HSL complex will bind to particular sites and expressed the virulence gene and other components [13,14]. If the cell density of signaling molecules is less, the level of molecules that reaches to the binding sites are insufficient to activate the LasR. Hence, the cell density of signaling molecules are high, the molecules level may range into threefold that reaches to activate site and LasR-ALs complex dimer will bind to particular sites of DNA, activated LasR protein may express the virulence gene and biofilm formation [15]. In addition, the *P.aeruginosa* also releases third intracellular signaling molecules 2-heptyl-hydroxy-4-quinolone, which may interact to AHLs (acyl-homoserine lactones). According to the Centers for Disease Control and Prevention ESKAPE pathogens survey, the *P.aeruginosa*, *Klebsilla pneumonia, Acinetobacter baumannii, Enterobacter* species are the foremost nosocomial infection caused throughout the world [16].

The current year, the development of novel, non-toxic and broad-spectrum of QQ drugs from microbes and medicinal plants are more fascinated. Plant-based compounds which are obtained from secondary metabolites such as a flavonoid, alkaloid, terpenoid, and phenolic have their own oxygen-substituted derivatives [17]. The synthesis of secondary metabolites plays an important role in the therapeutic and biological activities to defeat the quorum sensing pathogens. Though the compounds which were derived from secondary metabolites can inhibit the quorum sensing and the mechanism of antimicrobial effectiveness is still not clearly understood [18].

Biofilm formation and quorum sensing play significant deciding components in the development of acute and chronic infectious diseases, specifically caused by *P.aeruginosa.* It plays an essential character in microorganism persistence and reduces the sensitivity of antimicrobials. It's also resistant to a wide variety of antibiotics group due to the formation of biofilms on chronic infections. Based on those issues, the *P.aeruginosa* is chosen as a reference pathogen for further work. Here, the present work describes that the isolated 3, 5, 7-Trihydroxyflavone compound from *Alstonia scholaris* leaf extract that can inhibit both the production of QS-dependent virulence factors and biofilm formation of *P.aeruginosa* without affecting the bacterial growth.

2. Material and methods

2.1. Extraction and isolation of bioactive compound from A.scholaris leaf

Fresh leaves of *Alstonia scholaris* (L) R.Br. were collected from the campus of VIT, Vellore, Tamil Nadu, India. Later, the fresh leaves were washed with distilled water; shade dried and finely powdered for further analysis. The leaf powder was extracted through methanol and separation of flavonoid fraction was carried by standard protocol [19]. These extracted flavonoid fraction of *A.scholaris* was further purified, isolated and the major bioactive compound was identified by the spectroscopy method.

2.2. Bacterial strains

The selected *P.aeruginosa* (MCC-2080) strain was purchased from

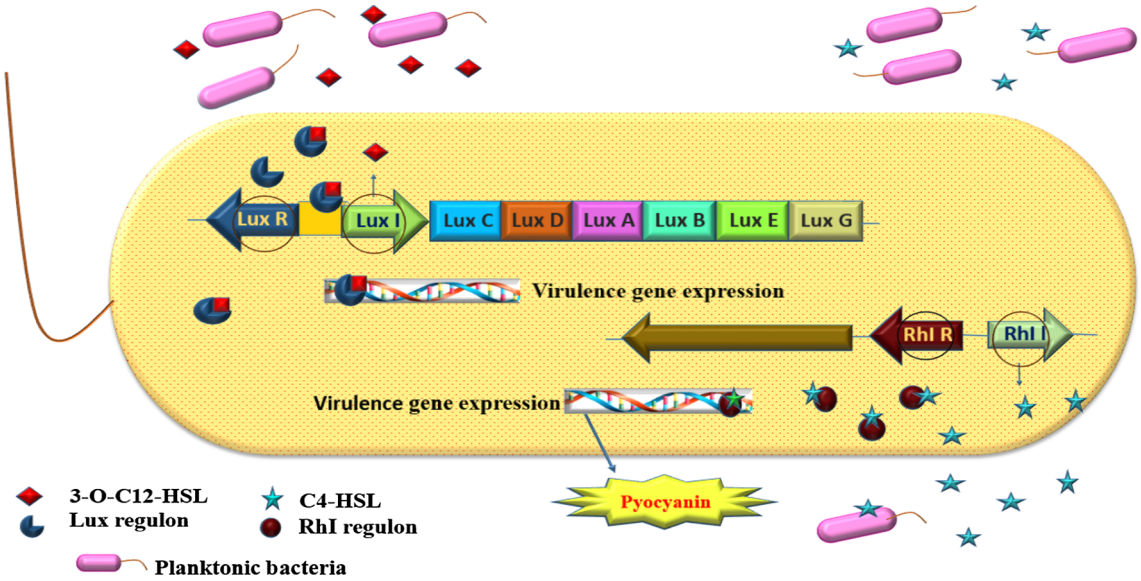

Fig. 1. Quorum sensing mechanism in *P.aeruginosa.*

the Microbial Type Culture Collection (MTCC), Chandigarh, Pune, India. The freeze-dried bacterial strain was routinely inoculated in nutrient broth and selective media for growth and incubated for 48 h at 37 °C. The glycerine stock was maintained and stored at 4 °C.

2.3. Inhibition of biofilm formation

The effect of the isolated TF from *A.scholaris* on biofilm forming *P.aeruginosa* strain was carried out by crystal violet assay [20,21]. The quantification of biofilm biomass density was observed under a microtitre plate reader at 595 nm. This assay was performed in triplicates. The percentage of biofilm inhibition was calculated.

$$\%\textbf{inhibition} = \text{OD growth control} - \text{OD sample}/\text{OD growth control} * 100$$

2.4. Inhibition of virulence factor regulated by QS

2.4.1. Quantitative analysis of pyocyanin production from P.aeruginosa

The effect of 3, 5, 7-Trihydroxyflavone from *A.scholaris* inhibits the formation of pyocyanin pigment in *P.aeruginosa* was quantified. The selected strains are grown in 10 mL of Kings A broth (KAB) was incubated for 37 °C for 48 h by few modifications [22]. After incubation, 100 µl of grown culture was inoculated in sterile KAB (100 mL) in a flask with or without flavones (100 µg/mL) and incubated for 37 °C for 48 h. Later incubation, the production of pigment-rich broth was collected and centrifuge for 10000 rpm for 10 min and supernatant was collected. Extraction of pigment from the supernatant was done by adding 6.0 mL of chloroform and mixed vigorously. The acidified chloroform forms blue bottom layer was collected and transfer to a new vial. Then add 2 mL of 0.2 M HCl and vortex for a few minutes, after acidified pink upper layer is produced. This pink layer was shifted to the cuvette and absorbance was observed under UV/Vis spectrophotometer at 520 nm.

2.4.2. Antimicrobial activity of pyocyanin produced by P.aeruginosa

The effect of pyocyanin produced from *P.aeruginosa* against various bacterial pathogens (*S.arueus, E.coli, P.vulgaris, B.subtilis, and P.aeruginosa*) was analyzed by a well diffusion method. The sterile Muller Hinton agar plates were solidified and swab with different test pathogens. Later, the different concentration of pyocyanin production (50,100,200 and 300 µg/mL) was seeded to each well and incubated for 24 h at 37C. After overnight incubation the agar plates were examined for the zone of inhibition against different bacterial pathogens.

2.4.3. Effect of swimming motility

Motility assay for *P.aeruginosa* was subsequently maintained on the soft agar medium (0.5% Agar, 0.5% peptone, 0.2% yeast extract and 1.0% glucose) was followed by standard protocol [23]. Later sterilized medium, with or without isolated TF compound of 100 µg/mL was supplementary to the warm soft agar medium before pouring into the Petri plates. After solidification, 5 µl of selected culture was inoculated on the center of the test and control medium. This plate was incubated through an upright position at 37 °C for 18 h. To visualize the viable pseudomonas motility across the surface of soft agar was compared to the zone of swarming motility in both test and control plates.

2.4.4. Extracellular protease activity by well diffusion method

To determine the selected bacterial culture have the ability of extracellular protease production using 1% (w/v) skimmed milk, casein, gelatin, and agar. After sterilization, the media was poured on Petri plates and solidified for a few minutes. The overnight culture of *P.aeruginosa* was diluted in 1:10 in LB broth contains with or without isolated compound (100 µl) of *A.scholaris* and incubated for 12 h at 28 °C. After the period of incubation, the production of an extracellular protease from the culture was collected using a centrifuge for 8900 rpm for 10 min. Later the supernatants of culture were collected and filtered with a 0.22 µm filter membrane to remove the cells. Further, the filtrate solution 100 µl of the solution was transferred through the wellbore on skim milk agar plates and incubated at 28 °C for 24 h. The clear zone of the diameter of the extracellular protease was measured. The experiment was repeated thrice [24].

2.5. Biofilm biomass assay

2.5.1. Biofilm metabolic activity assay

The influence of 3, 5, 7-Trihydroxyflavone compound from *A.scholaris* on metabolic activity of biofilm-forming *P.aeruginosa* was analyzed by the enzymatic reduction of MTT (3-(4, 5-Dimethylthiazol-2, 5-Diphenyltetrazolium Bromide) assay [25]. The grown culture with or without compound is carefully transferred to the microtubes contains 1 mL of sterile NaCl (0.85% w/v) and vortex for a few minutes to separate the biofilm and air dried. 100 µl of MTT solution was pipetted into each tube and incubated at 37 °C for 3 h under anaerobic conditions. After incubation, the yellow tetrazole converts to insoluble purple formazan acquired by enzymatic hydrolysis of MTT by dehydrogenase enzyme found in the living cells. The insoluble purple formazan was additional dissolved in 100 µl of DMSO in each microtube and incubated for 20 min at the dark condition. Later incubation, the DMSO solutions was tapped gently and transfer to the microplate reader and absorbance is measured at 540 nm. The result of metabolic activity mostly depends on higher absorbance of reaction is related to higher formazan concentration present, it specifies the higher metabolic activity of the biofilm formation. The percentage of cell inhibition of biofilm metabolic activity in the presence of a particular compound were calculated

$$\%\ \text{of cell inhibition} = [\text{At}-\text{Ab}/\text{Ac}-\text{Ab}] * 100$$

where, At- Absorbance of the test compound, Ab- Absorbance of blank, Ac- Absorbance of control.

2.5.2. Inhibition of EPS production

The Extrapolysaccharide matrix production from *P.aeruginosa* biofilm was inhibited by the isolated compound of *A.scholaris* was quantified as previously method [26]. The biofilm formed test pathogen was grown in the presence or absence of *A.scholaris* compound on the surface of sterile glass coverslip slides was immersed in 24 well microtiter plate. Then the plates were incubated for 16 h at 37 °C. After incubation, the glass coverslip is removed and washed with 0.5 mL of NaCl (0.9%). Subsequently, add an equal volume of 5% phenol and 5 mL of concentrated H_2SO_4 was also added to the cell suspension. Gentle mix the suspension and incubate in dark condition for 1 h. Later the cell suspension was centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was measured by UV–Vis spectrophotometric at 490 nm.

2.5.3. Determine the inhibition of biofilm formation by light Microscope

To visualize the effect of the isolated TF compound could inhibit the biofilm-forming bacteria through light microscopy. The sterile glass coverslip was immersed in 5 mL of LB broth with or without sub-MIC concentration (100 µl/mg) in 6 well plates. Subsequently, add 10 µl of an overnight grown culture of *P.aeruginosa* and incubated for 48 h at 28 °C for biofilm formation on glass slides. Later 48 h of incubation, the coverslips were removed and washed twice with sterile distilled water to remove the planktonic free bacteria. The adherent biofilm cells were immersed in methanol for 15 min, later the coverslips were stained with 2% crystal violet solution for 5 min. Eventually, the coverslips were washed with sterile distilled water to eliminate the excess stains and dried for 30 min at 30 °C. Later the coverslips were visualized under the light microscopy.

2.6. In-silico molecular docking analysis

The Ramachandran plot is a technique to visualize the energetic region were the backbone conformation of dihedral angles against amino acid residues of protein structure by Phi/Psi plot acquired in the RAMPAGE server.

The 3D structure of the *P.aeruginosa* QS transcriptional activator protein such as AHL- (*N*-Acyl Homoserine Lactone Lactonase), Las I (Acyl-homoserine-lactone synthase), Rhl I (Acyl-homoserine-lactone synthase), Las R (Transcriptional activator protein) (Receptor protein) and isolated compound ligand binding domain were all obtained from the Protein Data Bank (PDB code: 2BR6, 1RO5, 1KZF, 2UVO) was docked using AutoDock Tool version 1.5.6 software respectively.

The isolated compound and gentamycin (std) were docked with different proteins of QS such as synthase (Las I and Rhl I), transcription (Las R) and inhibition of autoinducer N-Acyl Homoserine Lactone (AHLase). The GLIDE (Grid-based Ligand Docking) is a program that pre-calculates the interaction energies of different atom types within the macromolecules such a protein, DNA or RNA. These type of protein-ligand interaction pocket site can view, whether the QS inhibited by ligand binding site. Finally, the optimized ligands were docked using the ligand docking module of GLIDE and were examined based on their GLIDE docking score and interaction of intermolecular. The confirmation of protein-ligand interaction with high binding energy was confirmed as the best docking position and docked interaction was examined through Discovery Studio Visualizer 2017.

2.7. Statistical analysis

All the experiments were performed as a mean ± standard deviation with triplicates. One-way ANOVA table is used to analyze the statistically significant difference between the tested groups using GraphPad Prism version 5.0 (GraphPad Software, Inc. San Diego, CA). The statistical significance was calculated with $P < 0.05$.

3. Results

3.1. Inhibition of biofilm formation

This method was implemented to inhibit both initial cell attachment and adherence of mature biofilm production of *P.aeruginosa* in the presence or absence of the isolated compound. The quantification was examined by 96 well microtiter plate using crystal violet assay and absorbance was measured through UV–Vis spectrophotometric at 595 nm. The crystal violet dye is not only used for staining, but it also used to smudge the cell adherence molecules of biofilm production on the surface. The different concentration (0.39, 0.78, 1.5, 3.12, 6.25, 12.5, 25, 50 and 100 μg/mL) of TF compound of *A.scholaris* can inhibit the biofilm formation of *P.aeruginosa* and also compared with negative (untreated) and positive (Gentamycin) control. Here, the efficacy of the TF compound showed 85% of biofilm formation was inhibited by an increase with the increasing concentration of 1 mg/mL. The concentration of 100 μg/mL of isolated TF compound reveals a more significant (85 ± 0.47) inhibition of biofilm eradication. On the other hand, the percentage of biofilm eradicated was decreased with decreasing concentration of compound (Fig. 2). In addition, the isolated TF compound can inhibit the growth of planktonic bacteria and also have the ability to reduce the exopolysaccharides.

3.2. Quantitative analysis of pyocyanin production

The *P.aeruginosa* produces some virulence factors which were regulated by the QS system. This system produces the secondary metabolites are well-known as pyocyanin a blue-green phenazine pigment. The extraction of pyocyanin production with or without sub MIC concentration (100 μg/mL) in cell frees supernatants of *P.aeruginosa.* The extracted pyocyanin pigment was quantified using UV–Vis spectrophotometric under the observance of 520 nm (Fig. 3). As per the result, the significant reduction of pyocyanin production of *P.aeruginosa,* after treated with or without sub-MIC concentration of the compound and compared to the control.

3.3. Antibacterial activity for pyocyanin

The susceptibility of pyocyanin production of *P.aeruginosa* was analyzed by agar well diffusion assay against Gram-positive and Gram-negative bacteria. Mostly, the result reveals that the antibiotic action of pyocyanin extraction using different concentration (50, 100, 200 and 300 μg/mL) are susceptible to Gram-positive bacteria (*S.aerues*, *B.subtilis*) than the Gram-negative bacteria (*E.coli, P.vulgaris, and P.aeruginosa*) (Table 1). Among the various bactericidal effect, the zone of inhibition was observed in *S.aureus and B.subtilis* is more sensitive to pyocyanin extraction. The effect of the antimicrobial activity of pyocyanin against *E.coli, P.vulgaris, and P.aeruginosa* was very low than comparing to the Gram-positive bacteria.

3.4. Effect of swimming motility activity

The effect of isolated TF compound shows a more significant reduction on swimming motility of *P.aeruginosa* at various concentration (25, 50, 75 and 100 μg/ml), which has been assessed as an indicator of quorum sensing inhibition (Fig. 4). Basically, the free-floating bacterial motility plays an important role in cell proliferation, surface colonization, and scattering on the surface of bacteria and formation of biofilm. Generally, the *P.aeruginosa* sustains the flagellar-mediated form of swarming motility, which is mostly disseminated through hyperflagellation and type-IV pili. The significant inhibition of swimming motility of *P.aeruginosa* on agar plates was exhibited after treated with TF at a concentration of 100 μg/mL. The result shows that the effect of different concentration of TF might inhibit the flagellar motility of *P.aeruginosa*, based on the increasing concentration of the compound (Table 2).

3.5. Extracellular protease activity by well diffusion method

The effect of extracellular protease activity of sub MIC concentration of isolated compound against *P.aeruginosa* was analyzed by 1% of skim milk agar plates (Fig. 5). After 48 h of incubation, the particular concentration of compound that inhibit/reduce the clear zone of a diameter which was produced by *P.aeruginosa*. The production of protease by *P.aeruginosa*, which shows the clear hydrolysis zone on the casein agar plates, it was based on the high production of enzymes in culture. The rate of inhibition may range up to 52%, while they are treated with 0.1 μl/ mL concentration of the compound and also compared with a blank group (Table 3). This result indicates that the isolated TF compound might inhibit the extracellular protease regulated by the QS system in *P.aeruginosa*.

3.6. Biofilm metabolic activity

The isolated TF compound of *A.scholaris* was treated against with *P.aeruginosa* biofilm for the reduction of metabolic activity by MTT assay. The effect of TF compound inhibition was mostly interrelated with dose dependents (Fig. 6A). After 24 h of incubation, the TF compound was only capable to significantly reduce the metabolic activity (76%) against the tested pathogen at a particular concentration (100 μg/mL) as compared to the untreated control (Fig. 6B). Moreover, higher absorbance is associated with high formazan concentration, whereas it indicates greater metabolic activity was detected in biofilm forming *P.aeruginosa*. Hence, the treated TF compound against the tested pathogen reveals the statistically significant variance ($P < 0.05$). Therefore, the effects of TF compound destructed the

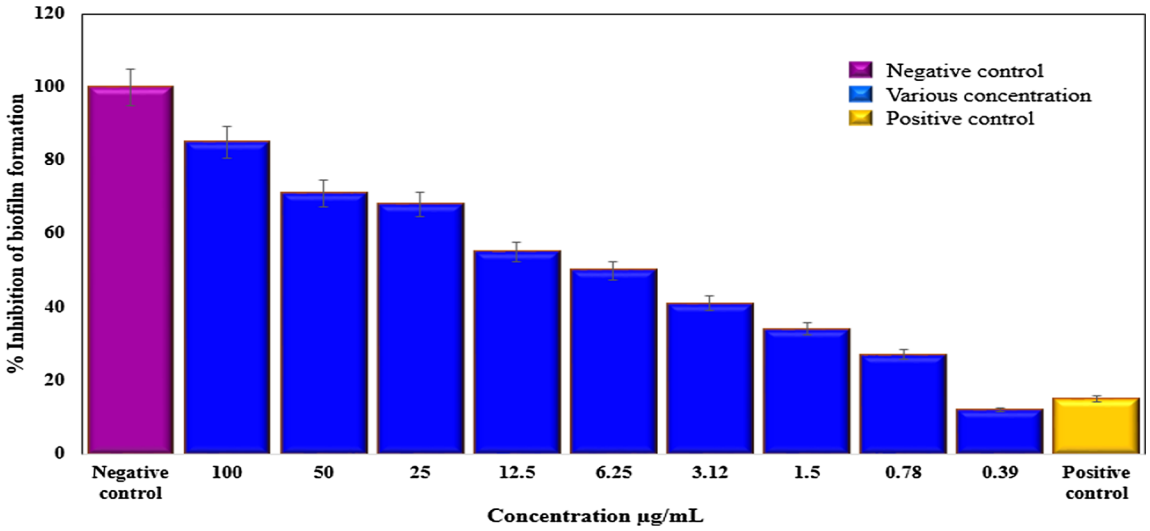

Fig. 2. Percentage inhibition of biofilm formation of *P.aeruginosa* after 24 h incubation in the presence or absence of TF compound. Values are expressed in Mean ± SEM.

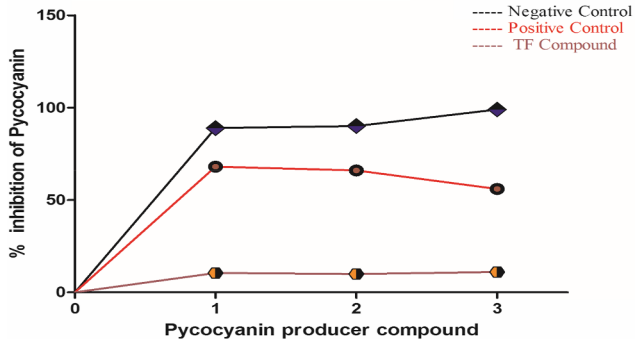

Fig. 3. Effect of the TF compound of *A.scholaris* can inhibit the production of the virulence factor of *P.aeruginosa.* These data are expressed in mean ± SD. The One-wave ANOVA analysis of P-value < 0.0001 are a significant difference at P < 0.05 ***.

Table 1
Antimicrobial zone of inhibition against various bacterial pathogen for *P.aeruginosa* pyocyanin extraction by agar well diffusion.

Organism	Zone of Inhibition (mm)			
	50 (µg/mL)	100 (µg/mL)	200 (µg/mL)	300 (µg/mL)
S.aurues	13.5 ± 0.40	15 ± 0.41	16.5 ± 0.401	18.3 ± 0.63
B.subtilis	9.5 ± 0.40	14.4 ± 0.65	15.9 ± 0.25	16.5 ± 0.70
P.vulgaris	–	–	–	–
E.coli	9.2 ± 0.65	10 ± 0.40	11.5 ± 0.57	12.56 ± 0.2
P.aeruginosa	–	–	–	–

These values are expressed in triplicate and Mean ± SD.

biofilm formed by *P.aeruginosa* and also inhibit/reduce the metabolic activity respectively.

3.7. Inhibition of EPS production

The influence of isolated TF on extra polysaccharide formation of *P.aeruginosa* strains was absolutely correlated with biofilm production. The extraction of EPS formation was quantitative by the compound was treated or untreated with tested cultures. This result reveals that the concentration of the compound increases the reduction of EPS formation during the biofilm will be decreased (Fig. 7). Therefore, the dose-dependent isolated TF (100 µg/mL) compound can inhibit the EPS formation in *P.aeruginosa* (74.5%*)* as compared to the control. The result reveals that the inhibition of biofilm and EPS production of the tested pathogen was treated with TF compounds are mostly depends on the concentration of biomass reduction. The EPS productions are typically correlated with potent biofilm forming planktonic bacteria. The inhibition of EPS forming bacteria reveals that the maximum reduction was observed in 74.5% at a concentration of 100 µg/mL.

3.8. In situ visualization of biofilm formation through light microscopy

The visualization of light microscopic images shows the untreated slides portrayed a well-developed thick and continuous lawn of biofilm formation by tested pathogens (Fig. 8a). Though the effect of isolated TF (100 µg/mL) was treated with a tested pathogen, reveals that the significant reduction of biofilm as compared to that of the untreated slide (Figure- 8a). Based on the light microscopy analysis, the efficacy of a particular concentration of TF compound from *A.scholaris* shows the maximum reduction of biofilm-forming planktonic cells and the production of EPS against tested strains. Therefore, the gentamycin which acts as a positive control also shows very less reduction of biofilm as of compared to the treated compound slide (Fig. 8b, c).

3.9. Molecular Docking

The analysis of the Ramachandran plot is used to examine the distribution of amino acid residues, where the Phi and Psi angles that searches for stereo-chemical parameters of the selected protein sequence structure of *P.aeruginosa*. These Ramachandran graphs examine the 1D-3D structure of the selected protein model of *P.aeruginosa* by using RAMPAGE programs (Fig. 9). Based on the RAMPAGE assessed the selected model's graph was displayed that 98.3% and 97.9% of the residues are most favorable energy region and a limited number of residues are found in the allowed region (Table 4). In disallowed regions, there is no amino acid residue was founded in the Ramachandran diagram [27].

In order to examine the potential of the biofilm formation mechanism, the nature of isolated TF compound interaction between the quorum sensing gene (LasI, LasR, rhlR,) and AHLase in Gram-negative bacteria (*P.aeruginosa)*. The autoinducers which are produced during the QS mechanism is 3-oxo-C12-HSL and C4-HSL. To block the signaling molecules and expression of virulence factors gene was docked with specific ligand with the various activated receptor (LasI, LasR, rhlR, and AHLase) through a molecular dock program. The three-

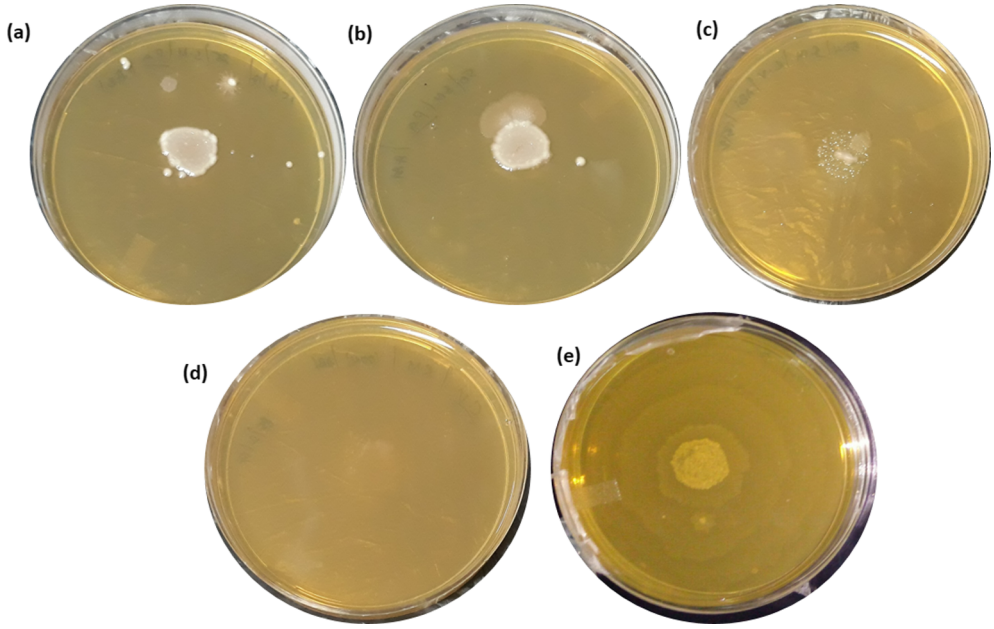

Fig. 4. The influence of isolated TF at different concentration for quorum sensing inhibition against the tested pathogen by swimming motility. (a) 25 µg/mL (b) 50 µg/mL: (c) 75 µg/mL: (d) 100 µg/mL (e) control without TF.

Table 2
Percentage inhibition of swimming motility of *P.aeruginosa.*

S.no	3, 5, 7-Trihydroxyflavone (TF) Concentration (µg/mL)	Swimming motility diameter (mm)	Inhibition of motility (%)
1.	25	17.3 ± 0.47	62
2.	50	15.8 ± 0.62	66
3.	75	11.6 ± 0.47	75
4.	100	4.33 ± 1.23	86
5.	Control (without compound)	45 ± 0.81	–

Values are expressed in Mean ± SD, n = 3,

Table 3
Percentage inhibition rate for Extracellular protease activity.

Additive	Protease activity	Inhibition rate (%)
Control	24.5 ± 0.5	100%
CG	11.7 ± 0.3 **	52%

** Significant difference of ($P < 0.05$), Mean ± SD triplicates.

dimensional structure of LasI, LasR, rhlR and AHLase protein sequence of *P.aeruginosa* was collected from Protein Data Bank (PDB) based on the crystal structure of domain protein at 1.80 Å and 2.3 Å resolution [28]. The database of PCBSum is used to analyze the secondary structural component. The search of the NCBI CD database for protein shows that it contains autoinducer domain from residues up to 20–160 for the transcription regulator process [29,30]. This present study reveals that the isolated TF compound of *A.scholaris* has the potential to inhibit the quorum sensing phenotypes of *P.aeruginosa* (Table 5). The isolated TF is effectively bound to the particular sites of QS protein (LasI, LasR, rhlR, and AHLase) of *P.aeruginosa (*Fig. 10). The docking affinity of TF with different protein complex was compared to the docking score of protein-Std (Gentamycin). This docking interaction study mostly depended on the phytochemical compounds which contain the keto group (C═O) and an amino group (NH) on the amino acid of protein supports the H-bond interaction. The docking interaction between the isolated TF with the LasR complex had a stronger binding affinity of −8.03 Kcal/mol towards the hydrogen bonding at SER155 (Fig. 10C). This inference of the isolated TF might inhibit the antibiofilm properties through the particular binding site of the QS gene.

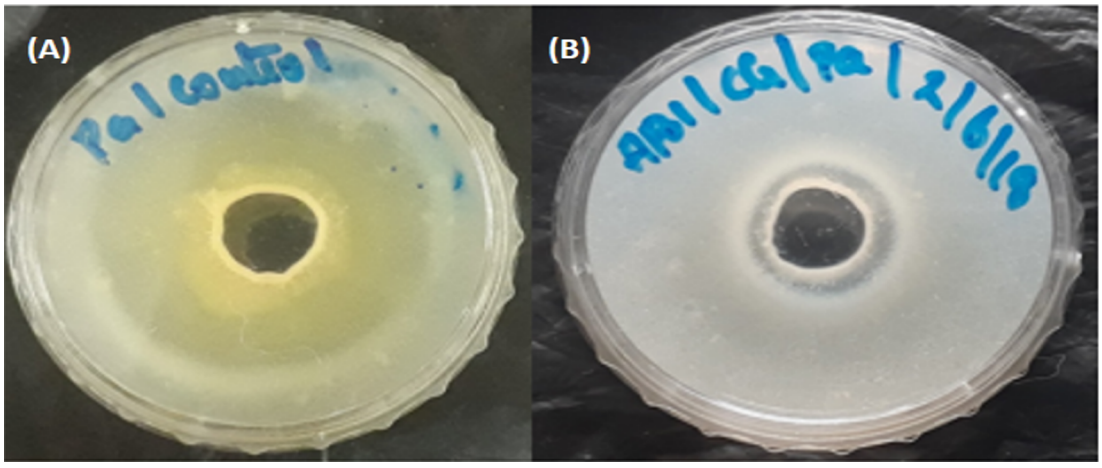

Fig. 5. Inhibition of extracellular protease activity by skim milk agar plates incubated for 48 h. A) Untreated (control) and B) 0.1 µl/ mL of isolated TF.

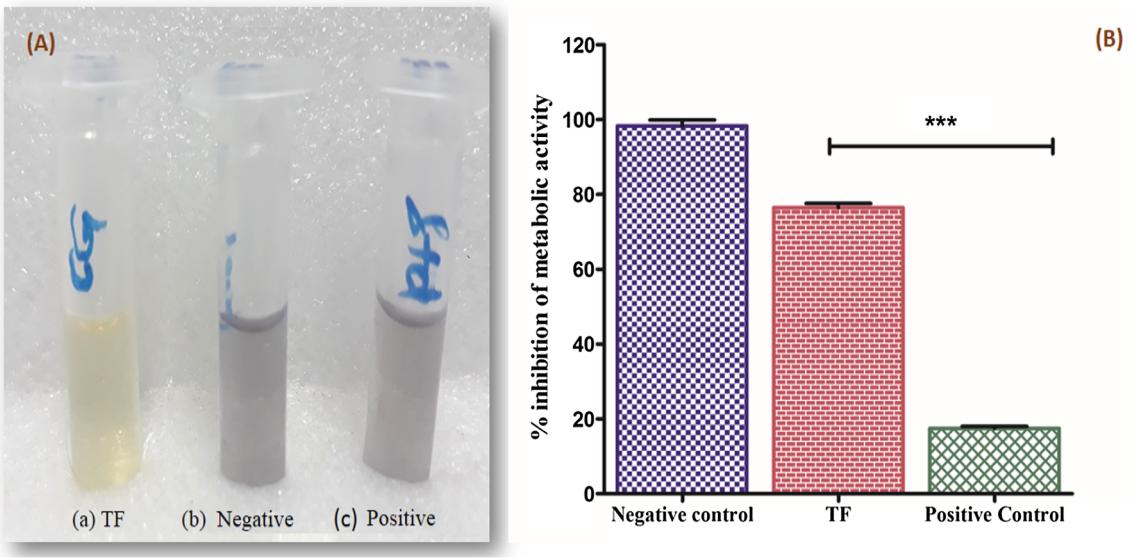

Fig. 6. Reduction of metabolic activity in *P.aeruginosa* cells was treated with TF for interruption of biofilm formation. (A) Conversion of tetrazole into insoluble formazan image. (B) % inhibition of metabolic activity using MTT assay. One way ANOVA test indicates that the significant difference between control and TF compound. Mean ± SD triplicates, the asterisk indicates the significance of $p < 0.05$ and P-value < 0.0001.

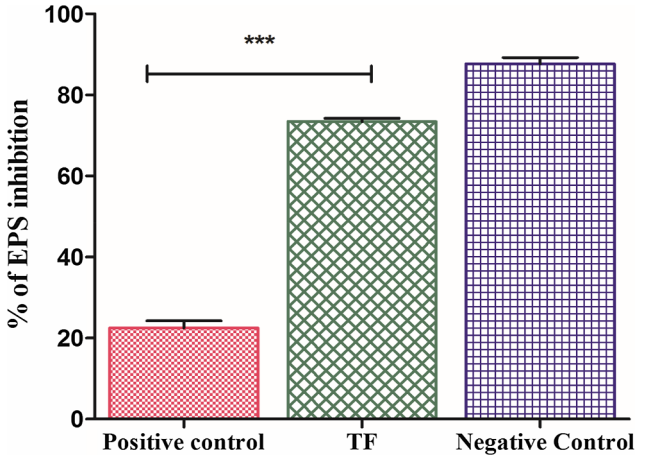

Fig. 7. Percentage inhibition of exopolysaccharide production of *P.aeruginosa* by particular concentration (100 µg/mL) of TF. One way ANOVA test indicates a significant difference between control and TF compound. Mean ± SD triplicates, the asterisk indicates the significance of $p < 0.05$ and P-value < 0.0001.

4. Discussion

The current study was focused on the effect of isolated 3, 5, 7-Trihydroxyflavone from *A.scholaris* can inhibit the biofilm formation and quorum sensing against *P.aeruginosa.* Mostly, the various plant's based compounds have a more biologically active system at low concentration. The bacteria which causes chronic infections to human are mostly due to the biofilm formation [31]. These type of planktonic biofilm production may lead to an increase the resistance against antimicrobial treatment and also favor the microorganism to grow in suboptimal environments [32,33]. Basically, the antibiotic which has a higher concentration is essential to destroy the bacteria adherence during the phase of biofilm production than they reach to planktonic counterparts [34]. Therefore, the effect of various concentration of 3, 5, 7-Trihydroxyflavone compound of *A.scholaris* shows the inhibition of biofilm production and also analysis the antibiofilm activity. The efficacy of the isolated compound for 1 mg/ml is significantly ($p < 0.05$) inhibit the biofilm biomass of *P.aeruginosa* bacteria. Though, the tested pathogen was treated with different concentration of isolated compound was examined at 50 and 55% reduction of biofilm biomass at a low concentration of 6.25 and 12.5 µg/mL. Hence, the compound with

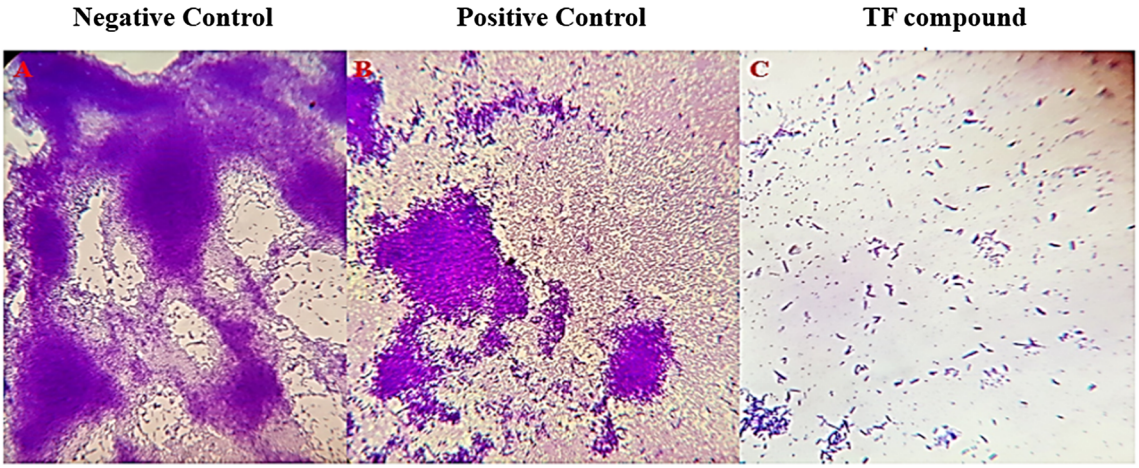

Fig. 8. *In situ* light microscopic images (40X) of *P.aeruginosa* inhibit forming biofilm with or without TF (100 µg/mL). (A)Negative Control (untreated slide) (B) standard drug (Gentamycin) and (C) treated with TF slide.

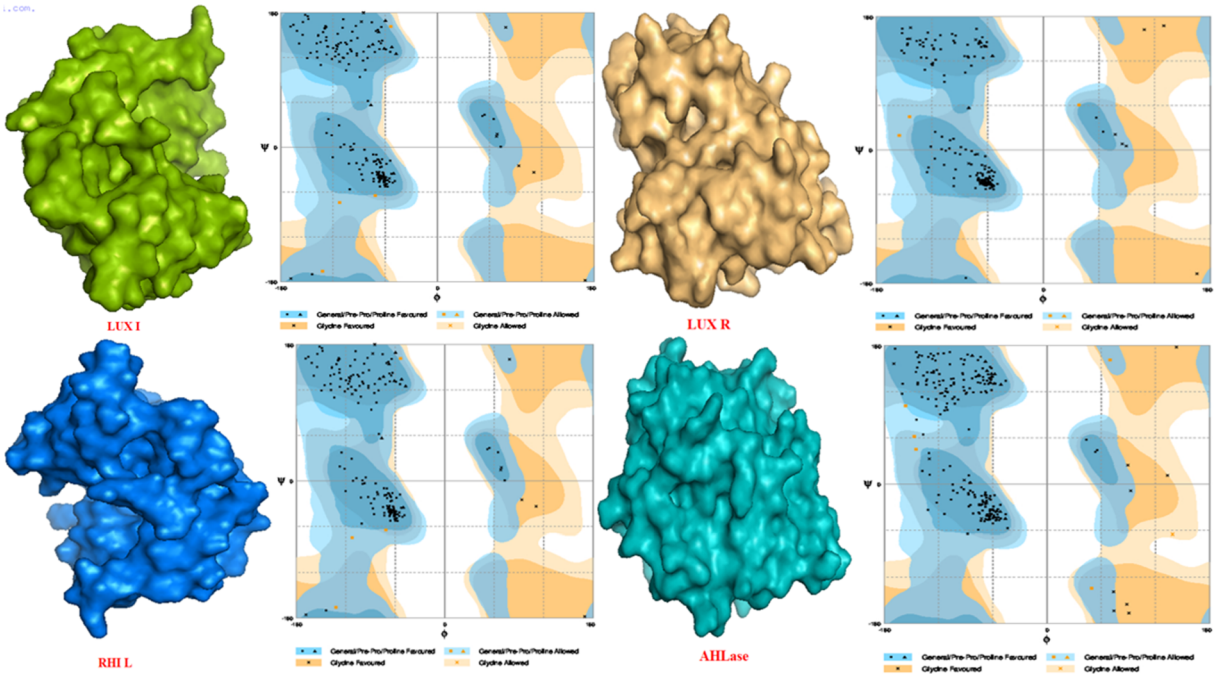

Fig. 9. Dispersions of amino acid residues of the selected protein using the Ramachandran graph. (a) LasR (b) LasI (C) *rhlI* (d) AHLase.

Table 4
Ramachandran plot viewer for selected protein.

Protein Name	Favored region	Allowed region	Dis-allowed region
Las I	97.9%	2.1%	0
Las R	98.3%	1.7%	0
rhl I	97.9%	2.1%	0
AHLase	97.5%	2.5%	0

increased concentration shows 85% of biofilm inhibition at the starting phase of adhesion and irradiate the microcolonies which are produced by bacterial pathogen was compared to the control.

The *P.aeruginosa* is an opportunistic pathogen which causes the virulence factor regulated by Quorum Sensing (QS) system. The QS bacteria accumulated and release the chemical signal molecules called autoinducer (AI) these may increase the concentration of cell density. Quorum sensing of *P.aeruginosa* possesses two autoinducer molecules such as *Las* and *Rhl* system. The Las homologs are responsible for the synthesis of *LasI* and *rhlI* system respectively. The QS system of *rhl* consists of RhlI, which was encrypted and synthase of diffusible C4-HSL signal molecules. Subsequently, these signaling molecules interact with *rhlR* transcriptional activator protein to initiate the pyocyanin pigment and rhamnolipid production. The Pyocyanin is water soluble phenazine pigments which are produced in enormous quantities by the active culture of *P.aeruginosa.* Therefore, the inhibition of pyocyanin biosynthesis production and neutralize its toxicity may be needful in the treatment of cystic fibrosis and other infection caused by the organism.

In this antimicrobial study, the quantified pyocyanin production against various Gram-positive and Gram-negative bacteria was examined by agar well diffusion method. Specifically, the extracted pyocyanin pigment production from *P.aeruginosa* was more sensitive to *S.aureus and B.subtilis* than other bacteria. The microbial acts of *P.aeruginosa* are resistance to pyocyanin pigment. Whereas, those were due to the Gram positive and Gram negative cell walls have a difference in the lipid content, so it can be linked with different extent of susceptibilities of pyocyanin against the inhibition of bacteria. The susceptibility of bacterial inhibition was due to pyocyanin at various concentration may slightly increase due to increasing concentration. The biofilm acts as a physical barrier for inhibiting the phenazine pigment production by *P.aeruginosa,* which was caused by its unique redox potential. The redox–active of pyocyanin, which might kill the mammalian and bacterial cells along the generation of reactive oxygen intermediates. The effect of inhibiting the pigment may cause during the electron transport in the respiratory chain and the formation of oxygen free radicals. The pyocyanin (N-methyl-1-hydroxyphenazine) also have antibiotic activity against various microorganisms. The toxic effect of pyocyanin from *P.aeruginosa* can protect by itself through a different mechanism, where higher enzymes are produced such as Hydrogen peroxide (H_2O_2), and superoxide dismutase than the aerobic microorganism.

The result reveals that the different concentration of isolated TF has the ability to distract the motile group of particular bacteria biofilm formation. Based on the previous report of cinnamaldehyde, the degradation or inhibition of QS molecules (3-hydroxy-butanoyl-homoserine lactone) was achieved in *V.harveyi.* During biofilm formation, some of the virulence factors were initiated by the pathogen to promote

Table 5
The efficacy of TF interaction with different activator proteins for inhibition of Quorum sensing mechanism in *P.aeruginosa* based on Molecular docking score.

S.no	Activator protein	PDB code	AngstromsGrid map of XYZ	Hydrogen Bond	Docking score of 3, 5, 7-Trihydroxyflavone(TF) (kcal mol^{-1})	Docking score of Gentamycin (kcal mol^{-1})
1.	AHL (*N*-Acyl Homoserine Lactone Lactonase)	2BR6	34.5: 30.8: 36.8	GLY12, VAL35	−6.06	−4.83
2.	Las I (Acyl-homoserine-lactone synthase)	1RO5	32:29.8:35.7	ARG100, ASP48	−7.92	−2.9
3.	Las R (Transcriptional activator protein)	2UV0	36.8: 30.8: 33.8	SER155	−8.03	−1.89
4.	Rhl I (Acyl-homoserine-lactone synthase)	1KZF	32:29.8:35.7	ARG100, ASP48	−7.87	−3.24

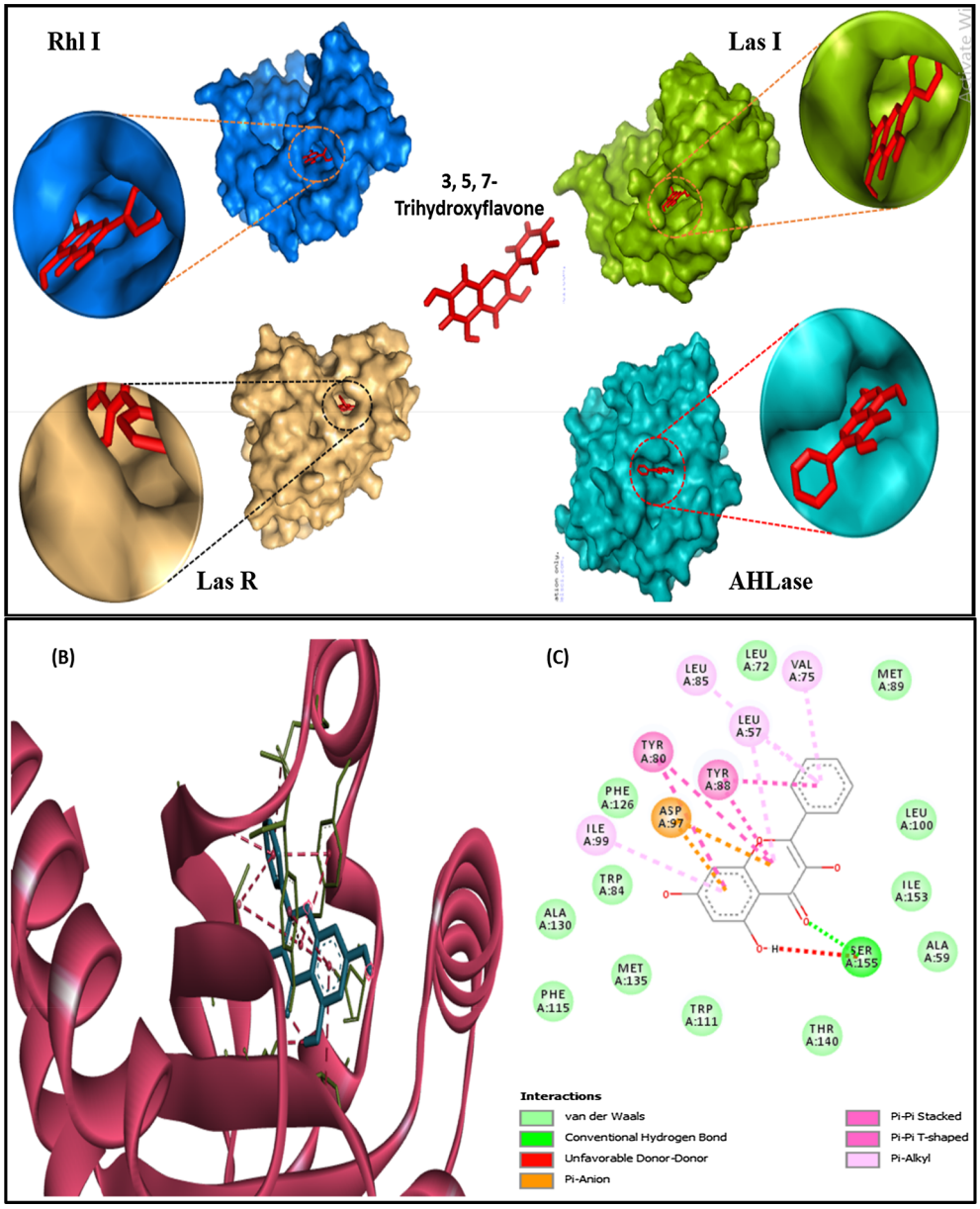

Fig. 10. (A) Three dimension structure of a docked protein with TF complex and (B) The structure of 3, 5, 7-Trihydroxyflavone binding pocket of the LasR receptor. (C) Illustration of 2D interaction between 3, 5, 7-Trihydroxyflavone complex with the LasR receptor.

the pathogenicity and also acts against the host immune system. Among the various virulence factor of *P.aeruginosa*, swarming motility plays a key factor for pseudomonas which was initiated by Las and *rhl* quorum sensing system. The *rhl*I gene synthase and produced C4-HSL signaling molecules, these molecules bind with *rhlR* for the transcriptional regulator and activate *rhlAB* complex forms rhamnolipid biosynthesis virulence gene [35]. The complex phenotype of motility was implicated with different regulatory components [36]. Consequently, the interruption of motility owing to some modulation of rhamnolipid biosynthesis in *P.aeruginosa* indicates that the quorum sensing inhibition occurs by isolated TF compound. The inhibition of swimming motility of *P.aeruginosa* was increased by increasing concentration of TF may be due to several up and downstream signaling molecules involved in the motility group.

Generally, the *P.aeruginosa* produce the extracellular protease was distinguished in the late log and in the stationary phase respectively [37]. The culture medium which contains the virulence factor may lead to the secretion of extracellular protease enzymes. The enzymes that execute the proteolysis, protein catabolism were activated by hydrolysis of peptide bonds in protein and polypeptides. Moreover, the protease can play a vital role in the production of exotoxins and also firmly regulated by the QS system of *P.aeruginosa*. Protease activity is also known as extracellular protease, this method was established by skim milk agar plates were loaded with supernatants and observed the clear zone of inhibition. Though, the calcium is one of the major factors which was presented in skim milk for the production of extracellular protease by *P.fluroscens* [38].

The metabolic activity of biofilm was prevented by an environmental condition where the microcolonies are attached to the surface and particular gene expression are induced by adhesion [39]. The effect of a compound that reduces the high cellular metabolism shows the statistically significant to planktonic bacteria that act as to form the biofilm phase. Hence, cellular metabolism is very low, the antimicrobial is resistance to the initial stage of bacterial growth. After the treatment of antimicrobial, the ability of the bacterial survival growth rate is slow and confirms the occurrence of persistent cells [40]. The present study

evaluated the efficacy of 3, 5, 7-Trihydroxyflavone compound that reduces the microcolonies load to form a biofilm. These MTT assay doesn't afford the evidence on cell viability occurrence in the biofilm. The result reveals that the reduction of metabolic activity of cells is treated with the particular compound, which is inhibiting the microbial loaded. The cellular metabolism is decreased due to the antimicrobial peptides effort through interacting with the planktonic bacterial cell surface as of interruption of cellular integrity [41]. The result of MTT assay shows that the *P.aeruginosa* biofilms, microbial cell have been highly reduced the metabolic activity due to the slow growth of nutrient limitation and oxygen supplementation. The inhibition of the metabolic activity of a compound is hydrophobic and still accompanying with cell wall rich in lipid content, potential increased the permeability of cell and cell death [42].

The Exopolysaccharide (EPS) productions were initiated by quorum sensing signaling molecules, which acts as a protective barrier for the host cells and enrich the formation of biofilm on the host surface [43]. The 3, 5, 7-Trihydroxyflavone compounds which inhibit the EPS production of *P.aeruginosa* were based on the impotent of planktonic bacteria adherence on the host surface for the biofilm formation and also susceptible to the host immune system to destroy the planktonic bacteria. The bacteria which produce the virulence factors during the quorum sensing signaling, this factor might assist the bacterium to promote the pathogenicity and furthermore acts against the host immune system [31]. Therefore, the eradication of EPS formation (74.5%) of test strain was observed in the presence of TF of *A.scholaris* at a concentration of 100 μg/mL. However, the *P.aeruginosa* shows the most significant inhibition of virulence factors, such as pyocyanin, swimming motility, elastase and the proteolytic gene for benefits of bacteria to stimulate the host immune response.

The mature biofilms symbolize to cause chronic infection to human that is highly resistant to antibiotics, while the planktonic bacteria are susceptible to a broad range of antibiotics [44]. Light microscopy analysis was achieved to elucidate the antibiofilm property with or without compound against the tested pathogen. The stained slides were observed under high resolution and magnification of light microscopy, the slide which shows a thick coating of biofilm biomass entrenched with EPS act as a control (untreated). On the other hand, the tested pathogen was treated with positive control (Gentamycin) revealed that a partial reduction of biofilm architecture. However, the elucidation of 3, 5, 7-Trihydroxyflavone (100 μg/ml) concentration-dependent compound can reduce the mature biofilm of *P.aeruginosa* can visualize through the microscope. Hence, the 3, 5, 7-Trihydroxyflavone compound with sub MICs (100 μg/mL) result shows the reduction of surface charge intensity may lead to inhibit the microcolonies during the planktonic biofilm formation by *P.aeruginosa* as also compared to the control. It was evidence that the formation of planktonic bacteria was inhibited at an early stage of attachment. The inhibition of cell attachment was also reported in milk constituents and chitosan [45].

Molecular docking is one of the most common techniques used in the structure-based drug design approach. This technique is generally used to understand the interaction of ligand molecules binds to the specific active site of the protein, and docking score of the ligand-receptor complex was analyzed by reference ligand score.

In-silico analysis of isolated 3, 5, 7-Trihydroxyflavone compound shows the inhibition of quorum sensing potential during the formation of biofilm. The molecular docking analysis of LasI, LasR, *rhlI,* and AHLase receptor protein reveals that the 3, 5, 7-Trihydroxyflavone compound binds compactly to the receptor and form the complex. The docked receptor-ligand complex shows high docking score as of compared to the signaling molecules in both conditions such as with or without flavone compound. Among, the interaction between TF and various receptor protein complex, the LasR-TF complex shows more stable, active binding sites and high docking score (-8.03 kcal mol^{-1}) than compared to the standard drug. Hence, the remaining interaction of the TF complex also shows significantly reduces the expression of QS genes like LasI, LasR, and AHLase that was initiated for the production of virulence factors in *P.aeruginosa*. The greater effectiveness of TF shows the stability based on the hydrogen bonds between the compound and receptor of LasR protein. It is well known as the lipophilic electron-donating aromatic ring. The solid interaction of the compound may due to the particular sites of binding groups, which facilitates the conformation changes to the receptor protein. The protein-ligand complex which shows high docking score have a potent ability for in vitro activities. Based on the molecular docking studies that the 3, 5, 7-Trihydroxyflavone can act as an anti-quorum sensing agent against *P.aeruginosa* and control the formation of biofilm bacteria.

5. Conclusion

The current study shows that the isolated 3, 5, 7-Trihydroxyflavone from *A.scholaris* might inhibit the QS-regulated virulence factor and biofilm formation in *P.aeruginosa*. *In-silico* molecular interaction studies of TF-LasR complex express higher binding attraction as compared to the standard drug gentamycin. Therefore, the efficacy of TF can inhibit the QS signaling (3-oxo-C12-HSL and C4-HSL) molecules, which was regulated to form a virulence factor during the biofilm formation. Hence, the isolated flavonol successfully suppressed the biofilm formation in *P.aeruginosa* by inhibiting the QS mechanism.

Conflict of interest

The author(s) declare that there is no conflict of interest in the publication of this paper.

Acknowledgment

The author expresses their gratitude to the Vellore Institute of Technology, Vellore for providing a good laboratory facility to carry out the research work.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.03.050.

References

[1] D.M. Livermore, M. Blaser, O. Carrs, G. Cassell, N. Fishman, R. Guidos, S. Levy, J. Powers, R. Norrby, G. Tillotson, R. Davies, S. Projan, M. Dawson, D. Monnet, M. Keogh-Brown, K. Hand, S. Garner, D. Findlay, C. Morel, R. Wise, R. Bax, F. Burke, I. Chopra, L. Czaplewski, R. Finch, D. Livermore, L.J.V. Piddock, T. White, Discovery research: the scientific challenge of finding new antibiotics, J. Antimicrob. Chemother. 66 (2011) 1941–1944, https://doi.org/10.1093/jac/dkr262.
[2] L.L. Silver, Challenges of antibacterial discovery, Clin. Microbiol. Rev. 24 (2011) 71–109, https://doi.org/10.1128/CMR.00030-10.
[3] R. Choudhary, S. Koppala, S. Swamiappan, Journal of Asian Ceramic Societies Bioactivity studies of calcium magnesium silicate prepared from eggshell waste by sol – gel combustion synthesis, Integr. Med. Res. 3 (2015) 173–177, https://doi.org/10.1016/j.jascer.2015.01.002.
[4] S.T. Rutherford, B.L. Bassler, Bacterial quorum sensing: its role in virulence and possibilities for its control, a012427 a12427, Cold Spring Harb. Perspect. Med. 2 (2012), https://doi.org/10.1101/cshperspect.a012427.
[5] P. Nadal Jimenez, G. Koch, J.A. Thompson, K.B. Xavier, R.H. Cool, W.J. Quax, The Multiple Signaling Systems Regulating Virulence in Pseudomonas aeruginosa, (2012). http://doi.org/10.1128/MMBR.05007-11.
[6] B.L. Bassler, Small talk: cell-to-cell communication in bacteria, Cell 109 (2002) 421–424, https://doi.org/10.1016/S0092-8674(02)00749-3.
[7] N.A. Whitehead, A.M. Barnard, H. Slater, N.J. Simpson, G.P. Salmond, Quorum-sensing in Gram-negative bacteria, FEMS Microbiol. Rev. 25 (2001) 365–404 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/11524130.
[8] S.A. Burt, V.T.A. Ojo-Fakunle, J. Woertman, E.J.A. Veldhuizen, The natural antimicrobial carvacrol inhibits quorum sensing in chromobacterium violaceum and reduces bacterial biofilm formation at sub-lethal concentrations, PLoS One 9 (2014) e93414, , https://doi.org/10.1371/journal.pone.0093414.
[9] T. Bjarnsholt, P.Ø. Jensen, T.H. Jakobsen, R. Phipps, A.K. Nielsen, M.T. Rybtke, T. Tolker-Nielsen, M. Givskov, N. Høiby, O. Ciofu, Scandinavian cystic fibrosis study consortium, quorum sensing and virulence of pseudomonas aeruginosa

during lung infection of cystic fibrosis patients, PLoS One 5 (2010) e10115, , https://doi.org/10.1371/journal.pone.0010115.
[10] J.P. Pearson, L. Passador, B.H. Iglewski, E.P. Greenberg, A second N-acylhomoserine lactone signal produced by Pseudomonas aeruginosa, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1490–1494 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/7878006.
[11] D.G. Storey, E.E. Ujack, H.R. Rabin, I. Mitchell, Pseudomonas aeruginosa lasR transcription correlates with the transcription of lasA, lasB, and toxA in chronic lung infections associated with cystic fibrosis, Infect. Immun. 66 (1998) 2521–2528 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/9596711.
[12] M. Schuster, C.P. Lostroh, T. Ogi, E.P. Greenberg, Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis, J. Bacteriol. 185 (2003) 2066–2079 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/12644476.
[13] P. Williams, M. Cámara, Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules, Curr. Opin. Microbiol. 12 (2009) 182–191, https://doi.org/10.1016/j.mib.2009.01.005.
[14] A. Latifi, M.K. Winson, M. Foglino, B.W. Bycroft, G.S. Stewart, A. Lazdunski, P. Williams, Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in Pseudomonas aeruginosa PAO1, Mol. Microbiol. 17 (1995) 333–343 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/7494482.
[15] K. Papenfort, B.L. Bassler, Quorum sensing signal–response systems in Gram-negative bacteria, Nat. Rev. Microbiol. 14 (2016) 576–588, https://doi.org/10.1038/nrmicro.2016.89.
[16] T.-F. Mah, B. Pitts, B. Pellock, G.C. Walker, P.S. Stewart, G.A. O'Toole, A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance, Nature. 426 (2003) 306–310, https://doi.org/10.1038/nature02122.
[17] M.M. Cowan, Plant products as antimicrobial agents, Clin. Microbiol. Rev. 12 (1999) 564–582 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/10515903.
[18] M. Gao, M. Teplitski, J.B. Robinson, W.D. Bauer, Production of substances by *Medicago truncatula* that affect bacterial quorum sensing, Mol. Plant-Microbe Interact. 16 (2003) 827–834, https://doi.org/10.1094/MPMI.2003.16.9.827.
[19] S.S. Subramanian, S. Nagarajan, Flavonoids of the seeds of Crotalaria retusa and Crotalaria striata, Cur Sci. (1969) (accessed December 13, 2018).
[20] A. Basson, L.A. Flemming, H.Y. Chenia, Evaluation of adherence, hydrophobicity, aggregation, and biofilm development of flavobacterium johnsoniae-like isolates, Microb. Ecol. 55 (2008) 1–14, https://doi.org/10.1007/s00248-007-9245-y.
[21] O.M. Vandeputte, M. Kiendrebeogo, S. Rajaonson, B. Diallo, A. Mol, M. El Jaziri, M. Baucher, Identification of catechin as one of the flavonoids from combretum albiflorum bark extract that reduces the production of quorum-sensing-controlled virulence factors in Pseudomonas aeruginosa PAO1, Appl. Environ. Microbiol. 76 (2010) 243–253, https://doi.org/10.1128/AEM.01059-09.
[22] D.W. Essar, L. Eberly, A. Hadero, I.P. Crawford, Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthases and evolutionary implications, J. Bacteriol. 172 (1990) 884–900 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/2153661.
[23] J. Tremblay, A.-P. Richardson, F. Lépine, E. Déziel, Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behaviour, Environ. Microbiol. 9 (2007) 2622–2630, https://doi.org/10.1111/j.1462-2920.2007.01396.x.
[24] Ponnuswamy Vijayaraghavan*, Samuel Gnana Prakash Vincent, A simple method for the detection of protease activity on agar plates using bromocresolgreen dye (accessed November 30, 2018), J Biochem Tech. 3 (2013) 628–630 https://pdfs.semanticscholar.org/c61d/40ff6de438214b73c6b69d686411c550b4ea.pdf.
[25] S. Koppala, S. Swamiappan, Materials and Manufacturing Processes Glowing Combustion Synthesis , Characterization , and Toxicity Studies of Na 2 CaSiO 4 Powders, (2015) 37–41. http://doi/10.1080/10426914.2015.1025973.
[26] C. Nithya, M.G. Devi, S. Karutha Pandian, A novel compound from the marine bacterium *Bacillus pumilus* S6–15 inhibits biofilm formation in Gram-positive and Gram-negative species, Biofouling 27 (2011) 519–528, https://doi.org/10.1080/08927014.2011.586127.
[27] N. Chowdhury, A. Bagchi, Molecular insight into the activity of LasR protein from Pseudomonas aeruginosa in the regulation of virulence gene expression by this organism, Gene. 580 (2016) 80–87, https://doi.org/10.1016/j.gene.2015.12.067.
[28] M.J. Bottomley, E. Muraglia, R. Bazzo, A. Carfi, Molecular insights into quorum sensing in the human pathogen *Pseudomonas aeruginosa* from the Structure of the virulence regulator LasR bound to its autoinducer, J. Biol. Chem. 282 (2007) 13592–13600, https://doi.org/10.1074/jbc.M700556200.
[29] A. Marchler-Bauer, S. Lu, J.B. Anderson, F. Chitsaz, M.K. Derbyshire, C. DeWeese-Scott, J.H. Fong, L.Y. Geer, R.C. Geer, N.R. Gonzales, M. Gwadz, D.I. Hurwitz, J.D. Jackson, Z. Ke, C.J. Lanczycki, F. Lu, G.H. Marchler, M. Mullokandov, M.V. Omelchenko, C.L. Robertson, J.S. Song, N. Thanki, R.A. Yamashita, D. Zhang, N. Zhang, C. Zheng, S.H. Bryant, CDD: a Conserved Domain Database for the functional annotation of proteins, Nucleic Acids Res. 39 (2011) D225–D229, https://doi.org/10.1093/nar/gkq1189.
[30] T.A.P. de Beer, K. Berka, J.M. Thornton, R.A. Laskowski, PDBsum additions, Nucleic Acids Res. 42 (2014) D292–D296, https://doi.org/10.1093/nar/gkt940.
[31] A.L. Spoering, K. Lewis, Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials, J. Bacteriol. 183 (2001) 6746–6751, https://doi.org/10.1128/JB.183.23.6746-6751.2001.
[32] D. Teepica, P. Darsini, P. Srinivasan, G. Guna, K. Manimekalai, In vitro anti biofilm activity of piper longum and piper nigrum against clinical isolates of *Streptococcus pyogenes* isolated from pharyngitis patients, Int. Res. J. Pharm. 6 (2015), https://doi.org/10.7897/2230-8407.06229.
[33] N. Takemura, Y. Noiri, A. Ehara, T. Kawahara, N. Noguchi, S. Ebisu, Single species biofilm-forming ability of root canal isolates on gutta-percha points, Eur. J. Oral Sci. 112 (2004) 523–529, https://doi.org/10.1111/j.1600-0722.2004.00165.x.
[34] H. Ishida, Y. Ishida, Y. Kurosaka, T. Otani, K. Sato, H. Kobayashi, In vitro and in vivo activities of levofloxacin against biofilm-producing Pseudomonas aeruginosa, Antimicrob. Agents Chemother. 42 (1998) 1641–1645 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/9660997.
[35] R. Daniels, J. Vanderleyden, J. Michiels, Quorum sensing and swarming migration in bacteria, FEMS Microbiol. Rev. 28 (2004) 261–289 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/15449604.
[36] J. Overhage, M. Bains, M.D. Brazas, R.E.W. Hancock, Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence factors and antibiotic resistance, J. Bacteriol. 190 (2008) 2671–2679, https://doi.org/10.1128/JB.01659-07.
[37] S. Rajmohan, C.E.R. Dodd, W.M. Waites, Enzymes from isolates of Pseudomonas fluorescens involved in food spoilage, J. Appl. Microbiol. 93 (2002) 205–213 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/12147068.
[38] R.C. McKellar, H. Cholette, Possible role of calcium in the formation of active extracellular proteinase by *Pseudomonas fluorescens*, J. Appl. Bacteriol. 60 (1986) 37–44, https://doi.org/10.1111/j.1365-2672.1986.tb01062.x.
[39] G.A. O'Toole, R. Kolter, Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis, Mol. Microbiol. 28 (1998) 449–461 (accessed November 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/9632250.
[40] K. Lewis, Persister cells: molecular mechanisms related to antibiotic tolerance, Handb. Exp. Pharmacol. (2012) 121–133, https://doi.org/10.1007/978-3-642-28951-4_8.
[41] K. Nawrocki, E. Crispell, S. McBride, Antimicrobial peptide resistance mechanisms of gram-positive bacteria, Antibiotics 3 (2014) 461–492, https://doi.org/10.3390/antibiotics3040461.
[42] S. Jadhav, R. Shah, M. Bhave, E.A. Palombo, Inhibitory activity of yarrow essential oil on Listeria planktonic cells and biofilms, Food Control. 29 (2013) 125–130, https://doi.org/10.1016/j.foodcont.2012.05.071.
[43] A.-C. Olofsson, M. Hermansson, H. Elwing, N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces, Appl. Environ. Microbiol. 69 (2003) 4814–4822, https://doi.org/10.1128/AEM.69.8.4814-4822.2003.
[44] V.B.P. Manickam Rajalakshmi*, Ponnuraj Srinivasan, Maria Francis Poffe, Rajendran Suresh, Crude fatty acid extracts of Streptomyces sps inhibits the biofilm forming Streptococcus pyogenes ATCC 19615 Manickam, J. Biochem. Tech. 2 (2014) 679–684.
[45] N. Sharon, I. Ofek, Safe as mother's milk: carbohydrates as future anti-adhesion drugs for bacterial diseases, Glycoconj. J. 17 (n.d.) (2000) 659–664 (accessed November 30, 2018).