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Bioactive Quorum Quenchers Antagonizing Pseudomonas Aeruginosa Biofilm

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Abstract

Quorum sensing is a generic regulatory mechanism of bacterial communication, allowing the launching of coordinated and unified response of bacteria in a population density-dependent manner to accomplish tasks, difficult to be carried out by an individual bacterium. It is based on secretion of signaling molecules called quorum signals to the environment, and plays a key role in controlling virulence, both in Gram-positive and Gram-negative bacteria. *Pseudomonas aeruginosa*, a Gram-negative bacterium, is an opportunistic pathogen, responsible for acute pulmonary infection and cystic fibrosis, as well as chronic infections, by virtue of natural drug resistance and capability of forming complex biofilms, using a highly complicated quorum sensing signaling mechanism, which is difficult to be cleared by antibiotic therapy. The quorum sensing system constitutes N-acyl homoserine lactone (AHL)-dependent regulatory circuits, modulated by a non-AHL related signal molecule, along with numerous regulators at the transcriptional and post-transcriptional level. Therefore, inhibition of quorum signals by using certain bioactive compounds, called quorum quenchers, especially phytochemicals, provides safe and effective remedial measure to eradicate infection caused by antibiotic-resistant *P. aeruginosa*. The promising quenchers make the biofilm more susceptible to antibiotics, thereby reducing virulence and mortality. They are either naturally found or can be synthetically designed through chemical engineering. Many of them are enzymes, degrading signaling molecules or impeding cellular signaling cascades. These quenchers are therefore attractive targets as next generation novel drugs that can be used in therapeutics to overcome the problem of antibiotic resistance issue of many bacteria due to careless and rampant use of increasing dosage of antibiotics.

Keywords: Quorum quenchers, Bioactive compounds, *Pseudomonas aeruginosa*, Quorum sensing, Biofilm.

Introduction

Quorum sensing (QS) refers to cell-to-cell communication utilized by many solitary bacteria to form a large consortium and high population density, either belonging to their own species or different species, by producing and perceiving certain diffusible signal molecules called quorum signals that coordinate virulence factor production, motility and biofilm formation. Biofilm is often commonly termed as 'city of microbes'. The bacterial communities within a biofilm are encased within a structural scaffold matrix of extracellular polymeric substance (EPS), comprising 85% of the total biofilm mass. The EPS is composed of biomolecules, exopolysaccharides and polypeptides forming hydrated polar mixture and extracellular DNA (eDNA). The EPS holds the cells together and protects them against physical factors as well as antimicrobial agents, including antibiotics (Femming et al. 2007). During biofilm formation, the planktonic bacteria are first reversibly adhered onto a surface suitable for growth. This is followed by irreversible bacterial attachment to form microcolonies in EPS matrix. The microcolonies expand progressively with time and their confluences develop more structured phenotypes with noncolonized spaces, which are in turn filled with bacteria covering the entire surface. Finally, the bacteria re-enter into planktonic state by dispersing from the sessile structure and spread and colonize other surfaces (Karatan and Watnick 2009).

Quorum signals coordinate the behavior of the bacteria in a population density-dependent manner. During growth, these signal molecules, secreted by the bacteria, accumulate in the surrounding environment with the increase in bacterial population density, until a critical threshold concentration is reached, following which certain sets of genes are activated. The term QS emphasizes the fact that a sufficient bacterial number or 'quorum' should be present to induce or repress expression of the target genes (Eber and Tümmler 2004). The quorum signals are small peptides in case of Gram-positive bacteria, acylated homoserine lactones (AHL) in the Gram-negative bacteria or autoinducer-2 (AI-2) in both. Natural AHLs mostly share conserved structural motifs, viz., a homoserine lactone ring unsubstituted at the β and γ positions, which is N-acylated at the R-position, with an acyl group derived from fatty acid biosynthesis. AHL synthesis occurs via a sequentially ordered reaction. The acyl side chain of AHL is synthesized by fatty acid biosynthesis pathway and homoserine lactone moiety is synthesized by S-adenosyl methionine, an amino donor for the formation of homoserine lactone ring which couples with acyl side chain to form AHL. Different proteins are involved in this pathway including acyl carrier protein, enoyl-ACP reductase, Fab I and AHL synthase. The AHL synthases are highly conserved enzymes in QS-regulating bacteria. Enoyl ACP is reduced by Fab I to produce acyl ACP which reacts with S-adenosyl methionine in the presence of AHL synthase enzyme to produce AHL. Another important protein involved in AHL biosynthesis is LuxR-type transcription factor which is involved in activating the expression and production of AHL (Rasmussen and Givskov 2006).

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Biofilm and QS in *Pseudomonas aeruginosa*

➢ Biofilm formation

Among the opportunistic pathogenic bacteria, *P. aeruginosa*, which produces several virulence factors, is an important human and plant pathogen, causing various infections in immunocompromised persons. *P. aeruginosa* causes the severe disease, cystic fibrosis and has the remarkable capacity of biofilm formation in diverse environment, thereby promoting chronic infectious diseases (Masak et al. 2014). *P. aeruginosa* produces at least three polysaccharides (alginate, Pel, and Psl) that determine the stability of the biofilm structure. Alginate, a linear unbranched polymer is composed of D-mannuronic acid and L-guluronic acid. They confer structural stability and protection to biofilms, as well help in water and nutrient retention. The Pel polysaccharide is mainly a glucose-rich matrix material, with still uncharacterized composition; Psl contains repeating pentasaccharide, made up of D-glucose, D-mannose and L-rhamnose. Both Pel and Psl serve as a structural scaffold for biofilm development and are involved at early stages of biofilm formation (Sutherland 2001). eDNA constitutes an important functional component of biofilm having the following functions (i) *P. aeruginosa* biofilm formation is prevented by exposure to DNase I; (ii) eDNA helps in twitching motility-mediated biofilm expansion by maintaining coherent cell alignments; (iii) biofilms deficient in eDNA are more sensitive to the detergent, sodium dodecyl sulfate; (iv) eDNA constitutes a nutrient source for bacteria during starvation; and (v) eDNA plays an important role in the initial and early development of *P. aeruginosa* biofilms (Gloag et al. 2013). The extracellular appendages, viz., flagella, type IV pili and cup fimbriae play adhesive roles in the cell-to-surface interactions and microcolony formation in biofilms.

➢ Quorum sensing

More than 9% of the assigned open reading frames (ORFs) of the fully sequenced *P. aeruginosa* genome encode known or putative transcriptional regulators and two-component systems. Presence of several regulators helps this bacterium to adapt to diverse environmental conditions. The QS system of *P. aeruginosa* constitutes two components, viz., LasR/I (transcriptional activator, LasR and the AHL synthase, LasI) and RhlR/I (transcriptional regulator RhlR and the AHL synthase RhlI). The LasI produces N-3-oxo-dodecanoyl-Lhomoserine lactone (3O-C12-HSL), which binds to the LasR protein at the threshold concentration, thereby regulating the expression of LasI, virulence factors and biofilm maturation. The 3O-C12-HSL molecule also helps *P. aeruginosa* to evade the host immune reactions. LasR positively regulates the expression of the extracellular virulence factor elastase, namely, LasB. *P. aeruginosa* employs AHLs as QS-signaling molecules which constitute either 3-oxo-C12-HSL or C4-HSL. The cells are freely permeable to C4-HSL, whereas active transport through the MexAB-OprM multidrug efflux pump is involved in the secretion of 3-oxo-C12-HSL. The *las* system was shown to be involved in the regulation of

various virulence factors, as well as *lasI* itself, thereby creating a positive regulatory feedback loop (Pearson et al. 1999).

The RhlI synthase in the Rhl system produces N-butanoyl-L-homoserine lactone (C4-HSL), which binds to the RhlR protein. The activated C4-HSL-RhlR complex stimulates the expression of RhlI, virulence genes and biofilm-associated genes. The LasR 3-oxo-C12-HSL complex positively regulates the transcription of RhlR and RhlI. The *rhlR* expression is dependent both on LasR and also on Vfr and RhlR itself, indicating that the gene is subject to negative autoregulation. RhlR forms a homodimer that could be dissociated into monomers by 3-oxo-C12-HSL. Since the *las* system is present at the top of the signaling cascade, QS systems in *P. aeruginosa* is regarded to be arranged hierarchically. The transcriptional regulation of the promoter region of the *rhlAB* operon encodes rhamnosyl transferase 1, the enzyme responsible for the biosynthesis of the surfactant rhamnolipid. RhlR binds to specific sequences, upstream of *rhlAB*, independently of the presence or absence of C4-HSL. However, transcription is activated in the former case, whereas RhlR represses transcription in the latter case. Such dual activator-repressor activities of LuxR-type regulators can fine tune QS in *P. aeruginosa* (Medina et al. 2003; Ventre et al. 2003).

There is a third *Pseudomonas* quinolone signal (PQS) system which functions via quinolone signal molecule, 2-heptyl-3-hydroxy-4(1H)-quinoline and provides a link between the *las* and *rhl* systems. On one hand, both the Las and the Rhl systems control the synthesis of PQS, while on the other, PQS controls the expression of RhlR and RhlI. The genes required for PQS synthesis are positively regulated by LasR, but negatively by the *rhl* system. Thus, the production of PQS is dependent on the ratio of 3-oxo-C12-HSL and C4-HSL, suggesting a delicate balance between the two QS systems. *P. aeruginosa* quinolone signal belongs to the family of 4-hydroxy-2-alkylquinolines (HAQs), well known for their antimicrobial activity. The biosynthesis of HAQs involves two operons, viz., *pqsABCDE* and *phnAB*. The *pqsABCDE* operon products synthesize 2-heptyl-4-hydroxyquinoline (HHQ), and the PqsH converts HHQ into PQS, which upon reaching a threshold concentration, binds to the regulator protein, PqsR and modulates the expression of virulence genes and synthesis of PQS itself, resulting in autoinduction. The *phnAB* encodes the two components of anthranilate synthase. The expression of *phnAB* and *pqsABCDE* is positively regulated by the virulence-related transcription factor, MvfR (pqsR), whereas 4-hydroxy-2-heptylquinoline (HHQ) is converted to PQS by PqsH, whose expression is controlled by LasR. Following the release of 4-hydroxy-2-heptylquinoline, it is again taken up by the cells, before it is converted to PQS. The quinolone signal constitutes an important part of the QS, as its exogenous addition promoted biofilm formation and positively affected production of multiple QS-controlled virulence factors, including lectins, pyocyanin and proteases (McKnight et al. 2000; Diggle et al. 2003). The regulation of three QS pathways are diagrammatically represented in Fig. 1.

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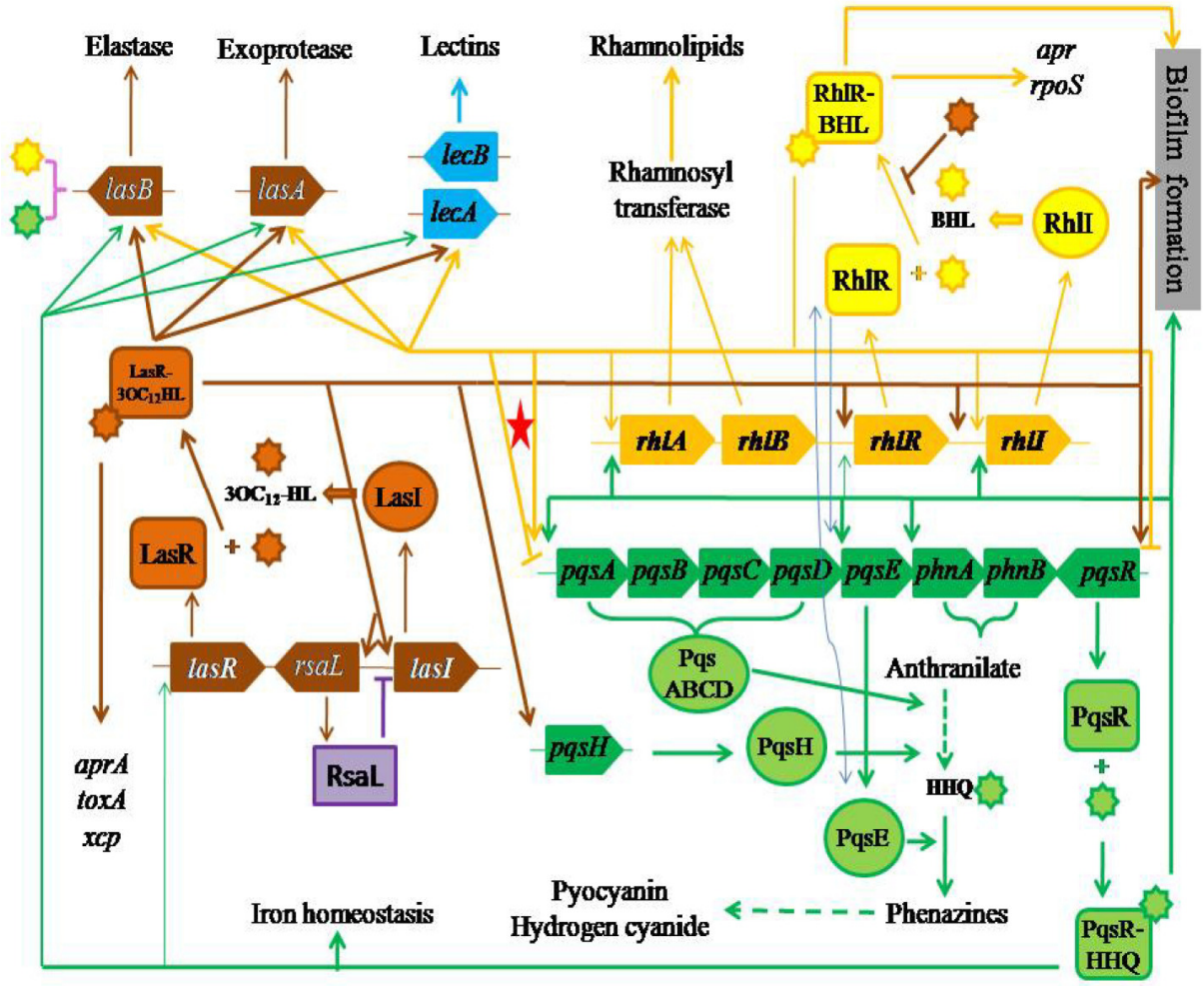

Figure 1.Quorum sensing pathways in *Pseudomonas aeruginosa* with their effects and regulatory pathways. The red star highlights bi-functional regulation of RhlI-RhlR system to PQS synthesis [adopted from Yan and Wu (2019), distributed under the terms of the Creative Commons Attribution License (CC BY)]

Quorum quenchers

The misuse and abuse of antibiotics creates selective pressure, leading to the development of resistant bacterial strains. Antibiotics also kill beneficial indigenous bacteria and are found to be less effective against biofilm-forming bacteria (English and Gaur 2010). Biofilms are difficult to be eradicated with antibiotics because of their resistance property which necessitates alternative way to get rid of the pathogenic micro-organisms involved in biofilm. To overcome these limitations of antibiotics, there is an increasing trend for the discovery and development of antimicrobial agents and exploration of their properties to efficiently control and manage bacterial diseases (Blackledge et al. 2013). Inhibition of bacterial virulence and/or biofilm formation by targeting the QS pathway is/are being increasingly explored. The present focus is to target the QS system by using chemical compounds that can inhibit the communication between microbes, either of the same or different species. Such

compounds are termed as quorum quenchers, which can be categorized into macromolecular quorum quenching enzymes and microparticulate quorum sensing inhibitors, on the basis of their molecular weight and chemical composition (Tang and Zhang 2014; Pejin et al. 2015a). Compared with the established treatments for infectious diseases, based on antibiotic compounds that kill or inhibit the growth of bacteria, non-toxic quorum quenchers do not kill or inhibit microbial growth and hence impose less selective pressure for the development of resistant strains. The quorum quenching phenomenon consists in the enzymatic degradation of signal molecules of the QS-system. These enzymes inhibit the production of autoinducers and virulence factors and expression of regulatory genes. An ideal quorum quencher should have certain desirable properties: (i) low molecular weight and chemically stable structure; (ii) highly specific to recognize and inhibit particular autoinducers; (iii) ability to decrease the expression of QS-regulatory genes; and (iv) lack of host cell cytotoxicity and metabolic neutrality (Kalia 2013). The common techniques for quantitative and qualitative analysis of quorum quenchers involve colorimetry, bioluminescence, chemiluminescence, fluorescence, chromatography-mass spectroscopy, and electrochemistry (Van der Meer and Belkin 2010). Various synthetic furanones, AHL analogs or other dissimilar compounds such as triphenyl compounds, salicylic acid, nifuroxazide, etc. have been observed to attenuate QS and its regulated phenotypes in Gram-negative bacteria such as *A. tumefaciens*, *P. aeruginosa*, *V. fischeri,* etc

➢ Sources of quorum quenchers

Natural quorum quenchers mostly originate from fungi and plants. Medicinal plants produce a wide spectrum of secondary metabolites such as terpenoids, phenolics, coumarins, tannins, flavonoids, saponins, quinones, alkaloids and polyacetylenes, against the QS-system (Pejin et al. 2014). The variation in inhibition potential of quorum quenchers depends on the structure and chemical composition of the compound. Curcumin, for example, produced from *Curcuma longa,* inhibits *P. aeruginosa* biofilm formation at early stages. The secondary metabolites like antibiotics are produced by *Penicillium* spp (Sarkar and Das 2019). However, bacteria are also known to harbor several enzymes that can inhibit QS, e.g., AHL-lactonase in *Agrobacterium tumefaciens* and lactonase in *Bacillus thuringiensis* can degrade AHL; AHL acylase in *P. aeruginosa* can degrade long chain AHLs, etc. Acylases from animal models can reduce biofilm formation in *P. aeruginosa*, while paraoxonases present in mammalian serum and human cell lines can hydrolyze esters and lactones (Kalia and Purohit 2011). The antibody XYD-11G2 has the ability to degrade 3OC12HSL of *P. aeruginosa* and hence acts as quorum quencher. The synthetic quorum quenchers are designed via several reactions, (i) substitution and alternate introduction in the acyl side chain to maintain the lactone ring, (ii) substitution and alternate introduction in the lactone ring to leave the acyl side chain unchanged, and (iii) extensive modifications of both acyl side chain and lactone ring. By introducing sulphur in the acyl side chain, instead of C3 atom, LuxR- and LasR-controlled QS was blocked (Rasmussen and Givskov 2006). Synthetic quorum quenchers

such as allyl cinnamate, cinnamyl alcohol and methyltrans-cinnamate, which are derivatives of cinnamic acid, inhibit violacein, the virulence factor produced by *Chromobacterium violaceum*. Biofilm formation can be also prevented in *Acinetobacter baumanii* by applying the analogues of AHL molecules that inhibit QS communication (Paluch et al. 2020).

➢ Mechanism of action of quorum quenchers

The quorum quenchers act in several ways. They cause degradation of QS signals enzymatically using three types of enzymes, namely (i) AHL-lactonase that hydrolyzes ester bond of the homoserine lactones ring in AHL, e.g., AHL-lactonase produced by *Bacillus cereus* VT96 directly controls exopolysaccharide production, biofilm formation and pyocyanin production in *P. aeruginosa* (Rajesh and Rai 2016); (ii) acylase that degrades amide bonds of AHL and forms 3-oxodecanoic acid and homoserine lactone (HSL); and (iii) oxidoreductase that modifies the activity of AHL, but do not degrade it. The bpib09 enzyme deactivates the 3-oxo C-12 homoserine lactone (3OC12HSL). All these enzymes, by inhibiting the production of autoinducers, prevent the production of virulence factors and biofilm production. Quorum quenchers may also suppress the biosynthesis of QS by hindering acyl-ACP generation, SAM synthesis or inactivating the enzyme responsible for synthesis. Analogues of SAM have proved to be potent inhibitors of the *P. aeruginosa* AHL synthase, RhlI. The holo-ACP, sinefungin, L/D-S-adenosylhomocysteine and butyryl-SAM are few examples of such compounds. The most effective, L-S-adenosylhomocysteine, was found to lower the activity of RhlI by 97% *in vitro* (Rasmussen and Givskov 2006). Anthranilate analog in *P. aeruginosa* inhibited PQS production (LaSarre and Federle 2013). Naringenin inhibited LuxR transcription factors by down regulating the expression of the *lasI* and *rhlI* genes, leading to reduced production of AHL molecules (Paluch et al. 2020). Another mechanism of action of quorum quencher is the inhibition of detected QS signal or blocking the signal transduction cascade, where non-productive signal-receptor complex inhibits the QS signal. The inductor antagonists compete with the inductors for the same binding site of the receptors; they block the inductor-mediated cell signal transmission by binding to the receptor non-competitively (Bodede et al. 2018). The kinase inhibitors like RWJ-49815, closantel and LY266500 result in inhibition of the QS signal molecules in Gram-positive bacteria. Two isothiocyanate-based probes, Itc-11 and Itc-12 covalently modify the Cys-containing ligand-binding, nucleophilic pocket of LasR in *P. aeruginosa*, thereby inhibiting QS (Sarkar and Das 2019). Savrin, a small molecule inhibitor interferes with AgrA, binds to DNA and inhibit RNAIII production (Sully et al. 2014). Sometimes, certain antibiotics may serve as quorum quenchers due to their alternate target behavior. Such mechanism of quorum quenching has been observed in *P. aeruginosa* where production of exotoxin A, elastase, proteases, DNase, leukocidin, and phospholipase C has been reported (LaSarre and Federle 2013). The endophytic bacteria like *Bacillus megaterium* strain B4, *Bacillus sp.* strain B3, *Bacillus sp.* strain B11 and *Brevibacillus borstelensis* strain B8,

separated from *Cannabis sativa* can disrupt QS signal in *C. violaceum* (DSM 30191) (Sarkar and Das 2019).

Quorum quenchers against *P. aeruginosa*: Source and action

➢ From microorganisms

Furanone derivatives are found naturally as pheromones, flavor compounds and secondary metabolites. Naturally occurring furanones such as halogenated furanones from Australian marine macroalga, *Delisea pulchra* inhibit bacterial infection and biofilm formation by *P. aeruginosa*. Unfortunately, most of these furanones contain halogens, are too reactive and toxic, making them unsuitable for human use (Hentzer et al. 2002). Malesevic et al. (2019) observed dose-dependent reduction of the virulence factors, viz., elastase, rhamnolipid and pyocyanin production by *P. aeruginosa* MMA83 and significant down regulation of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqs* and *mvfR* expression, using the quorum quencher *N*-octadecanoylhomoserine lactone (C18-HSL) present in the ethyl acetate extract of the Gram-negative bacterium, *Delftia tsuruhatensis* 11304. In another work by Sharma et al. (2019), hydrocinnamic acid, isolated from *Enterobacter xiangfangensis* PUFSTI26 attenuated pyocyanin production and repressed the expression of major QS-regulated genes and phenotypes, by interacting with LasR receptor, thereby acting as competitive inhibitor of autoinducer molecule, and averting autoinducer binding. Enzymes like AHL-lactonases, AHL-acylases and decarboxylases, identified in bacteria like *Erythrobacter, Labrenzia* and *Bacterioplanes*, that attack the lactone ring or degrade AHLs of different acyl chain lengths, particularly the 3-oxo-C12-HSL, inhibit the formation of *P. aeruginosa* PAO1 biofilm (Ghosh et al. 2019). The acetic acid and phenyl lactic acid found in probiotic bacterial strains like *Lactobacillus paracasei* subsp. *paracasei* CMGB and *Pediococcus acidilactici* M7 strain, isolated from newborn feces, were also found to be inhibit the QS-related genes and short-chain AHLs, elastase, protease and pyocyanin (Ghosh et al. 2019). The patulin and penicillic acid from *Penicillium* also acted as inhibitors of QS (El-Mowafy et al. 2014). The derivative of erythromycin (derived from *Saccharopolyspora erythraea*), viz., azithromycin, showed strong *P. aeruginosa* QS and biofilm inhibitory effect, along with lower production of the AHL signal molecules, C4-HSL and 3-oxo-C12-HSL. The alkylcyclopentanone terrein, isolated from *Aspergillus terreus*, antagonized QS-receptors and reduced virulence factors (elastase, pyocyanin, and rhamnolipids) in *P. aeruginosa* (Ghosh et al. 2019).

➢ Quorum quenchers from plant sources

From time immemorial, phytochemicals have been used as major bioactive compounds in the form of powders, decoctions and extracts against several diseases caused by microbial pathogens and have led to the development of many life-saving drugs. Various chemicals isolated from different plant parts have demonstrated the capability of interfering with the bacterial QS-signaling to resist biofilm formation and/or virulence to human systems. The

extract from *Terminalia bellerica* plant effectively inhibited the production of pyocyanin and EPS in *P. aeruginosa*. The compounds like peppermint oil and menthol (from *Mentha piperita*), eugenol (from *Syzigium aromaticum*, viz., clove), 6-gingerol (from ginger oil), rhein (from *Rheum palmatum*), nodakenetin (from *Peucedanum decursivum*), chrysophanol (from *Rheum officinale*), emodin (from *Rheum palmatum*), shikonin (from *Lithospermum erythrorhizon*), fraxin (from *Fraxinus chinensis*), alkaloids like caffeine (purine alkaloid) and derivatives of shikimic acid and polyphenols were all found to be effective as quorum quenchers against *P. aeruginosa*, of which emodin inhibited biofilm formation in *P. aeruginosa* and caused proteolysis of an AHL binding protein (Tham and Yeo 2012). Anti-biofilm activity was also observed for *Ananas comosus* (pineapple) extract or *Musa paradisiaca* (banana) water extracts which prevented the synthesis of *P. aeruginosa* virulence factors such as proteases, elastases and pyocyanin (Musthafa et al. 2010). Several quorum quenchers have also been recognized in some plants from South Florida like *Chamaesyce hypericifolia*, *Bucida buceras*, *Callistemon viminalis*, *Conocarpus erectus*, *Tetrazygia bicolour*, *Quercus virginiana*, *Nymphaea tetragona* and *Callistemon viminalis*, all of which were found to inhibit QS-controlled factors like LasA protease, LasB elastase and pyoverdin, and reduce the expression of *LasI, LasR, RhlI, and RhlR* genes in *P. aeruginosa* (Adonizio et al. 2008). Further studies with quorum quenchers in *P. aeruginosa* unveiled novel plant sources such as *Rhizophora* spp., *Ananas comosus*, *Manilkara zapota*, *Musa paradisiaca*, *Ocimum sanctum,* etc. whose extracts were capable to interfere with the AHL systems and virulence of the pathogen. The table underneath lists some medicinal plants, along with their active principles, that target *P. aeruginosa* and inhibit the QS-signals.

Source plant	Quorum quenchers	Target against	References
Conocarpus erectus	Ellagitannins, such as vescalagin and castalagin	Biofilm formation LasA and LasB elastase, pyoverdin	Gala and Desai (2014)
Garlic	Ajoene, an unsaturated disulphide compound (allyl sulfide); S-phenyl-L-cysteine sulfoxide and its derivatives, notably diphenyl disulfide	Inhibit rhamnolipids; sulfoxide derivative seems to interfere with both *las* and *rhl* systems, whereas the diphenyl sulfide only disturbs the *las* system.	Gala and Desai (2014)
Combretum albiflorum	Three flavonones, viz., naringenin, eriodictyol and taxifolin along with flavan-3-ol-catechin	Reduce the expression of QS genes, *lasI, lasR, rhlI* and *rhlR*, and further attenuate virulence factors such as pyocyanin, elastase and biofilm formation; naringenin also reduces the production of C4-HSL and 3-oxo-	Gala and Desai (2014)

		C12-HSL	
Scutellaria baicalensis	Baicalin (a flavonoid)	Reduce LasA protease, LasB elastase, pyocyanin, rhamnolipids, and exotoxin A production and down regulate the QS-regulatory genes, including *lasI, lasR, rhlI, rhlR, pqsR* and *pqsA*	Gala and Desai (2014)
Armoracia rusticana	Iberin, an isothiocyanate is chemically isothiocyanato-3-(methyl-sulfinyl) propane	Specifically and effectively targets the LasIR and RhlIR QS-networks and down regulate the QS-controlled production of rhamnolipids	Jakobsen et al. (2012)
Broccoli	Sulforaphane and erucin (isothiocyanates)	Inhibit the *P. aeruginosa las* and *rhl* system as well as biofilm formation	Gala and Desai (2014)
Terminalia	Phenolic compounds (tannins, ellagic acid)	Down regulate *lasIR* and *rhlIR* genes expression and inhibit AHL systems	Gala and Desai (2014)
Brassica oleracea	Natural isothiocyanates, sulforaphane and erucin	Inhibit AHL-regulated virulence and biofilm formation	Gala and Desai (2014)
Tinospora cordifolia	Ethyl acetate extract	Short and long chain AHL production	Gala and Desai (2014)
Tea	Terpinen-4-ol	Bind to the active site of LasR, RhlR and PqsR receptors; down regulate the expression of QS-genes like *lasI, lasR, rhlI, rhlR*, *rhlAB*, *lasB*, *aprA*, *toxA* and *plcH* at sub-minimum inhibitory concentration (MIC); attenuate virulence factors and biofilm formation	Bose et al. (2020)
Lagerstroemia speciosa	Extract	Down regulate QS-related genes (*las and rhl*) and their respective signaling molecules, N-acylhomoserine lactones, without affecting the growth of bacteria; significantly inhibit virulence	Singh et al. (2012)

		factors: LasA protease, LasB elastase, and pyoverdin production	
Plantain herb	Extract	Inhibit the virulence factors like pyocyanin, rhamnolipids, protease and alginate	Li et al. (2019)
Medicago sativa	L-canavinine	Inhibit the production of violacein in *Chromobacterium violaceum*, which is a QS- regulated process with AHL	Keshavan et al. (2005)
Cinnamomum camphora	Cinnamaldehyde	Sub-inhibitory levels of cinnamaldehyde down regulate las, rhl and PQS QS-systems	Topa et al. (2020)
Grape fruits	Furocoumarins (dihydroxybergamottin and bergamottin)	Inhibit biofilm formation	Gala and Desai (2014)
Myristica cinnamomea	Malabaricone C (a diarylnonanoid)	Inhibit the QS-regulated pyocyanin production and biofilm formation	Gala and Desai (2014)
Dalbergia trichocarpa	Oleanolic aldehyde coumarate	Inhibit the formation/maintenance of biofilm and expression of the *las* and *rhl* QS-systems as well as *gacA* gene. Consequently, the production of QS-controlled virulence factors, including rhamnolipids, pyocyanin, elastase and extracellular polysaccharides is reduced.	Gala and Desai (2014)
Platostoma rotundifolium	Cassipourol and β-sitosterol (triterpenoids)	Inhibit QS-regulatory gene expression in *las* and *rhl* systems	Gala and Desai (2014)
Forsythia suspense	Phillyrin	Decline the production of virulence factors such as rhamnolipid, pyocyanin and elastase	Zhou et al. (2019)
Centella asiatica	Flavonoids	Inhibit swarming and twitching motility and production of pyocyanin and biofilm	Vasavi et al. (2016)

➢ Other natural and Chemically-Synthesized Quorum Quenchers

A natural 2-(5H)-furanone, ascorbic acid, has been identified as a QS-analogue. Ascorbic acid provides a degree of protection against adhesion and colonization by some uropathogens. Sub-minimum inhibitory concentration (MIC) of sodium ascorbate led to significant depression of elastase, protease and haemolysin activities, together with inhibition of pyocyanin production, attenuation of biofilm formation and repression of QS-regulatory genes, *lasI, lasR, rhlI, rhlR, pqsR* and *pqsA* (El-Mowafy et al. 2014). Aspirin is widely used as antipyretic, anti inflammatory and thrombolytic agent. Aspirin (6 mg/ml) showed significant reduction of biofilm production and QS-signals in *P. aeruginosa*, including expression of elastase, total proteases and pyocyanin without affecting bacterial viability, and decreased the expression of *lasI, lasR, rhlI, rhlR, pqsA* and *pqsR* genes by 38%, 72%, 69%, 72%, 74% and 43% respectively. Moreover, the expression of *Pseudomonas* toxins exoS and exoY was reduced by 47% and 55% respectively (El-Mowafy et al. 2014). Chemically synthesized quorum quenchers include AHL analogs such as phenylpropionyl homoserine lactones and phenyloxyacetyl homoserine lactones. A series of cyclopentanols and other furanone derivatives also have quenching activity. However, all these compounds are toxic with limited use for mammalian cells (El-Mowafy et al. 2014). Hentzer et al. (2002) showed that a synthetic halogenated furanone compound, which is a derivative of the secondary metabolites produced by *Delisea pulchra*, was capable of interfering with AHL-mediated QS in *P. aeruginosa* and reducing the production of important virulence factors, indicating a general effect on target genes of the *las* QS-circuit. Kalaiarasan et al. (2017) chemically synthesized two novel QS-analogues, viz., N-[4-(4-fluoroanilno) butanoyl]-L-homoserine lactone (FABHL) and N-[4-(4-chlororoanilno) butanoyl]-L homoserine lactone (CABHL), which disabled the circuits of the QS-systems, reduced the expression of *rhlR* and particularly *lasR*, and served as anti-biofilm agents. Norspermidine a type of polyamine, was reported to reduce the expression of *lasI, lasR, rhlI, rhlR*, and *mvfR* genes that are involved in the QS system in *P. aeruginosa*, leading to reduced attachment to the surface and limited biofilm production (Qu et al. 2016). Combined treatment of cinnamaldehyde revealed an additive effect with the antibiotic colistin and tobramycin, with regard to more efficient biofilm inhibition and pre-formed biofilm dispersion, as compared to individual treatments, showing that cinnamaldehyde can increase the success of the antibiotic treatment (Topa et al. 2020).

Conclusion and future perspectives

Quorum quenchers have gained prime importance in designing novel drugs against bacterial infections, unable of being cured by the antibiotic treatment, or which require excessively high concentrations of antibiotics for prolonged durations. The organism, *P. aeruginosa* is emerging as a dreadful and notorious nosocomial pathogen, with its evolving antibiotic resistance and amazing adaptability to diverse environmental conditions. Pulmonary infections caused by *P. aeruginosa* biofilm are often too severe and fatal to be removed by antibiotic therapy. Therefore, it is indispensable to develop new drugs, not only to control

infections, but also to counteract the resistance mechanism (Pejin et al. 2015b). A huge number of plant-derived active metabolites have quorum quenching potentiality. It is necessary to establish the mode of action of theses quenchers and categorize them as narrow-spectrum or broad-spectrum quenchers. The narrow-spectrum quenchers will only target specific pathogens where this may be useful to specifically target a type of pathogen in a polymicrobial environment, but with limited clinical value. Because such quenchers can disrupt the biofilm integrity, the susceptibility of bacteria towards antibiotics is enhanced, so that the infections are removed much readily at lower antibiotic dosage. In that way, the quorum quenchers can play a magical role in therapy in the coming generations, either singly or in combination with antibiotics. However, one of the probable reasons for the failure of quorum quenchers in clinical studies under 'real life' could be evolution of bacterial resistance to quorum quenching compounds. Quorum quenching resistant strains, with lower uptake or higher efflux of QS-inhibitors, have already been reported either from laboratories or clinical samples. On the brighter side, such resistance to QS inhibition appears at a much slower rate than that noted for conventional antibiotics, since quorum quenchers generated a selective pressure only under conditions where QS is essential, whereas antibiotics generate a very strong selective pressure under all environmental conditions (Remy et al. 2018). The resistance mechanism also depends on the type of quorum quencher and its impact on bacterial growth. Many QS inhibitors have toxic activities and need to enter the cells in order to be active. To limit quorum quenching resistance, the quenching agents should be carefully chosen to keep growth deleterious effects minimal. Multitherapies appear as promising approaches against pathogens for limiting their proliferation, virulence and resistance emergence (Grandclément et al. 2016). The in vivo potential of the newly identified quorum quenchers, single or combined, should be tested along with multiple clinical trials in order to build up a database of the quencher phytochemicals for *P. aeruginosa*. Some of these like garlic, *C. erectus*, and *P. ginseng* have already shown significant in vivo potential of treating *P. aeruginosa* infections. Till date, most of the work has focused upon targeting AHL systems; however, disruption of the quinolone signaling in *P. aeruginosa* should also be attempted as an important anti-virulence target. An earlier effort to inhibit PQS system by using the enzyme 1H-3-hydroxy-4-oxoquinaldine 2, 4-dioxygenase (Hod) produced by the bacterium *Arthrobacter nitroguajacolicus* Ru61a, failed due to the cleavage of Hod protein by the extracellular proteases of *P. aeruginosa*, so that the enzyme proved to be ineffective as a quorum quencher (Pustelny et al. 2009). AHL mimics have been found that act as competitive inhibitors of Lux homologous proteins, which clearly suggest the probability for the existence of quinolone inhibitors in plants (Teplitski et al. 2000). The latest trend in the inhibition of QS is to use nanomolecules like Ag or ZnO-based compounds as quorum quenchers, since they can inhibit microcolony and biofilm formation. The nanoparticles possess potent antibacterial action, inhibit AI synthesis and degrade receptor proteins, thereby decreasing the production of virulence factors, such as elastase, pyocyanin and biofilm components. Future research should focus upon identification and exploration of more such nanoparticle-mediated quorum quenchers to eradicate *Pseudomonas* infections.

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