Repurposing phytochemicals as anti-virulent agents to attenuate quorum sensing-regulated virulence factors and biofilm formation in *Pseudomonas aeruginosa*

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Summary

Unregulated consumption and overexploitation of antibiotics have paved the way for emergence of antibiotic-resistant strains and ‘superbugs’. *Pseudomonas aeruginosa* is among the opportunistic nosocomial pathogens causing devastating infections in clinical set-ups globally. Its artillery equipped with diversified virulence elements, extensive antibiotic resistance and biofilms has made it a ‘hard-to-treat’ pathogen. The pathogenicity of *P. aeruginosa* is modulated by an intricate cell density-dependent mechanism called quorum sensing (QS). The virulence artillery of *P. aeruginosa* is firmly controlled by QS genes, and their expression drives the aggressiveness of the infection. Attempts to identify and develop novel antimicrobials have seen a sharp rise in the past decade. Among different proposed mechanisms, a novel anti-virulence approach to target pseudomonal infections by virtue of anti-QS and anti-biofilm drugs appears to occupy the centre stage. In this respect, bioactive phytochemicals have gained prominence among the scientific community owing to their significant quorum quenching (QQ) properties. Recent studies have shed light on the QQ activities of various phytochemicals and other drugs in perturbing the QS-dependent virulence in *P. aeruginosa*. This review highlights the recent evidences that reinforce the application of plant bioactives for combating pseudomonal infections, their advantages and shortcomings in anti-virulence therapy.

Introduction

Perpetual consumption of antibiotics for antimicrobial therapy has led to the widespread emergence of antimicrobial-resistant bacterial strains worldwide (Chadha and Khullar, 2021). Today, antimicrobial resistance (AMR) is one of the graver public health issues. Pathogens have evolved into multi-drug-resistant (MDR) varieties as a consequence of overexploitation and unregulated usage of antibiotics (Chadha, 2021). Confining to this, the ESKAPE pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* sp., have posed a severe challenge in combating infectious diseases (Mulani et al., 2019). The race between the discovery of newer antibiotics and superbug evolution is inclined towards the latter end. According to the statistics of the UN Ad hoc Interagency Coordinating Group on Antimicrobial Resistance, global drug-resistant infections could inflict 10 million deaths each year by 2050 and impel 24 million people into extreme poverty by 2030 (World Health Organization, 2019). This scenario is grimly reflected in developing countries such as India, where the antibiotic consumption rates have nearly doubled (Taneja and Sharma, 2019). Alarmingly, one-fifth of the world’s AMR-associated deaths have been projected to occur in India by 2050 (Dixit et al., 2019). Realizing this, the United Nations General Assembly at New York (2016) conducted a high-level meeting to address the global AMR issue (Laxminarayan et al., 2016). Therefore, it is a global need to armour alternative intervention and therapeutical stratagems against the rising burden of AMR. In this direction, secondary plant metabolites and bioactive phytochemicals have regained immense prominence among the scientific community. The roots of herbal medicine originate from the Ayurveda, Siddha and Unani, the ancient healthcare systems of Indian medicine derived from the Indian ancient literature: ‘Vedas’ (Sen and
Chakraborty, 2017). Research on traditional medicines has proven to be a cyansure for pharmaceutical industries as plants harbour repertoires rich in secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, saponins and polyphenols, which have been shown to resist pathogens and simultaneously boost the immune system (Behl et al., 2021). Their antimicrobial potential has proved to be beyond comprehension, with promising efficacy at extremely low doses (Chadha et al., 2021c). The antimicrobial properties of plants have been favoured worldwide due to their natural existence, easy and economic availability, formidable pharmacological properties and negligible cytotoxicity with the least probability of developing resistance compared to antibiotics (Cheesman et al., 2017). Despite the advent of molecular docking, drug discovery development and the rise of the ‘omics era’, bioactive phytochemicals prove to hold strong and fertile ground for their therapeutic application in medicine.

Among the ESKAPE pathogens, Pseudomonas aeruginosa is an extensively versatile gram-negative opportunistic pathogen associated with nosocomial infections (Kerr and Snelling, 2009). It is highly invasive and toxicogenic, and possesses an arsenal of diverse virulence factors ranging from the secretion of extracellular enzymes to exotoxins and biofilms to alluring but disarmig pigments (Chadha et al., 2021a). Nevertheless, its ability to be ubiquitous, furnished with remarkable tissue tropism and a multitude of intrinsic and acquired antimicrobial resistance mechanisms make it an obdurate and notorious pathogen (Pang et al., 2019). These factors synergistically orchestrate the manoeuvring of the host immune system and destruction of host tissues leading to high morbidity and mortality in immunocompromised individuals. P. aeruginosa is well known to be associated with respiratory tract infections, particularly pneumonia and chronic bronchitis in patients suffering from cystic fibrosis, acquired immune deficiency syndrome (AIDS), severe burn wounds, and gastrointestinal, osteomyelitis, bone and joint infections (Bodey et al., 1983). A major proportion of pseudomonal manifestations result from urinary tract infections infected due to the insertion of medical devices such as urinary catheters (Cole et al., 2014). Its role in the development of devastating eye infections such as keratitis, conjunctivitis, endophthalmitis, dacryocystitis and corneal ulcers with permanent loss of vision is well documented (Ferreiro et al., 2017). Apart from this, P. aeruginosa is also linked with septic shock, bacteraemia, meningitis, endocarditis and brain abscess in patients undergoing neurological procedures (Shah et al., 1999).

The pathogenicity of P. aeruginosa is regulated by a multitude of concatenated mechanisms, which in turn are stringently governed by a phenomenon called quorum sensing (QS) (Lee and Zhang, 2015). This resilient pathogen communicates and harmonizes at the intercellular level by releasing diffusible signalling molecules called autoinducers (AIs). The intricacy of the QS system in P. aeruginosa lies in the fact that the synthesis of AIs depends on the pathogen’s population density, which in turn corresponds to the expression of virulence factors, biofilm formation, progression and severity of the disease (Erickson et al., 2002). We have augmented the findings based on pre-existing and recent literature and illustrated the QS circuitry of P. aeruginosa in Fig. 1 along with the virulence machinery each QS system activates, driving the progression of bacterial infection.

This magnificent bacterial communication system has drawn tremendous attention from microbiologists because of its involvement in acute and chronic human infections. In this context, the global effects of the three QS systems in regulating the QS-associated virulence hallmarks of P. aeruginosa have been described recently (Chadha et al., 2021a). Thus, targeting the QS system can be a possible strategy to combat pseudomonal infections since it would directly impact the downstream virulence factors. This includes the QS inhibitors (QSIs) that target signal output, accelerating the degradation of AIs, or inhibit signal reception by directly associating with AIs, thereby armouring anti-QS response called quorum quenching (QQ). Since QS contributes like an auxiliary factor towards regulating virulence elements in P. aeruginosa, its abrogation using QSIs does not exert any selection pressure for the emergence of any anthropogenic resistance, which is generally seen upon conventional antibiotic administration (Rasmussen and Givskov, 2006). Additionally, a fascinating aspect of QSIs is that they attenuate the QS circuits of a particular pathogen at subbiotal concentrations (sub-MICs) without posing any stress or survival threats (Kalia et al., 2019). Hence, this seems to be a promising anti-virulence therapy to tackle pseudomonal infections. A plethora of traditional medicinal plants, herbs and their constituent phytochemicals that have been elucidated to exhibit prolific QQ potential against P. aeruginosa is also considered safe for therapeutic use (Vandeputte et al., 2010; Kumar et al., 2015; Lou et al., 2019; Topa et al., 2020). QSIs derived from various natural and synthetic origins have been documented to attenuate the virulence machinery in P. aeruginosa (Kalia, 2013). Their deployment in the biotechnological arena to counter human infections has recently been described (Kalia et al., 2019). Considering the colossal literature and tremendous potential of phytochemicals in therapeutics, this review is a humble attempt to recapitulate the findings by implicating the anti-virulent properties of plant bioactives in attenuating QS and virulence in P. aeruginosa.
Quorum Sensing circuitry: the dictator for pseudomonal virulence

Intercellular communication in bacteria stimulates coordinated responses to display a multitude of virulence traits that are not possible to achieve for individual cells. The signalling network in *P. aeruginosa* is perhaps one of the most complex yet profoundly studied examples among all the QS systems. It consists of multiple concatenated signalling pathways that synergistically regulate bacterial virulence, making *P. aeruginosa* a highly notorious and deadly human pathogen (El Zowalaty et al., 2015). The QS circuitry in *P. aeruginosa* represents a global regulatory system that directly or indirectly controls the expression of more than 10% of its genome (Schuster and Greenberg, 2006) and about 20% of the bacterial proteome (Deep et al., 2011). QS in *P. aeruginosa* is mediated through three main systems, termed Las, Rhl and Pqs. The LasR/LasI and RhlR/RhlI QS systems regulate the synthesis and signal transduction via N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) and N-butyryl-L-homoserine lactone (C4-HSL) AIs respectively. *LasI* synthesizes 3-oxo-C12-HSL, which activates the cytoplasmic receptor, LasR, which regulates the expression of genes associated with biofilm formation, haemolysins, proteases, elastases and exotoxin A production (Rutherford and Bassler, 2012). Similarly, *RhlI* synthesizes the C4-HSL, which associates with its cognate receptor RhlR and drives the expression of virulence genes responsible for producing pyocyanin, hydrogen cyanide, siderophores, elastases and alkaline protease, and regulating bacterial motility (Papenfort and Bassler, 2016), while the *Pqs* system is modulated using non-AHL signalling molecules called alkyl-4-quinolones. PQS, structurally known as 2-heptyl-3-hydroxy-4-quinolone, is chemically isolated from the AHL-mediated Las and Rhl systems (Reyes et al., 2020). It modulates the expression of genes involved in biofilm formation, inducing swimming motility and production of pyocyanin, proteases, elastases, rhamnolipids, pyocyanin, lectins and rhamnolipids (Jander et al., 2000; Cao et al., 2001; Duggleby et al., 2003). Although most of the virulence determinants of *P. aeruginosa* are regulated through QS, others such as type III secretion system (T3SS) and lipopolysaccharide (LPS) production are not (De Kievit et al., 2001; Pena et al., 2019). Apart from these three QS systems, the last decade witnessed speculations over the involvement of a fourth QS system in *P. aeruginosa*, called the integrated quorum sensing (IQS) pathway. However, studies have recently discredited its role in QS and asserted that IQS is an aeruginaldehyde and a by-product of pyochelin biosynthesis or degradation, putting an end to the previous conceptions of IQS to be...
a product of ambBCDE genes (Cornelis, 2020). Nevertheless, these three QS circuits play a quintessential role in the fortification and augmentation of virulence factors in *P. aeruginosa*.

Over the years, scientists have proposed different models describing the cross-talk between different branches of the QS signalling network. These have been in the limelight and sparked critical debates over the interaction of various QS systems in *P. aeruginosa*. In this regard, Lee and Zhang (2015) proposed that the three QS systems in *P. aeruginosa* are interconnected hierarchically, with the Las system occupying the keystone position regulating the signalling hierarchy. It has been shown to regulate both Rhl and Pqs systems (Lee and Zhang, 2015). Interestingly, a circular model establishing the interconnections between the QS systems in *P. aeruginosa* has been proposed recently (Maura et al., 2016). It was documented that MvfR (PqsR) directly controls the expression of lasR and rhlR during early phases of bacterial growth with RhlR acting as a direct repressor of the MvfR (PqsR) QS system during late exponential and stationary phases (Maura et al., 2016). On the contrary, binding of MvfR to lasR induces mvrR expression and the PqsH-assisted conversion of HHQ into PQS, thereby increasing MvrR activity. These mechanisms were shown to function as stringent autoregulatory loops, dictating the QS circuitry in *P. aeruginosa*. Nevertheless, the QS systems of *P. aeruginosa* communicate with one another via chemical molecules known as quorum sensing signalling molecules (QSSMs). The LasR-3-oxo-C12-HSL complex multimerizes and activates the transcription of rhlR, rhlI, lasI, and other pseudomonal virulence genes, resulting in a positive feedback loop (Kiratisin et al., 2002). The RhlR-C4-HSL complex also dimerizes and activates the expression of RhlR and its own regulon, thereby generating a second positive feedback loop (Ventre et al., 2003). LasR-3-oxo-C12-HSL complex regulates PqsR, the transcriptional regulator of pqsABCD operon stimulating the biosynthesis of PQS and the transcription of pqsH, the gene encoding a probable monooxygenase (Schröter et al., 2010). Additionally, PQS has been found to enhance the transcription of rhlI, thereby influencing the C4-HSL output and cumulative impact of the Rhl system (McKnight et al., 2000). Also, the expression of pqsR and pqs operon is inhibited by RhlR-C4-HSL, suggesting that the concentration ratio between 3-oxo-C12-HSL and C4-HSL plays a decisive role in the dominance of the Pqs signalling system (Cao et al., 2001). Although the Las and Pqs systems regulate the Rhl pathway like a QS-command workhorse, most virulence factors dependent on QS are principally triggered by the RhlR-C4-HSL complex. Therefore, these systems represent a cascade mechanism commanding the virulence artillery of *P. aeruginosa* after achieving a quorum. This magnificent bacterial communication system has drawn tremendous attention from microbiologists because of its involvement in acute and chronic human infections. Deciphering the precise molecular mechanisms and potential targets behind these communication systems can help scientists in developing novel antimicrobial drugs that will help in targeted and combative therapy.

**Orchestrating bioactive phytochemicals as potential therapeutics against *P. aeruginosa*: evidences from studies**

With the rise of advanced molecular techniques, countless researches across the globe are attempting to unravel the potential of novel antimicrobials. Unprecedented explorations during the 21st century have successfully established the mantle of bioactive phytochemicals in combating *P. aeruginosa* by impeding its QS, thereby attenuating its virulence factors and abrogating biofilm formation. Virulence factors and QS may be targeted by plant bioactives at distinct levels and/or multiple steps. Diverse plant bioactives and their derivatives have also been documented to attenuate the swimming, swarming and twitching motilities, which play a pivotal role in bacterial adhesion and biofilm formation in *P. aeruginosa* (Khan et al., 2019). We have depicted the chemical structures of bioactive phytochemicals reported to exhibit QQ, anti-virulence and anti-biofilm activities against *P. aeruginosa* in Fig. 2. It is evident from the figure that most QQ phytochemicals share a heterocyclic ring structure similar to that of AHL molecules. This structural confirmation may permit stable interactions with the QS receptors and their ability to degrade signal receptors (Kalai, 2013). For instance, ajoene, allicin and curcumin have structures similar to AHL side-chains but with different oxygenation levels, while in 6-gingerol, zingerone, eugenol, carvacrol and cinnamaldehyde, the lactone ring is replaced by an aromatic moiety making it challenging to open up. A similar trend can be seen with naringin, quercetin, naringenin, vitexin and baicalein, which share a complex polycyclic structure. Moreover, *in silico* studies have revealed that these phytochemicals strongly associate with the different QS receptors of *P. aeruginosa* (Kumar et al., 2015; Rathinam et al., 2017; Quecan et al., 2019; Bose et al., 2020a). However, these have not been confirmed by any interaction studies due to the non-proteinaceous nature of bioactive phytochemicals. Nevertheless, findings from molecular docking experiments have been positively correlated with the downregulation of essential QS genes in numerous studies, both *in vitro* and *in vivo* (Kumar et al., 2015; Xu et al., 2019; Bose et al., 2020b; Sharma et al., 2020b). This has repurposed plant-based bioactives as multifaceted...
compounds holding no bar for their deployment as futuristic anti-virulence therapies.

6-gingerol

Ginger oil, derived from *Zingiber officinale* (ginger), contains this phenolic bioactive that activates spice receptors located on the tongue. Kim et al. (2015) established the anti-quorum potential of 6-gingerol against *P. aeruginosa* using standard assays *in vitro*. At a concentration of 100 µM, 6-gingerol significantly downregulated the expression levels of *lasR* and *rhlR* alongside rhamnolipid production by about 60%. It was also predicted that 6-gingerol could directly associate with the QS master regulator LasR protein *in silico*, abrogating its functional role in the production of exoproteases, pyocyanin and pseudomonal biofilm. Moreover, the transcription of PQS operons, *phnAB* and *paqsB-E*, T3SS-related genes, QS-regulated ExoT and elastase was also repressed by 6-gingerol. More recently, 6-gingerol analogs have been employed as biofouling agents in reverse osmosis (RO) systems (Ham et al., 2019). Gingerol analogs disrupted QS activities and inhibited biofilm formation on RO membranes at significantly higher rates than 6-gingerol itself (~37% analogs vs. 22% gingerol), without altering them chemically or physically. The binding affinity of gingerol analogs to QS protein receptor LasR was also enhanced coinciding with the repression of *lasB* gene by 78%.

α-Terpineol

It is a terpene alcohol and an integral component of essential oils extracted from pine, tea tree, etc. acclaimed for its application in the fragrance industry. Preliminary screening of plant volatiles provided the first insight into a possible role of α-terpineol in QQ (Ahmad et al., 2015a). It was reported to inhibit violacein production in an established QS biosensor strain of *Chromobacterium violaceum* by more than 90%. In recent years, this hypothesis was affirmed by Bose et al. against *P. aeruginosa* using free and nanostructured lipid carriers (NLCs) containing formulations of α-terpineol (Bose et al., 2020b). Expression levels of key QS genes (*las, rhl*) and virulence-encoding genes including *rhlAB, aprA, lasB, toxA* and *plcH* were significantly

![Chemical structure of plant bioactives reported to exhibit quorum quenching and anti-virulence properties against *P. aeruginosa*. Structure of phytochemicals cited in this review has been drawn using ChemDraw Professional 17.0 (Perkin-Elmer, Shelton, CT, USA).](image-url)
diminished upon treatment with \( \alpha \)-terpinol. Swimming motility and biofilm formation were greatly reduced, as with the case of EPS, haemolysin, elastase and pyocyanin production. In silico analysis also suggested a strong binding affinity of \( \alpha \)-terpinol towards the QS receptors, LasR, RhlR and PqsR, accounting for its far-reaching effects in vitro. Furthermore, attempts to investigate the therapeutic role of \( \alpha \)-terpinol have been undertaken in vivo. A follow-up study in the murine keratitis model reported diminished bacterial count in corneal tissues with improved corneal histopathology and decreased inflammatory markers such as myeloperoxidase and reactive nitrogen intermediates upon treatment with nanolipoidal \( \alpha \)-terpinol (Bose et al., 2021). Interestingly, treatment with nanolipoidal \( \alpha \)-terpinol exerted an anti-biofilm effect on \( P. \) aeruginosa and stimulated immunomodulatory responses by enhancing the production of inflammatory cytokines, IL-2, TNF-\( \alpha \) and macrophage inhibitory protein-2 in the infected eyes.

**Ajoene**

Ajoene, an organosulfur bioactive derived from *Allium sativum* (garlic), has been extensively studied as a potent inhibitor of QS and QS-derived virulence factors. The first study in this regard surfaced when Harjai et al. (2010) showed that crude extracts of garlic significantly reduced the synthesis of QS signals and virulence machinery in *P. aeruginosa* in vitro. Consequently, *P. aeruginosa*-induced pyelonephritis mice with orally administered garlic extracts exhibited lower renal bacterial counts with their kidneys protected from any tissue destruction as compared to their untreated counterparts. However, this bioactive was identified from garlic extracts by Jakobsen and colleagues (Jakobsen et al., 2012). It was determined that ajoene downregulated the expression of key QS-regulated genes: lasA, chiC, lecA, rhlA, rhlB and pqsL in a concentration-dependent manner in vitro. Moreover, the anti-QS activity of ajoene was examined using reporter assays. Ajoene (100 \( \mu \)g ml\(^{-1} \)) attenuated biofilm production in *P. aeruginosa* by rendering them rhamnolipid deficient, thereby completely abolishing the lytic necrosis of PMNLs. It was even demonstrated in a murine model of pulmonary infection, where administration of ajoene (12.5\( \mu \)g g\(^{-1} \)) resulted in clearance of bacterial cells as compared to untreated mice.

Combination anti-virulence therapy using ajoene–ciprofloxacin against pseudomonal biofilms and biofilm-associated murine acute pyelonephritis has been investigated (Vadekeetil et al., 2016). The compounds were reported to function synergistically in vitro by inhibiting biofilm formation and swimming, swarming and twitching motilities in *P. aeruginosa*. Also, in vivo efficacy of this combinational administration was proved to be prolific with significantly reduced bacterial load in the renal tissue of treated mice in comparison with that of their infected controls and solo drug treatments. Alternate molecular mechanisms underlying the QS inhibition by ajoene have also emerged. Ajoene was reported to repress QS in *P. aeruginosa* by the virtue of small regulatory RNA (sRNA) inhibition (Jakobsen et al., 2017). Using reporter assays and qRT-PCR, the expression of RsmY and RsmZ sRNAs was found to be greatly reduced by ajoene in a dose-dependent manner during the early-stationary phase of bacterial growth. Recently, structural analogs of ajoene have also come into the limelight as potent inhibitors of QS mechanisms. These ajoene analogs were reported to successfully target QS activity, thereby inhibiting the virulence factors including the production of elastase, pyocyanin and rhamnolipid *in vitro* (Fong et al., 2017). The compounds were also shown to impede and weaken biofilm formation on implants by *P. aeruginosa* in a murine infection implant model.

**Allicin**

Garlic is a rich source of antioxidants, and anti-inflammatory and antimicrobial compounds. Another principle bioactive phytochemical derived from garlic is allicin (diallylthiosulfinate), an organosulfur compound also called as the ‘heart of garlic’. *In vitro* effects of allicin on the production of QS-controlled virulence factors and biofilm formation in *P. aeruginosa* were assessed using standard assays and fluorescence microscopy, respectively, by Lihua et al. (2013). The findings indicated that allicin (128 \( \mu \)g ml\(^{-1} \)) inhibited early bacterial adhesion up to 9 h following treatment, while EPS production was reduced by over threefold in a dose-dependent manner. Biofilm architecture also underwent a transition to a thinner and looser form. The production of exotoxin A and elastase was downregulated by threefold and 10-fold, respectively, while pyocyanin and rhamnolipid production was completely abrogated under subinhibitory concentration of allicin. Lately, the mechanisms underlying allicin-mediated virulence attenuation in *P. aeruginosa* were unearthed (Xu et al., 2019). It was revealed that allicin inhibited the production of pyocyanin (phzB2 and phzM genes), pyoverdine, elastase and rhamnolipids (*rhlA*, *rhlB* and *rhlC* genes) in a concentration-dependent manner. Concisely, qRT-PCR analysis showed prominent inhibition of *rhl*, *pqsA*, *pqsD* and *pqsR* transcripts, without affecting the las system. The suppression of *rhl* and *pqs* systems was substantiated when supplementation with C4-HSL and PQS rescued the pathogenic phenotype of *P. aeruginosa* PAO1 strain by restoring the production of virulence factors. These studies have...
established the ethnopharmacological importance of alli- 
cin as a therapeutic drug that can be exploited to disarm 
the QS-dependent virulence determinants of P. aerugi- 
 nosa.

Terpinen-4-ol
Terpinen-4-ol is a naturally occurring monoterpene, an 
isomer of α-terpineol that forms the principle bioactive in 
teat tree oil. Thyme essential oil containing numerous 
phytochemicals, including terpinen-4-ol, has been shown to 
suppress QS, thereby reducing twitching motility and 
pyocyanin production (~74%) in P. aeruginosa (Bukvički et al., 2016). The terpene alcohol negated biofilm formation 
and exhibited prolific anti-quorum activity by inhibiting 
the production of violacein (>90%) in the AHL- 
mediated QS reporter strain, C. violaceum (Kerekes et al., 2013). Further, Noumi et al. (2018) examined the 
QQ potential of terpinen-4-ol on swimming motility in 
PAO1. Migration capabilities of the PAO1 strain were 
majorly inhibited by 25% in the presence of terpinen-4- 
ol. Moreover, this monoterpene was also shown to abro- 
gate biofilm production and simultaneously inhibit the 
adhesion of Staphylococcus aureus to polystyrene and 
glass surfaces.

Most recently, an elaborate investigation shed light on 
the potential of terpinen-4-ol to attenuate QS pathways, 
virulence determinants and biofilm formation in P. erugin- 
osna (Bose et al., 2020a). The QQ ability of terpinen-4- 
ol was assessed in silico with QS receptors, providing 
strong evidence for its interaction with LasR, RhlR and 
PqsR. Subinhibitory levels of terpinen-4-ol were found to 
downregulate the expression of lasI, lasR, rhl, rhlR, 
rhlAB, lasB, aprA, toxA and plcH QS transcripts in P. aeruginosa PAO1 strain. Further, the production of alginate, elastase, haemolysin and pyocyanin was found to be 
significantly reduced along with swimming, swimming and 
twitching motilities upon treatment with terpinen-4-ol, 
corroborating the findings of virulence gene expression 
profile. Interestingly, biofilm architecture was disrupted 
leading to fragile biofilm with significantly reduced bacte- 
rial adherence. Nevertheless, a novel combinational 
approach using terpinen-4-ol and ciprofloxacin proved to act synergistically against P. aeruginosa and its associ- 
ated virulence factors, paving way for the innovation of 
novel anti-virulence therapies.

Zingerone
Zingerone, also called vanillylacetone, is another bioac- 
tive derived from ginger responsible for imparting a 
sweet flavour. Researches over the years have pro- 
claimed its diverse pharmacological properties as a 
potent anti-inflammatory, anti-diarrhoeic and anti-diabetic 
agent (Ahmad et al., 2015b). Nonetheless, the use of 
zingerone for silencing QS mechanisms and negating 
biofilm formation in P. aeruginosa has been assessed by 
various investigators. Supplementation with zingerone at 
sub-MICs impeded swimming, swimming and twitching 
moieties by more than 50% as compared to its absence 
(Kumar et al., 2013). SEM analysis revealed that zinger- 
one prevented biofilm formation by P. aeruginosa on 
catheter surface without affecting bacterial planktonic 
growth. Also, a combination of ciprofloxacin and zinger- 
one was shown to completely eradicate preformed bio- 
film of P. aeruginosa at significantly higher rates. In a 
follow-up study, Kumar et al. (2015) demonstrated that 
zingerone drastically truncated the production of QSSMs: 
C12-HSL, C4-HSL and PQS in clinical isolates of P. aeruginosa and standard strain PAO1. In consequence, 
the production of virulence factors such as pyocyanin, 
haemolysins, elastases, proteases and rhamnolipids was 
also inhibited. This QQ capability of zingerone was 
adjudged by in silico studies, where its affinity towards 
QS receptors was determined. Interestingly, molecular 
docking showed profound interaction between zingerone 
and receptor binding pockets of LasR, RhlR and PqsR 
attributed to the inhibition of these QS systems in P. aeruginosa.

In an attempt to enhance drug delivery with high 
specificity and efficacy, the development of nanoformula- 
tions is on the move. In this regard, zingerone-loaded 
chitosan nanoparticles (Z-NPs) have been developed with 
promising anti-virulence properties against P. aerugin- 
osna. The Z-NPs were reported to be stable under vary- 
ing temperatures, inhibiting the production of QSSMs in 
P. aeruginosa using a reported assay employing 
Agrobacterium tumefaciens A136 strain (Sharma et al., 
2020b). The production of alginate, elastases, haemolys- 
sins, proteases and pyocyanin was drastically reduced 
when P. aeruginosa was passaged under sub-MIC of Z- 
NPs as compared to zingerone alone. Moreover, all the 
bacterial motilities were inhibited by more than fourfold, 
while qRT-PCR studies indicated that expression of 
lasR, rhl and rhlR was downregulated by more than two- 
fold in the presence of Z-NP at sub-MIC level. As a 
result, an anti-biofilm response was observed with Z- 
NPs, which successfully prevented biofilm formation and 
eradicated preformed biofilms of P. aeruginosa. 
Recently, the ex vivo and in vivo efficacies of Z-NPs 
were examined against P. aeruginosa in a murine model 
of acute pyelonephritis (Sharma et al., 2020a). Z-NPs 
were reported to be nontoxic to HEK-293 cells (~94% 
survival rate), reducing the pathogenicity of P. aergui- 
osna and ensuring the elimination of bacteria from renal 
tissues with better recovery. Additionally, Z-NPs 
enhanced the serum bactericidal property, accelerated 
the phagocytic uptake and killing of P. aeruginosa cells

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by mouse macrophages ex vivo and induced an anti-
biofilm response in vitro. These evidences have outlined
the significance of zingerone as an alternative to antibi-
otics in combating pseudomonal infections.

Curcumin

Curcumin is the major bioactive present in Curcuma
longa (turmeric) imparting a bright yellow colour to the
modified stem. It is extensively used for food flavouring
and colouring, and as a cosmetic ingredient. Apart from
possessing broad-range antimicrobial activity, curcumin
has been reported to exhibit profound QS property against
P. aeruginosa. Studies pioneered by Rudrappa and Bais
reported that sub-MICs of curcumin (3 µg ml⁻¹) inhibit bio-
film formation, production of QSSMs and QS-regulated
virulence elements including protease (50%), elastase
(50%) and pyocyanin (~70%) in PAO1 (Rudrappa and
Bais 2008). Supplementing exogenous curcumin signifi-
cantly prolonged survival rates and rescued killing in
PAO1-infected C. elegans. These outcomes were sub-
stantiated by transcriptome analysis, which indicated the
downregulation of 31QS genes in the presence of curc-
min sub-MICs. Similarly, curcumin displayed anti-QS
activity by inhibiting violacein production by ~89% in the
C. violaceum biosensor (Packiavathy et al., 2014). At 100 µg ml⁻¹, curcumin effectively dislodged pseudomonal
biofilms by 89%, retarding swimming and swimming motil-
ities of P. aeruginosa by sevenfold and fivefold, respec-
tively, alongside decreasing the production of alginate
and rhamnolipids by more than 50%.

In an attempt to target P. aeruginosa, the synergistic
effect of curcumin-natural honey (ChC) mixture on QS
and virulence factors was examined in vitro (Jadaun
et al., 2015). ChC significantly lowered the production of
C12- and C4-AHLs, alginate, rhamnolipids, haemolysins,
pyocyanin, pyoverdine, pyochelin, LasB elastase and
LasA protease than honey or curcumin alone. Using an
enzyme-coupled reporter assay, the expression of piv-
otal las and rhl QS genes was reduced by more than
60% and 50% respectively. Interestingly, biofilm for-
mation was completely abolished with ChC, thereby
increasing the susceptibility of bacterial cells to car-
bapenems and cephalosporins. In the same direction,
Bahari et al. (2017) showed that supplementing cur-
cumin at sub-MIC levels during bacterial growth drasti-
cally reduced the MICs of azithromycin and gentamicin
against P. aeruginosa. The synergistic interaction
between curcumin at sub-MICs and antibiotics also
quenched QS signals, resulting in downregulation of las
and rhl genes, accompanied by a reduction in the
swarming and twitching motilities and biofilm formation
capability of PAO1. Also, nanocomposites of zinc–cur-
cumin have been developed to enhance the delivery and
bioavailability of this QQ bioactive inside P. aeruginosa
(Prateeksha et al., 2019). The nanof ormulations were
reported to vanquish LasR and RhlR QS systems,
thereby protecting PAO1-infected mice and lung epithe-
lial cells by diminishing the expression of virulence fac-
tors, biofilm formation without hindering bacterial growth.
Moreover, curcumin-coated medical implants have been
developed to combat nosocomial infections. Coating of
curcumin-loaded gold nanoparticles on surgical sutures
and chitosan–curcumin complexes on stainless-steel
substrates showed promising antimicrobial activities with
high stability and sustained release of curcumin (Sunita
et al., 2017; Virk et al., 2019). Therefore, curcumin can
be exploited as a potent bioactive against P. aeruginosa,
laying a strong foundation for its application in anti-
virulence therapy.

Cinnamaldehyde

Cinnamaldehyde, derived from Cinnamomum, is a
phenylpropanoid and the major component of cinnamon
oil that imparts the spice its distinct flavour and aroma.
In preliminary studies, it was shown to effectively inhibit
the AHL-mediated QS systems of Vibrio harveyi and bio-
film formation in P. aeruginosa (Niu and Gilbert 2004;
Niu et al., 2006). An elaborate study has unravelled the
QQ potential of cinnamon oil in vitro (Leoni et al., 2015).
The authors indicated that cinnamon oil quenched the
synthesis of AHLs, thereby inhibiting biofilm formation,
swarming motility and production of pyocyanin, alginate
and proteases in PAO1. In the same light, sub-MICs of
cinnamaldehyde were reported to inhibit swarming motil-
ity and disrupt pseudomonal biofilms by lowering the
levels of intracellular cyclic di-GMP (Topa et al., 2018).
A breakthrough study by Ahmed and colleagues
reported the anti-QS response of trans-cinnamaldehyde
at sub-MIC levels against P. aeruginosa (Ahmed et al.,
2019). Cinnamaldehyde (2.2 mM) stimulated the down-
regulation of las and rhl QS systems, particularly lasR
and lasI transcripts by sevenfold and 13-fold, respecti-
vely, during the stationary phase of bacterial growth.
This accounted for the reduced production of elastase,
pseudomonal biofilms, pyocyanin and proteases by
22%, 26%, 32% and 65%, respectively, and repressed
rhamnolipid expression (rhlA) by 100-fold in PAO1. Fur-
ther, the combinational effect of cinnamaldehyde and
antibiotics on QS was evaluated recently (Topa et al.,
2020). Synergism was confirmed with colistin (as anti-
microbial) and tobramycin (as QSI). Cinnamaldehyde
(1.5 mM) alone induced the repression of lasB, rhlA and
pqsA genes by ~50, 60% and 45% respectively. The effect
was further enhanced in the presence of tobramycin by ~70%
for the three test genes. On the contrary, combinational effect with colistin

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and tobramycin enhanced the anti-biofilm efficacy by 75% and 84%, respectively, compared with cinnamaldehyde alone (~ 30%). Additive activities also accelerated the dispersion of preformed PAO1 biofilms by ~ 90% in case of both antibiotics. Interestingly, cinnamaldehyde-encapsulated chitosan nanoparticles at sub-MIC levels were developed to attenuate QS and biofilm formation in P. aeruginosa (Subhaswaraj et al., 2018). The nanofluid formulations were shown to hinder the swimming and swarming motilities of PAO1 with notable inhibition of pyocyanin, LasA protease, EPS, biofilm and rhamnolipid production as compared to cinnamaldehyde alone. Since this bioactive exhibits magnificent QQ activity, it is most likely to be the game-changer towards the fight against this notorious pathogen.

Eugenol

Eugenol is a phenolic component obtained from various plant products, majorly spices, herbs and oils. Its broad-range antimicrobial properties have been known for years, while its QQ ability has recently surfaced in the last decade. Eugenol was identified as the primary component in clove oil responsible for inhibiting QS-controlled gene expression (Zhou et al., 2012). Sub-MICs of eugenol (200 µM) were reported to diminish violacein production (~ 50%) in C. violaceum F026 biosensor along with QS-controlled virulence factors: elastases (~ 32%), pyocyanin (~ 60%) and biofilm formation (~ 36%). These downstream effects were reported to be an outcome of las and pqs QS system inhibition by eugenol. Similarly, sub-MICs of methyl eugenol (30 µg ml⁻¹) were shown to inhibit the swimming (~ 66%) and swarming (~ 24%) motilities, and biofilm formation in PAO1 by 90% (Packiavathy et al., 2012). Another study affirmed the eugenol-mediated attenuation of PAO1 QS system and QS-associated virulence elements by abrogating the synthesis of QSMMs (C12-HSL, C4-HSL) and directly repressing the transcription of lasR, lasI, rhlR and rhl QS genes (Rathinam et al., 2017). In silico analysis coupled with in vitro reporter assays confirmed the competitive binding of eugenol to the LasR QS receptor, thereby driving the repression of QS-regulated genes. Recently, eugenol nanoemulsions were developed by Lou et al., (2019) with high QQ efficacy against P. aeruginosa, repressing the transcription of lasI and rhl genes and inhibiting the production of violacein, pyocyanin, rhamnolipids and pseudomonal biofilms at a significantly higher rate than eugenol alone.

Citral

Citral is a terpenoid that constitutes about 70–80% of lemongrass oil and imparts the herb its characteristic aroma and taste. Preliminary investigations using reporter assays have pointed towards the inhibition of C12-HSL-mediated QS systems of P. putida by citral at relatively higher concentrations (Colorado et al., 2012). Citral has been reported to inhibit violacein production by 96.18% in C. violaceum biosensor, thereby asserting its role as potent QSI (Rueda and Salvador 2020). For P. aeruginosa, sub-MIC of citral (840 µg ml⁻¹) was shown to inhibit swarming motility by 93% with a significant reduction in the levels of pyoverdine production. For enhanced efficacy against QS and biofilm formation, various derivatives of citral (CD1, CD2 and CD3) have been developed and tested in vitro. These derivatives abrogated biofilm production and downregulated the expression of QS genes in C. violaceum, without altering bacterial growth (Batohi et al., 2021). This can serve as a template that can be tested against QS systems and virulence factors of P. aeruginosa.

Interestingly, a study focused on enhancing citral’s solubility and QQ potential by synthesizing its glycomonoterpeine via conjugation with glucose (Patil et al., 2016). The conjugate was shown to abolish QS by inhibiting the production of violacein, pyoverdine and pseudomonal biofilms at significantly lower concentrations than citral itself, signifying enhanced bioavailability and delivery of citral to attenuate the virulence of P. aeruginosa. A virtual screening study recently identified citral as a potential QSI against P. fluorescens (Ding et al., 2019). Molecular docking revealed that citral exhibited a high docking score (121.52) with the active site LuxR receptor and speculated this interaction to be stabilized via hydrogen bonds, carbon-hydrogen bonds and π-alkyl bonds. In vitro experiments showed that citral exhibited promising anti-QS activities against P. fluorescens, thereby inhibiting the swimming motility, biofilm formation, and production of extracellular proteases and siderophores, without hindering bacterial growth. However, the precise mechanism that citral employs for QQ remains to be explored.

Coumarins

Coumarins are secondary plant metabolites that constitute a large family of fused benzene and pyrone rings. Few coumarins that exhibit antimicrobial activity have also been regarded as phytoalexins, produced in response to pathogenic infection. Furrocoumarins present in grapefruit juice were shown to repress the activities of AI-1 and AI-2 by more than 95% in V. harveyi reporter strain indicating their anti-QS potential (Girennavar et al., 2008). Moreover, biofilms of P. aeruginosa were inhibited by 18% and 27% by furrocoumarins dihydroxybergamottin and bergamottin, respectively, without altering bacterial growth. In a virtual screening study, the coumarins,
et al. nalling by lowering its cellular levels by 36.4% compared
ingly, coumarin led to the disruption of cyclic di-GMP sig-
0.9 and 7.9 mM inhibited
cated that carvacrol at concentrations ranging between
P. aeruginosa
et al. P. aeruginosa
and substantiated with subsequent experi-
detecting anti-QS compounds (Fakunle
P. aeruginosa
that its sub-MICs inhibit QS and bio
gent odour. Early investigations on carvacrol showed
derived from the oregano plant and bears a warm, pun-
fi
virulence in
P. aeruginosa
was also reduced upon exposure to coumarin and
its derivatives. Recently, the precise mechanism of
coumarin-mediated QQ in P. aeruginosa was unearthed
(Chen et al., 2016; Peng et al., 2019). Recently, baicalein at subinhibitory concentrations
in vitro
P. aeruginosa
also showed a significant reduction in the synthesis of pyocyanin, LasA protease and LasB elas-
tase were diminished (Rodriguez et al., 2019). The find-
ings were further substantiated when carvacrol was
reported to interact with the amino acid residues of LasR and LasI active domains, repressing lasR and inhibiting
LasI activity. This establishes its potency as a novel quo-
rum quencher against pseudomonal virulence factors at both enzymatic and gene levels.

Baicalein
Baicalein is a trihydroxyflavone derived from the roots of
Scutellaria baicalensis, renowned for its use as a tradi-
tional herbal medicine with antioxidant and antimicrobial
activity. It has been shown to attenuate virulence in S.
aureus and E. coli by abrogating QS mechanisms and
biofilm formation in vitro (Chen et al., 2016; Peng et al., 2019). Recently, baicalein at subinhibitory concentrations
has been elucidated to attenuate the QS circuitry in P.
aeruginosa. The synthesis of QSSMs, C4-HSL and C12-
HSL was lowered by twofold along with significant down-
regulation of both las and rhl QS genes (Seleem et al., 2017).
This resulted in the global attenuation of virulence factors such as swimming, swimming and twitching motil-
ities, biofilm formation, and the production of alginate,
LasB elastase, LasA protease, pyocyanin and rhamno-
lipids. Interestingly, the production of inflammatory
 cytokines, IL-1β, IL-6, IL-8 and TNF-α, in P. aeruginosa-
infected macrophages was also reduced significantly
alongside suppression of MAP kinase and NF-κB sig-
alling pathways (Luo et al., 2016). This indicated baica-
lein’s potential as an anti-virulence drug against P.
aeruginosa. In a similar study, Luo et al. (2016) illus-
trated the effects of baicalein sub-MICs on QS and viru-
ence factors in vitro alongside in vivo investigations with P. aeruginosa-infected C. elegans and murine model of
peritoneal implant. Baicalein was reported to repress the
expression of pivotal QS-regulatory genes, including lasl, lasR, rhl, rhlR, pqsA and pqsR, thereby limiting the pro-
duction of QSSMs resulting in the downregulation of glo-
bal virulence machinery and biofilm formation of P.
aeruginosa in vitro. Baicalein-treated C. elegans showed
greater survival rates, while it accelerated the clearance of
bacterial cells from the implants of infected mice com-
pared with the untreated group. Administration of baica-
lein instigated inflammatory responses via activation of
Th1-induced immune response to enhance the elimina-
tion of pathogen, augmenting its anti-QS agent and anti-
biom potential.

Naringenin and naringin
Naringenin is a colourless flavone, an integral bioactive
component of citrus fruits, predominantly grapefruit. Its
O-glycosylated derivative, naringin, is another principle phytochemical that imparts bitterness to citrus fruits. Both these flavones have been reported to exhibit QQ activities against \textit{P. aeruginosa}. Naringenin was first reported to quell the production of QS-regulated virulence factors of \textit{P. aeruginosa} (Vandeputte et al., 2011). The production of elastase and pyocyanin was inhibited by 46% and 87% in the presence of naringenin respectively. This reduction occurred by the virtue of downregulated \textit{lasI}, \textit{lasR}, \textit{rhl}, \textit{rhlR} and \textit{lasB} expression, sharply declining the production of QSSMs, C4-HSL and C12-HSL in naringenin-treated cultures of \textit{P. aeruginosa} \textit{in vitro}. Intriguingly, QS inhibition by naringenin was not limited to the reduction in QSSMs but also related to the defective functioning of the \textit{RhlR}-C4-HSL complex. A study pointing towards the precise molecular mechanism of naringenin-mediated QQ has recently surfaced. It was revealed that naringenin outcompeted C12-HSL for binding to its cognate LasR receptor in a time-dependent manner (Amado et al., 2020). LasR inhibition was heightened when naringenin was directly associated with the nascent receptor form than its activated form. Amusingly, naringenin disrupted the expression of QS genes and QS-regulated virulence genes during the early phase of bacterial growth, while the inhibitory effect was lost upon transition to the stationary phase. This suggested that competition between C12-HSL and naringenin for the LasR receptor is a function of bacterial growth.

Naringenin has also been shown to exert similar anti-QS effects. It was initially demonstrated to disarm the QS machinery in the enteric pathogen, \textit{Yersinia enterocolitica}, by inhibiting swimming and swarming motilities along with biofilm formation (Truchado et al., 2012). Naringenin has recently been shown to effectively eradicate pseudomonal biofilms on inanimate and catheter surfaces, inhibiting swimming motility and production of EPS matrix (Dey et al., 2020). In contrast, naringenin’s antibiofilm and anti-virulence efficacy against \textit{P. aeruginosa} was appreciably augmented upon supplementation with antibiotics, ciprofloxacin and tetracycline compared with their solo treatment. These evidences establish that both naringenin and naringin are potential QQ and anti-biofilm agents against \textit{P. aeruginosa}.

Quercetin

Quercetin is a pentahydroxyflavone recovered from pericarps of fruits, seeds and leafy green vegetables with extensive anti-inflammatory, antioxidant and anti-cancerous properties. Early investigations reported the ability of quercetin at sub-MICs to impede biofilm formation (~95%) and twitching motility in \textit{P. aeruginosa} (Pejin et al., 2015). The adverse effect of quercetin at sub-MICs and its synergism with various antibiotics (at sub-MIC) against pseudomonal biofilms has also been elucidated (Vipin et al., 2019). Several studies on this aspect have discretely pointed towards the QQ potential of quercetin, abrogating the virulence determinants of \textit{P. aeruginosa}. A breakthrough study indicated that quercetin did not hinder bacterial growth but triggered the inhibition of biofilm formation (50%) and the production of elastase, protease and pyocyanin significantly (Ouyang et al., 2016). Quercetin at 16 µg ml⁻¹ led to the notable repression of essential QS genes including \textit{lasI}, \textit{lasR}, \textit{rhl} and \textit{rhlR} by 34%, 68%, 57% and 50% respectively. This laid down the foundation for exploring quercetin as a potential therapeutic.

Further concrete evidences have been produced to prove this point from multiple investigations by independent research groups. Quercetin at sublethal concentrations (5–40 µg ml⁻¹) inhibited the production of alginate by 9–65%, bacterial biofilms by 8-80% and EPS matrix by 15–73% (Gopu et al., 2015). Swimming and swarming motilities were reduced significantly with quercetin (80 µg ml⁻¹) by 50% and 75% respectively. Molecular docking of LasR receptor with quercetin revealed strong interactions, abrogating QS by a potential change in the receptor conformation upon quercetin binding. Quecan et al. (2019) reported the QQ potential of quercetin aglycone and quercetin 3-[3]-D-glucoside against \textit{P. aeruginosa}. The bioactives inhibited swarming motility and production of violacein \textit{in vitro}, while biofilm formation remained unaffected. However, in \textit{vivo} analysis suggested effective binding of the two quercetins with the AHL-binding domain of LasR receptor of PAO1, providing a rationale for its anti-virulence effects. It has also been proposed that quercetin retards biofilm formation in \textit{P. aeruginosa} via inhibition of \textit{vfr} and biofilm-related genes (pelA, pslA), regardless of the presence of QS genes (Ouyang et al., 2020). This was speculated to be a consequence of \textit{lasI}/\textit{lasR} inhibition by quercetin, affecting the expression of \textit{vfr} gene, resulting in abrogation of pseudomonal biofilm. Thus, quercetin represents a promising candidate that requires precise evaluation in animal models for its deployment in anti-virulence therapy against \textit{P. aeruginosa}.

Caffeine

Caffeine, or 1,3,7-trimethylxanthine, is a natural ingredient in beverages such as coffee and tea and is often proclaimed for its CNS stimulation and antidepressant properties. Although caffeine has been in use for centuries since its discovery in 1819, its anti-QS potential was revealed in the last decade. The incentive study reported caffeine to impede violacein production in \textit{C. violaceum} in a dose-dependent manner without exerting any antibacterial activity (Norizan et al., 2013). Caffeine
did not stimulate the degradation of AHLs but reduced the violacein production induced by PAO1 AHLs indicating its role in repealing the synthesis of short-chain AHLs (C6-HSL) in PAO1. Swarming motility of PAO1 was also inhibited by caffeine (300 μg ml⁻¹) with short and undefined tendrils. In a similar study, caffeine was identified as the major constituent of fenugreek seed extract responsible for QQ activities (Husain et al., 2015). At 200 μg ml⁻¹, caffeine reduced violacein production by 87% in C. violaceum and diminished elastase, protease and pyocyanin production by 68%, 78% and 74% in PAO1 respectively. Caffeine was also shown to inhibit swarming motility (70%) and biofilm formation (64%) in PAO1. The latest study on caffeine’s potential to target QS corroborated the previous findings using bench-based experiments and in silico studies. It was confirmed that caffeine at sub-MIC levels (80 μg ml⁻¹) inhibited the production of proteases (~65%) and pyocyanin (~60%), retarded biofilm formation by 50%, and disrupted swarming motility at significant rates in P. aeruginosa (Chakraborty et al., 2020). Additionally, molecular docking analysis confirmed these findings by suggesting strong interactions between caffeine and LasR, LasI proteins attributing this bioactive as a potential anti-QS compound.

Apart from the phytochemicals mentioned above, other bioactives have also been shown to possess QQ and anti-virulence properties. Findings related to these bioactive phytochemicals are summarized in Table 1. However, these account for preliminary evidences and require further validation to develop them into potential anti-QS agents. In summary, investigations have reported noteworthy findings for repurposing plant-derived bioactives as an anti-virulence therapy against P. aeruginosa. To epitomize the anti-virulence nature of phytochemicals as per the scientific explorations cited in this review, a mechanistic overview illustrating the virulence machinery of P. aeruginosa and how phytochemicals target them is depicted in Fig. 3.

Other inhibitors of P. aeruginosa QS systems

Apart from bioactive phytochemicals, different QSIs have been identified, ranging from antibiotics to synthetic drugs (analogs) and even microbial enzymes. These have been widely explored for their QQ potential in medical and biotechnological arenas (Kalai et al., 2019). Antibiotics such as azithromycin (Tateda et al., 2001) and ciprofloxacin (Gupta et al., 2016) have been reported for their QQ and anti-virulence properties against P. aeruginosa. Even niclosamide (anthelmintic) and itaconimides (antifungal) have been repurposed for their QQ abilities to disarm the virulence factors in P. aeruginosa (Imperi et al., 2013; Fong et al., 2019). Also, the anti-QS activities of compounds such as salicylic acid and sodium ascorbate have been documented with a monumental decrease in biofilm formation and the expression of QS genes in PAO1 (El-Mowafy et al., 2014; Ahmed et al., 2019). With advancements in structural biology and structure–activity relationships, a plethora of synthetic compounds, including novel conjugates and drug analogs with QQ quenching abilities, have been developed over the last two decades. In silico analysis accompanied by bench-based investigations with novel thiazolidinedione-type compounds has been shown to significantly reduce QSSM production and biofilm formation in P. aeruginosa without hindering its growth (Lidor et al., 2015). Methyl anthranilate, an analog of anthranilate moiety of 2-heptyl-3-hydroxy-4-quinolone, has been reported for repressing the production of PQS and LasB elastase in PAO1 under sublethal concentrations (Calfee et al., 2001). Similarly, halogenated anthranilate analogs inhibited PQS biosynthesis and PQS-driven gene expression, and restricted the systemic dissemination of P. aeruginosa in a burn wound model (Lesic et al., 2007). N- Decanoyl cyclopentylamide has also been reported to attenuate QS and virulence in PAO1 by abrogating the interaction between LasR and RhlR response regulators and their autoinducers (Ishida et al., 2007). Conjugate compounds consisting of an antibiotic or phytochemical chemically linked to a QSI have also been synthesized and shown to augment the QQ activities of the QSI against P. aeruginosa. Recently, a chemically synthesized LasR QSI conjugated with ciprofloxacin (ET37) has been demonstrated to decrease the production of QS-associated pyocyanin and elastases, biofilm formation and antibiotic tolerance in clinical isolates of P. aeruginosa (Bortolotti et al., 2019). This approach has been further extended in the form of an emerging ‘Trojan Horse’ strategy that employs the conjugation of a potent drug with either antibiotic(s) or pathogen-encoded virulence factors for deceitful drug delivery. In this context, a novel pyochelin–zingerone conjugate has been recently developed to target the QS circuitry and virulence in P. aeruginosa (Nosran et al., 2021). The conjugate reportedly mimicked the function of pyochelin and successfully associated with its cognate receptor (FptA), allowing its entry into the pathogen by deception and attenuating QS circuits, biofilm formation and downstream virulence factors in PAO1.

Further, researchers have designed novel AI mimics that interact with the QS receptors and behave as antagonists, thereby repressing the QS-regulated virulence genes. Small molecule inhibitors or antagonists have been developed to closely associate and target the different QS receptors of P. aeruginosa, thereby preventing signal reception (Suneby et al., 2017; Horita et al., 2018). Numerous antagonists have been shown to strongly interact and stabilize LasR, preventing its dimerization and making it incapable of binding DNA (Suneby et al., 2017). Similarly, antagonists targeting QscR (an
### Table 1. Studies showing preliminary evidence that bioactive phytochemicals demonstrate curative potential against quorum sensing and virulence factors in *P. aeruginosa*.

| Name of bioactive phytochemical | Alginate production | LasB protease | LasA protease | Pyocyanin production | Pyoverdine production | Bacterial motility | Biofilm formation | Rhamnolipid production | Other remarks | References |
|---------------------------------|---------------------|---------------|---------------|----------------------|-----------------------|-------------------|------------------|---------------------|--------------|------------|
| Betulin (125 µg ml\(^{-1}\))    | ↓ 88%               | ↓ 17%         | ↓ 74%         | ↓ 75%                |                       | Swarming motility | ↓ 57% with EPS matrix ↓ 31% and ↓ 20% | ↓ 20%               | Mortality in infected *C. elegans* by 12%. Molecular docking suggested interactions with LasR and RhlR QS receptors | (Rajkumari *et al.*, 2018a) |
| Betulinic acid (125 µg ml\(^{-1}\)) | ↓ 55%              | ↓ 8%          | ↓ 84%         | ↓ 75%                |                       | Swarming motility | ↓ 33% with EPS matrix ↓ 36% and eDNA release ↓ 20% | ↓ 22%               | Survival rate in infected *C. elegans* by 21%. Moderate interactions with RhlR by in silico analysis | (Rajkumari *et al.*, 2018a) |
| Chlorogenic acid (2.56 mg ml\(^{-1}\)) | ↓ 38%              | ↓ 40%         | ↓ 25%         |                      |                       | Swarming motility | ↓ 52%                       | ↓ 30%               | Expression of QS genes: lasI 85.09%, lasR 48.63%, rhlI 27.98%, rhlR 34.7%, pqsA 73.06%, and pqsR 45.85%. Survival rate in infected *C. elegans* by 16%. Wound closure in infected mouse model by 84% with reduced bacterial load | (Wang *et al.*, 2019) |
| Cinnamic acid (250 µg ml\(^{-1}\)) | ↓ 50%              | ↓ 71%         | ↓ 22%         | ↓ 81%                |                       | Swarming motility | ↓ 42%                       | ↓ 17%               | Survival rate in infected *C. elegans* by 59%. Strong binding towards LasR and RhlR QS receptors by in silico studies | (Rajkumari *et al.*, 2018b) |
| Hordenine (750 µg ml\(^{-1}\))  | ↓ 50%              | ↓ 46%         | ↓ 40%         | ↓ 48%                | ↓ 42%                 | Swarming motility | ↓ 50% with eDNA release ↓ 25% and EPS production ↓ 32%. Biofilm thickness ↓ 12 µm | ↓ 40%               | QS Signals: C4-HSL ↓ 74%, C12-HSL ↓ 40%. Transcript levels of las and rhl ↓ 50% | (Zhou *et al.*, 2018) |
| Mosflavone (125 µg ml\(^{-1}\)) | ↓ 57%              | ↓ 36%         | ↓ 60%         |                      |                       | Swimming and swarming motility ↓ 50% | ↓ 52% and EPS production ↓ 47% | ↓ 34%               | QS Gene and QS-target transcripts: lasI 61%, lasR 92%, rhlI 57%, rhlA 48%, rhlR 22%, lasB 36%, toxA 61%, aprA 58% and exoS 78%. Survival rate in infected *C. elegans* by 37% | (Hnamte *et al.*, 2019) |
Table 1. (Continued)

| Name of bioactive phytochemical | LasB production | LasA production | Biocidal activity | Rhamnolipid production | O-C8-HSL production | Other remarks |
|---------------------------------|-----------------|-----------------|-------------------|------------------------|---------------------|--------------|
| Phthalic acid                   | ↓ 75%           | ↓ 55%           | ↓ 40%             | ↓ 55% with marked EPS matrix | C. albicans isolate 5, 6, 10 and 11 | (Kalia et al., 2019) |
| Thymol                          | ↓ 40%           | ↓ 55%           | ↓ 40%             | ↓ 55% with marked EPS matrix | C. albicans isolates 5, 6, 10 and 11 | (Kalia et al., 2019) |
| Eugenol                         | ↓ 40%           | ↓ 55%           | ↓ 40%             | ↓ 55% with marked EPS matrix | C. albicans isolates 5, 6, 10 and 11 | (Kalia et al., 2019) |
| Carvacrol                       | ↓ 40%           | ↓ 55%           | ↓ 40%             | ↓ 55% with marked EPS matrix | C. albicans isolates 5, 6, 10 and 11 | (Kalia et al., 2019) |
| Anethol                         | ↓ 40%           | ↓ 55%           | ↓ 40%             | ↓ 55% with marked EPS matrix | C. albicans isolates 5, 6, 10 and 11 | (Kalia et al., 2019) |
| Pulegone                         | ↓ 40%           | ↓ 55%           | ↓ 40%             | ↓ 55% with marked EPS matrix | C. albicans isolates 5, 6, 10 and 11 | (Kalia et al., 2019) |
| carveol                         | ↓ 40%           | ↓ 55%           | ↓ 40%             | ↓ 55% with marked EPS matrix | C. albicans isolates 5, 6, 10 and 11 | (Kalia et al., 2019) |

Down arrow (↓) indicates the significant reduction in the levels of particular virulence factor tested. Upward arrow (↑) indicates the significant increase in the particular attribute.

Effects observed against QS and various virulence factors of P. aeruginosa

Bridging the gap between laboratory and market: data from clinical and field trials

The mammoth literature on the anti-virulence prospects of bioactive phytochemicals is majorly collated from bench-based experiments and investigations with animal
models. However, there is a lack of scientific knowledge from clinical settings and field trials discussing their safety and possible toxic effects. In contrast, most of the field studies circumstance around other types of QSI, including antibiotics and synthetic drugs. A pilot-scale clinical trial was undertaken to evaluate the therapeutic efficacy of garlic oil macerate as a QSI against *P. aeruginosa* in 26 cystic fibrosis patients (Smyth et al., 2010). Garlic capsules at concentrations lower than the toxicity dose for humans (656 mg) were consumed daily for 8 weeks, and the clinical outcomes were measured at baseline and post-treatment. Patients from the treatment group displayed improved pulmonary function with a significant reduction in disease symptoms. However, a small fraction of patients exhibited abnormal liver function and minor adverse effects (Smyth et al., 2010). In a controlled study, the anti-QS potential of azithromycin was assessed in intubated patients colonized by *P. aeruginosa* (Köhler et al., 2010). Azithromycin was administered intravenously (300 mg per day) for 20 days, and tracheal aspirates were collected each day. Following treatment with azithromycin, the expression of QS genes (*lasI* and *rhlA*) in tracheal samples was significantly reduced. However, this treatment strategy remained dubious as it may drive microbial evolution leading to the selection of resistant strains that are unresponsive to the QSI. In a similar direction, a randomized controlled trial was on intubated patients vulnerable to ventilator-associated pneumonia caused by *P. aeruginosa* (van Delden et al., 2012). *P. aeruginosa* was drastically reduced following treatment with azithromycin (300 mg per day for 20 days). The QS-regulated virulence of colonizing *P. aeruginosa* isolates remained low, and azithromycin treatment resulted in a fivefold reduction in the incidence of high-level rhamnolipids producing isolates among the high-risk patient subgroup. Interestingly, Wahjudi and colleagues developed a novel inhalable system by freeze drying an AHL acylase (PvdQ).
with anti-QS capabilities to attenuate the virulence of colonizing *P. aeruginosa* in cystic fibrosis patients (Wahjudi et al., 2013). The formulation was found to be stable up to four weeks even at 55°C. However, it warrants clinical investigations to assess its therapeutic and safety index. Fimbrolide-coated contact lenses were monitored for their clinical safety against multiple human pathogens, including *P. aeruginosa* in guinea pigs and human subjects, for 30 days and 24 h respectively (Zhu et al., 2008). The contact lenses exhibited QQ properties and showed that bacterial adherence was reduced by 67–92%, asserting its bioremedial ability. In clinical set-ups, indwelling medical devices act as a focal point for initiating health care-associated infections, particularly catheter-associated urinary tract infections (Saini et al., 2017). Urinary catheters functionalized by coating with 5-fluorouracil, a previously characterized QSI (Ueda et al., 2009), have demonstrated effective antimicrobial properties with no episodes of catheter-related bacteraemia or any adverse effects in ICU patients (Walz et al., 2010).

Cumulatively, these trials can be considered imperative in their attempts to translate bench-based researches on QSI. Such investigations can encourage further trials that may deal with a wide range of QSI, specifically QQ phytochemicals, their potential toxicity and therapeutic applications in the medical sector. Collaborative efforts from the pharmaceutical industry and research-directed academia can help bridge this gap for repurposing bioactive phytochemicals for anti-virulence therapy. Until then, studies in this avenue are still in their infancy.

### Weighing the outcomes on scales: benefits and pitfalls of using QQ phytochemicals

The discovery of antibiotics was considered to be a pinnacle in the field of medicine. Unfortunately, indiscriminate use and unregulated consumption of these antimicrobial drugs have led to the emergence of MDR pathogens (Chadha et al., 2021c). Notably, the COVID-19 pandemic and the emergence of SARS-CoV-2 variants (Chadha et al., 2021b) have increased the consumption of multiple antibiotics globally (Adebisi et al., 2021). This sudden ‘antibiotic rush’ can accelerate the pace at which bacterial resistance evolves and severely compromise the global commitment to curb AMR (Pelgrene et al., 2021). Despite the advent of new generation antibiotics, bacterial infections continue to rise, posing a continuous risk to the global healthcare system (Kalia et al., 2007). With the onset of the post-antibiotic era, anti-virulence drugs (QSIs) have caught the attention of the scientific community. These ‘wonder drugs’ include bioactive phytochemicals that interfere with the expression of virulence genes without killing the pathogenic bacteria or impeding their growth. Hence, this strategy exerts a relatively lower selection pressure as compared to antimicrobial drugs. Contrarily, since evolution is considered to be the only constant in the microbial world, it is believed that these QQ compounds may also impose selection pressure resulting in the development of drug resistance (Kalia et al., 2019). Although there is very little evidence to support this concern (Kalia et al., 2014; Koul et al., 2016), this line of thought seems critical as no treatment strategy be considered full-proof. However, a combinational therapy employing QQ phytochemicals and antibiotics can increase the success rate of drug repurposing by targeting multiple bacterial signalling pathways, thereby reducing the selection pressure. In this regard, studies illustrating the synergistic effects of anti-virulence phytochemicals and antibiotics have surfaced recently (Sharma et al., 2020a; Bose et al., 2021). Nonetheless, more insights are warranted in this avenue to substantiate the use of potent phytochemical–antibiotic regimens.

In addition, bacterial pathogens may reiterate their metabolic pathways or select mutations that confer resistance to QSIs. Clinical strains of *P. aeruginosa* isolated from cystic fibrosis patients were shown to harbour resistance against QSIs such as brominated furanone by inducing genomic alterations that enhance bacterial efflux capabilities (Maeda et al., 2012). Hence, bacterial pathogens can evolve artilleries that disrupt QQ by different mechanisms. Interestingly, reports have identified QS mutants of *P. aeruginosa* that are unresponsive to the QSSMs (Sandoz et al., 2007). These QS mutants are termed ‘social cheaters’ and display altered fitness with reduced chances of survival. However, such mutants can deceive QSIs, belittling their therapeutic value and lowering their efficacy (Gerdt and Blackwell, 2014). Therefore, like any other anti-virulence drug, QQ phytochemicals can also share the same fate. Another dimension to pseudomonal virulence is the presence of three distinct but interconnected QS mechanisms, making it an uphill task for targeting by QQ phytochemicals. Moreover, the presence of multiple QS systems increases the possibility that heterodimers of transcriptional regulators can associate with different promoters indiscriminately, driving differential gene expression in bacteria as a countermeasure to evade QSIs (Koul and Kalia, 2017). Just like the pseudomonal Pqs system that regulates the expression of virulence factors that drive apoptosis, which in turn induces the release of eDNA, conferring survival advantages to the pathogen (Hazen et al., 2016). Bacterial pathogens also can modify their QS receptors for enhanced binding by substituting the amino acid residues of the QSSM-binding pocket (Hawkins et al., 2007). Hence, QSIs exhibiting a relatively high affinity towards the QS receptors will be more effective in disarming the pathogen’s virulence. This may
prove to be a setback when employing natural anti-QS phytochemicals but advantageous with chemically modified ones with desired functional groups that facilitate stronger interactions.

On the positive front, bioactive phytochemicals play an imperial role in modulating the host immune system by activating humoral and cell-mediated responses, supplying antioxidants, impeding inflammatory responses and autoimmune disorders, and maintaining a healthy gut microbiome (Yin et al., 2019; Behl et al., 2021). Although they offer endless benefits, their role in modulating the diversity, abundance and equilibrium of the human microbiome has not been well documented. Considering the far-reaching effects of this versatile organ (human microbiome) in various diseases, including cancers, its role in shaping human well-being cannot be undermined (Chadha et al., 2021d). Therefore, QQ phytochemicals can be anticipated to impact human health by modulating the microbiome of different organs of the body. Other major limitations of using phytochemicals in anti-virulence therapy are their cytotoxic effects, poor solubility in water and inadequate bioavailability. Nevertheless, these can be regulated for human benefit by fabricating suitable delivery systems for achieving a high therapeutic index. Therefore, the desirable properties of an ideal QQ phytochemical can be enlisted as follows: (i) low molecular weight; (ii) high specificity towards QS machinery with no toxic or off-target effects in the bacterial pathogen; (iii) high stability and adequate bioavailability; (iv) resistant to enzymatic degradation within the host system; (v) minimal or no cytotoxicity for the host, i.e. safe for human use; and (vi) no exertion of harmful effects over the host microbiome. Nevertheless, such naturopathic QSSIs warrant more scientific explorations in clinical studies and their ability to withstand the sands of time by overcoming the risk of antimicrobial resistance. It will then be a matter of time before such potent anti-virulence drugs enter into clinical practices.

Conclusion and prospective outlook

With the indiscriminate use of antibiotics in clinical settings, P. aeruginosa has emerged as a highly notorious and multi-drug-resistant pathogen posing a serious threat to public healthcare systems. Since antimicrobial therapies work by inhibiting bacterial growth or killing them, they continue to pose an intense selection pressure favouring the selection of resistant variants in the population. The spurring fidelity between ever-evolving AMR and advancements in antimicrobial therapies has rattled clinicians worldwide to seek alternative medicine that attenuates pathogen virulence without compromising microbial growth. Also, considering the slow pace at which newer drugs get the safety and administrative approvals, drugs that exhibit both antimicrobial and anti-virulent traits may serve as an ideal intervention strategy for future action. The ‘disarm-don’t kill’ anti-virulence approach can also be revitalized to develop effective, robust, pathogen-specific drugs. Therefore, targeting the QS machinery that supervises virulence in P. aeruginosa can be vital to limiting the severity of infections in clinical set-ups. In this context, bioactive phytochemicals from plant varieties have come to the rescue. These natural anti-virulence elixirs have unravelled newer prospects for researchers to explore and develop novel therapeutics against P. aeruginosa that disarm its QS mechanisms, attenuating its virulence determinants. The last two decades have witnessed unprecedented research on this aspect and indicated the QQ prospects of numerous plant bioactives. Secondary metabolites that influence the pathogenesis of P. aeruginosa with anti-QS and anti-biofilm potential have also been recognized from bench-based studies in vitro and experiments with animal models in vivo. However, these require further validation for their successful transition and translation into applied medicine for combating pseudomonal infections in clinical set-ups. Rather than designing new antibiotics, scientific experimentations should be directed to identify or repurpose natural products, bioactive compounds, synthetic drugs, etc. with anti-virulent properties and the development of compatible nanocarriers for better delivery and bioavailability. These compounds may be subjected to chemical or structural modification(s) for improved binding to putative drug targets of the bacterial QS systems. Moreover, greater emphasis must be laid to boost large-scale clinical trials on the effects of QSSIs on various infectious diseases. This will generate concrete and reliable data before this anti-virulence strategy can be exploited for the benefit of mankind. It can be said that the mammoth scientific journey for these novel bioactives has crossed its halfway mark. With the advent of advanced molecular techniques, the age of molecular medicine is leading to the revival and modernization of Ayurvedic medicine. This has opened another vast avenue augmenting plant bioactives and nanotechnology for enhanced and targeted drug delivery. The future outlook may include translational approaches such as the ‘Trojan Horse’ strategy for developing novel conjugates of QQ phytochemicals and bacterial virulence factors for targeted and improved delivery. This can prove to be path-breaking in circumventing the menace of AMR. Also, it is vital to study the prevalence of QSSM-unresponsive mutants of P. aeruginosa using large-scale screening studies. By estimating the clinical prevalence of such QS mutants, a dual drug regimen harbouring both antimicrobial and anti-virulence properties may be employed to counter these cell populations. Presently, there is not enough evidence to support the development of QSI...
induced resistance in pathogenic microorganisms. Since the mechanism of the QSI also influences the possibility of developing resistance to QSI, it becomes essential to discretely investigate how anti-virulence drugs can induce anthropogenic resistance in pathogenic bacteria. Since pseudomonal biofilms on medical devices are the grim instigator in most nosocomial infections, fabricating multilayer coatings of bioactive phytochemicals and their nanoformulations can boost the biocurative potential of such nanoscale materials. Also, hybrid nanocoatings of multiple phytochemicals and/or QQ enzymes such as acylases, paraoxonase and lactonases on indwelling medical devices can be developed for imparting antibiofilm activities holistically. These advancements can aid in reducing the global reliance on antibiotics. Therefore, dedicated investigations from academia and attention from the pharmaceutical industries can boost the innovation and recognition of novel plant-derived anti-pseudomonal drugs. Overall, enacting such initiatives can help reduce the incidence of drug resistance and the global medical burden imposed by P. aeruginosa, thereby reducing hospital stays and improving the quality of patient life. This will also provide substantial societal and economic returns, essential to improving lives today and for future generations.

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Conflict of interest

None declared.

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