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In vitro and *in silico* screening for *Andrographis paniculata* quorum sensing mimics: new therapeutic leads for cystic fibrosis *Pseudomonas aeruginosa* biofilms

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Abstract

The clinical management and definitive treatment of cystic fibrosis (CF) biofilm-mediated chronic *Pseudomonas aeruginosa* lung infection remains a challenge. The methanol extract of the popular ethanomedicinal herb *Andrographis paniculata* L., which is traditionally used to treat respiratory illnesses, was shown to have growth and biofilm inhibitory activities against clinical isolates of *P. aeruginosa* from CF patients. Here, we report the antibiofilm activity of *A. paniculata* methanol extract on quorum sensing (QS) inhibition using both *in vitro* and molecular docking studies. The inhibitory activity of the extracts on exopolysaccharide and pigment production by the CF isolate and the QS indicator organism *Chromobacterium violaceum* ATCC 12472 and the interaction between the phytoconstituent, biofilm and QS proteins were determined. GC-MS analysis detected 32 plant phytochemicals of diverse nature that may account for its antibiofilm activity and its traditional use in the treatment of many illnesses, including respiratory infections. A number of compounds such as carbamic acid, N-phenyl-, 2-methylphenyl ester, and 2-methoxy-4-[(1-phenylpropan-2-ylamino)methyl]phenol, were found to possess better binding affinity ($\geq$ -5) to no less than two *P. aeruginosa* QS proteins without violation of Lipinski's rule. Furthermore, several compounds exhibited high drug-like molecular activity scores indicating their bioavailability and possible activity. These results indicate a significant affinity between the antibiofilm and quorum quenching activities and a probable use for the development of novel CF biofilm specific therapeutic strategies using *A. paniculata* extracts in the future. Moreover, the results also support the use of *A. paniculata* in traditional medicine.

Introduction

Globally, more than 1,407 pathogenic microorganisms are recognized as causative agents of human illness (Woolhouse and Sequeria, 2005). Currently among these agents, only a few pose more peril and challenges, including those with increasing resistance to current therapeutics, high morbidity and mortality rates and a large socioeconomic burden. Biofilm formation by pathogenic bacteria is a key virulence factor because it leads to antibiotic tolerance and the development of chronic infection (Landini et al., 2010). The therapeutic treatment of many clinical and device-associated infections is a major concern because of biofilm-mediated antibiotic resistance. The normal exposure of pathogenic bacteria to sub-inhibitory concentrations of antibiotics during treatment serves as an environmental signal that initiates biofilm formation (Murugan et al., 2013). The extraordinary resistance of *Pseudomonas aeruginosa* against nearly all antibiotics due to the interactions between intricate intrinsic and mutation-driven resistance pathways compromises the infection treatment options. The biofilm mode of growth further increases these resistance mechanisms (Mulet et al., 2011). Past scientific approaches have not adequately addressed these challenges, and consequently which warrant new research strategies and tactics for minimizing these threats and improving global health (Aderem et al., 2011).

Any compound, including the current antibiotics, which inhibits bacterial growth and/or cell viability exerts selective pressure for the development of resistance. Additionally, even the normal exposure of pathogenic bacteria to a sub-inhibitory concentration of antibiotic during treatment serves as an environmental signal to initiate biofilm formation (Murugan et al., 2013). Therefore, compounds that inhibit quorum sensing have received considerable attention as a novel class of potential antimicrobial agents (Sifri, 2008) because they attenuate bacterial virulence rather than killing the bacteria. Currently, the rich phytochemical repertoires of plants are being continuously explored in the quest for novel antimicrobials and antipathogenic agents. Although a number of controlled studies have revealed a rich new therapeutic reservoir of plant phytochemicals, the precise mechanism of antimicrobial activity is not completely known for many of these compounds (Al-Hussaini and Mahasneh, 2009). Previous studies on plant phytoconstituents have also focused on planktonic bacteria, which place less emphasis on the highly resistant organisms of biofilms. The search for inhibitors of biofilm development and biofilm-related cellular processes using either activity- or structure-based screening has led to the identification of a number chemicals and therapeutic compounds (Landini et al., 2010). The potential

severity of infection by the human opportunistic versatile pathogen *Pseudomonas aeruginosa* and optimal therapy selection for multidrug resistant (MDR) isolates as well as the identification and implementation of effective infection prevention strategies are not only vital but also a primary concern (Kerr and Snelling, 2009). The most noted pathogenic ability of *P. aeruginosa* is its colonisation in patients with chronic obstructive pulmonary diseases (COPD) (Ramsey and Whiteley, 2004). *P. aeruginosa* is a model organism for studying group-related behaviour, which engages in two types of group behaviour, quorum sensing (QS), which is intercellular signalling, and the formation of biofilms, which are surface-associated communities. Both QS and biofilm formation are important in the pathogenesis of infection. The effectiveness of biofilms is its ability to co-ordinate activity as a group (Kirisits and Parsek, 2006). *P. aeruginosa* chronically colonises the airways of CF patients as sessile antibiotic-resistant biofilm populations even with aggressive antibiotic treatment (Ramsey and Whiteley, 2004). Therefore, eradication of *P. aeruginosa* from established chronic pulmonary infection appears impossible even with modern therapies (Lee, 2009). *Andrographis paniculata* Nees (Family: Acanthaceae), the 'king of bitters', is a popular medicinal herb in Southeast Asia, India and China. It is used in the treatment of a large variety of diseases, including meningitis, acute hepatitis, the common cold, and many other inflammatory conditions (Uttekar et al. 2012). Additionally, it has been used in Asian countries for more than two thousand years for the treatment of respiratory and urinary infections, rheumatoid arthritis, laryngitis, diarrhoea, and diabetes. More recently, clinical findings on the benefits of *A. paniculata* extract in treating cold and influenza-like illnesses have been demonstrated (Jiang et al., 2009). Previously, we determined earlier that multidrug-resistant *P. aeruginosa* KMS P03 and *P. aeruginosa* KMS P05 capable of causing cystic fibrosis and forming biofilms are susceptible to *A. paniculata* extracts (Murugan et al., 2011). These extracts were found to interfere with bacterial attachment to surfaces. We have also shown that these *A. paniculata* extracts and phytochemicals could be used for the green synthesis of silver nanoparticles with antibiofilm activity (Murugan et al., 2013a). Recently, it has been recognised that the antibiotic properties of medicinal plant phytochemicals may also have potential antipathogenic activity, which may not lead to the development of resistance because they primarily act on pathogenic gene expression mechanisms and attenuate these mechanisms by interfering with the communication system (Sybiya Vasantha Packiavathy et al., 2012). The exchange of information among single-cell organisms using cell-to-cell communication with small chemical molecules has been termed QS. Currently, QS represents a promising antivirulent therapeutic target (Kaufmann et al., 2008). In addition, inhibitors of QS possess anti-biofilm activity and may also counteract bacterial pathogenicity (Landini et al., 2010). *A. paniculata* extracts are known to inhibit the growth and biofilm formation of CF isolates. Therefore, in this study, the mode of action of the phytochemical constituents of the *A. paniculata* extracts on biofilm-specific events was determined using *in vitro* and *in silico* studies to develop a targeted biofilm therapy. Although QS plays a vital role in *P. aeruginosa* biofilm formation, particularly during the maturation steps, biofilm formation is also affected in a QS-independent manner by dozens of other regulatory networks. Therefore, in this study, three target proteins with different functions and different roles in *P. aeruginosa* biofilm formation were selected as target proteins for molecular docking to develop a targeted biofilm therapy. These proteins are LasR, the transcriptional regulator of the Las QS system, the QS-regulated virulence factor rhamnolipid synthesis RhlG enzyme and a QS-independent sialidase.

Results

P. aeruginosa biofilm implicated EPS and QS in vitro inhibition

The biofilm forming capacity and multidrug-resistant nature of both *P. aeruginosa* KMS P03 and *P. aeruginosa* KMS P05 isolates were confirmed by biofilm-specific investigation techniques in Congo red agar (CRA) plates. The methanol extract of *A. paniculata* inhibited the microtitre plate growth and biofilm development of both strains, and the rates were between 80-90%. The modified gradient-plate assay on the *A. paniculata* extract using CRA demonstrated the concentration-dependent inhibition of the plant extract and interference with bacterial exopolysaccharide synthesis and biofilm formation. During the growth of both CF test isolates, the colonies were black in areas with a lower concentration of plant extract, which is indicative of biofilm formation, whereas the same colonies became white and smooth along the increase in the plant extract concentration gradient. At lower concentrations, the plant extracts only inhibited *Chromobacterium violaceum* ATCC 12472 violacein pigment production and not growth. These results suggest a strong rationale for designing biofilm specific intervention strategies against these organisms using these extracts.

Bioactive phytochemical constituents of A. paniculata

GC–MS phytochemical screening chromatogram (Fig. 1) of the *A. paniculata* methanolic extract indicating the presence of 32 components. Among the phytochemicals, pyran-4-one, 3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one,(4Z)-4-((E)-but-2-enylidene)-3,5,5-trimethylcyclohex-2-en-1-one, 3,7,11, 15-tetramethyl2-hexadecen-1-ol (Phytol), hexadecanoic acid, 4H-1-benzopyran-4-one, and 5-hydroxy-6,7-dimethoxy-2-phenyl- were found in large quantities along with several compounds present in minor proportions. The relative percentages of these phytochemicals, their PubMed 2D chemical structures, and the contributions of important functional groups were obtained. The presence of many potent compounds, such as flavonoids, phenolics, saponins, and tannins support its traditional medicinal use and antibiofilm activity.

Phytoconstituent interaction with biofilm and QS target proteins

The binding energies of all *A. paniculata* natural ligand compounds with *P. aeruginosa* QS proteins were calculated and ranked to identify the more apposite and better QS inhibitors and are presented in Table 1. 6H-dibenzo (b,d)pyran-1-ol,3-hexyl-7,8,9,10-tetrahydro-6,6,9-trimethyl showed the highest binding energy of -7.62 with the *P. aeruginosa* Las QS system transcriptional regulator LasR. The same compound showed a high binding energy of -9.2 with the rhamnolipid biosynthetic pathway enzyme beta-ketoacyl reductase, an important *P. aeruginosa* QS regulated virulence factor in *in vivo* lung biofilm production. A number of compounds (Fig. 2) were found to possess better binding affinity $\geq$ -5 with no less than two QS implicated regulators.

Table 1.*A. paniculata* phytochemicals as well as the autoinducer3-oxo-C12-HSL docking scores with the active site of *P. aeruginosa* quorum sensing regulators and their predicted bioactivity.

S.No	Compound Name	PUBCHEM ID	Docking Energy for			miLogP	TPSA	MW	N ON	N.OHNH	N Violations	No..rot. bond	volume	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
			2B4Q	3JPU	2w38														
1	6H-Dibenzo(b,d)pyran-1-ol, 3-hexyl-7,8,9,10-tetrahydro-6,6,9-trimethyl-	SID 10476356	-9.21	-7.62	-9.2	6.972	29.462	328.496	2	1	1	5	340.769	0.38	0.03	-0.32	0.38	0.08	0.11
2	Methyl acetyllithofellate	CID 579601	-8.16	-6.89	-9.06	4.604	72.838	396.568	5	1	0	8	405.267	0.12	0.24	-0.43	0.59	0.08	0.44
3	2-methoxy-4-[(1-phenylpropan-2-ylamino)methyl]phenol	CID 586172	-7.38	-6.27	-6.93	2.969	41.489	271.36	3	2	0	6	268.41	0.11	0.03	-0.18	-0.24	-0.13	0.04
4	Hexadecanoic acid	CID 985	-7.14	-4.84	-5.71	7.059	37.299	256.43	2	1	1	14	291.422	0.02	0.06	-0.33	0.08	-0.04	0.18
5	2-methyl-5-(6-methylhept-5-en-2-yl)phenol	CID 520468	-7.07	-6.16	-7.48	5.735	20.228	218.34	1	1	1	4	236.153	-0.38	-0.09	-0.64	0.09	-0.61	-0.03
6	(4-cyanophenyl) 3-methylbut-2-enoate	CID 532359	-6.78	-6.01	-7.62	2.711	50.097	201.225	3	0	0	3	189.41	-0.68	-0.19	-0.58	-0.15	-0.59	-0.05
7	(5-methyl-2-propan-2-ylphenyl) acetate	CID 68252	-6.19	-5.31	-7.35	2.905	26.305	192.258	2	0	0	3	195.083	-0.82	-0.43	-1.11	-0.49	-0.99	-0.43
8	1-(4-hydroxy-2-methylphenyl)ethanone	CID 70133	-6.79	-4.98	-5.98	1.733	37.299	150.177	2	1	0	1	144.167	-1.07	-0.56	-1.41	-0.99	-1.38	-0.68
9	Carbamic acid, N-phenyl-, 2-methylphenyl ester	SID 52988543	-6.4	-6.21	-6.7	3.259	29.543	227.263	3	0	0	3	212.765	-0.14	-0.07	-0.32	-0.11	-0.37	0.15
10	3,7,11,15-Tetramethyl2-hexadecen-1-ol	CID 145386	-6.79	-4.27	-6.04	6.761	20.228	296.539	1	1	1	13	349.376	0.11	0.16	-0.32	0.35	0	0.31
11	(4Z)-4-((E)-but-2-enylidene)-3,5,5-trimethylcyclohex- 2-en-1-one	CID 6437599	-6.48	-6.01	-7.16	3.191	17.071	190.286	1	0	0	1	202.576	-0.79	-0.19	-1.65	0.44	-0.75	0.18
12	1-(4-hydroxy-3,5-dimethoxyphenyl)ethanone	CID 17198	-6.4	-5.19	-6.27	1.191	55.767	196.202	4	1	0	3	178.697	-0.79	-0.37	-0.88	-0.8	-0.98	-0.35
13	Carbamic acid, N-phenyl-, 2-methylphenyl ester	SID 52988543	-6.4	-6.21	-6.7	3.259	29.543	227.263	3	0	0	3	212.765	-0.14	-0.07	-0.32	-0.11	-0.37	0.15
14	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	SID 10509033	-6.33	-5.41	-5.98	1.497	26.305	182.263	2	0	0	0	185.645	-0.59	-0.25	-0.82	-0.35	-0.79	-0.26
15	4-[(E)-3-hydroxyprop-1-enyl]-2-methoxyphenol	CID 1549095	-6.2	-5.42	-6.78	1.371	49.69	180.203	3	2	0	3	169.843	-0.55	-0.05	-0.74	-0.3	-1	-0.08
16	3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one	CID 119838	-6.07	-5.2	-5.7	-0.457	66.761	144.126	4	2	0	0	123.404	-1.59	-0.96	-2.25	-1.6	-1.53	-0.65
17	2,3-Diaminophenol	CID 579937	-5.98	-4.28	-5.31	0.588	72.274	124.143	3	5	0	0	114.638	-2.14	-1.7	-2.01	-2.4	-2.39	-1.34
18	2-methylbenzoic acid	CID 8373	-5.94	-4.27	-5.5	1.91	37.299	136.15	2	1	0	1	127.606	-1.1	-0.61	-1.34	-1	-1.31	-0.63
19	Ethyl icosanoate	CID 29009	-5.92	-4.35	-6.43	9.016	26.305	340.592	2	0	1	20	392.959	-0.03	-0.05	-0.2	0.04	0.02	0.01
20	Phenyl vinyl ether	CID 69840	-5.89	-4.16	-4.63	2.297	9.234	120.151	1	0	0	2	120.759	-2.14	-0.98	-2.65	-2.29	-2.45	-1.99
21	2-Pentylphenol	CID 61084	-5.44	-5.22	-6.33	4.247	20.228	164.248	1	1	0	4	175.83	-0.55	-0.18	-0.93	-0.48	-0.79	-0.21
22	Methyl Tridecanoate	CID 15608	-5.27	-5.64	-5.17	5.851	26.305	228.376	2	0	1	12	258.545	-0.32	-0.1	-0.61	-0.33	-0.36	-0.06
23	3-hydroxy-2-methylpyran-4-one	CID 8369	-5.24	-4.08	-5.01	-0.243	50.439	126.111	3	1	0	0	109.174	-3.13	-2.62	-3.19	-3.25	-2.85	-1.81
24	2,5-dimethylpyrazine	CID 31252	-5.07	-4	-4.53	0.674	25.784	108.144	2	0	0	0	108.853	-3.01	-2.6	-2.98	-3.61	-3.26	-2.75
25	ethanethioic S-acid	CID 10484	-4.71	-3.21	-3.21	0.887	17.071	76.12	1	0	0	0	65.84	-4.78	-4.07	-5.83	-3.86	-3.15	-3.91
26	Bis(2dimethylamino) ethyl) ether	CID 18204	-3.81	-4.48	-3.61	0.357	15.171	160.261	3	0	0	6	180.639	-0.84	-0.38	-0.78	-1.2	-1.03	-0.55
27	3-oxo-C12-HSL	CID 127864	-3.54	-3.86	-	2.161	72.47	297.4	5	1	0	11	298.3	-0.07	-0.29	-0.64	-0.18	-0.13	-0.13

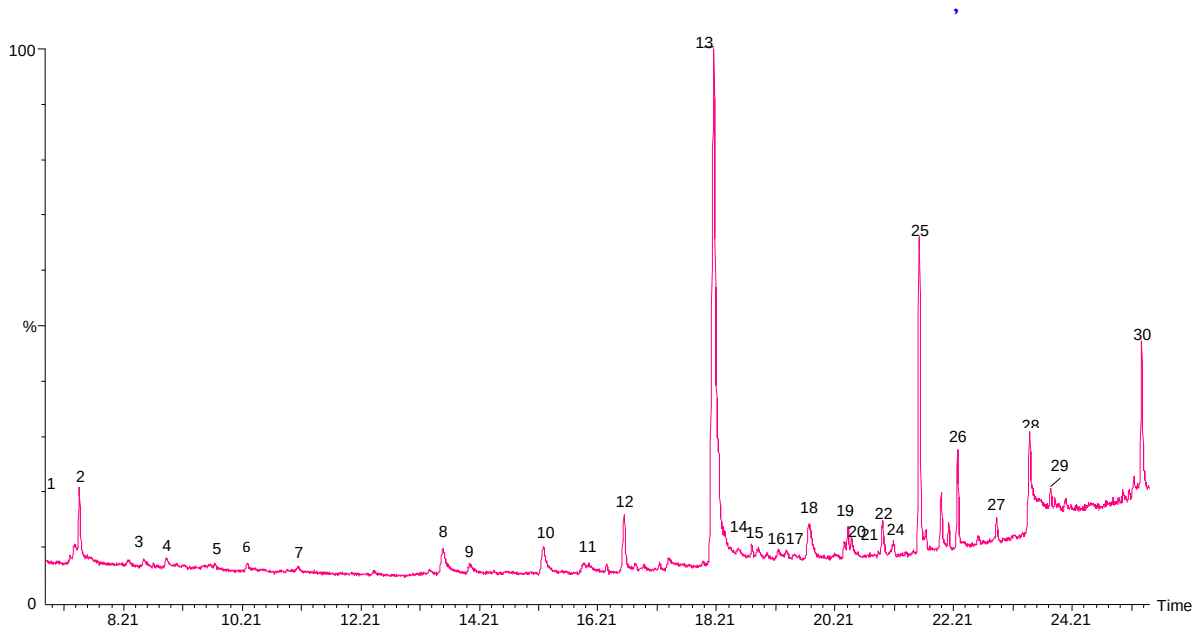

Fig.1. GC-MS chromatogram (RT 0-28 min**)** of antibiofilm methanol extract of *Andrographispaniculata* showing the compounds (Table 1) and their percentage of abundance.

A Molinspiration-based ADME (absorption, distribution, metabolism, and excretion) computational study revealing the *A. paniculata* phytochemical bioavailability was performed (Table 1). The calculated molecular hydrophobicity (miLogP) and topological polar surface areas (TPSA) revealing the cell permeability of *A. paniculata* phytochemicals are presented in Table 1. The values suggest that a number of *A. paniculata* bioactive compounds should be able to penetrate the cell membranes. A number of *A. paniculata* bioactive compounds also showed high binding energies with *P. aeruginosa* QS response regulators and obeyed Lipinski's Rule of Five. Therefore, many compounds should show good absorption or permeability properties through the intestine or other biological membranes. The drug-like molecule activity scores allowing the separation of active and inactive molecules calculated for GPCR ligands, kinase inhibitors, ion channel modulators, nuclear receptor ligands, protease inhibitors and other enzyme targets are also included in Table 1. The results indicate that a number of *A. paniculata* bioactive molecules have high activity scores, suggesting a maximal probability of activity.

Discussion

The results of this study confirm earlier observations of the antibiofilm activity and susceptibility of biofilm-forming cystic fibrosis isolates *P. aeruginosa* KMS P03 and *P. aeruginosa* KMS P05 to *A. paniculata* methanol extracts. Our previous studies showed that among six extracts, hexane, chloroform, ethanol, aqueous and methanol, the methanol extract exhibited the highest inhibitory activity compared to the others and the standard antibiotic amikacin (Murugan et al., 2011). Although the biofilm confers resistance, the antimicrobial resistance mechanism is not well understood. Many studies have demonstrated the multifactorial nature and lack of communication among biofilms (Araújo et al., 2011). The presence of over 34 bioactive components belonging to various groups was detected by GC-MS analysis. This validated the effectiveness of these plants in traditional medicine and their use for the treatment of uncomplicated acute upper respiratory tract infections (Poolsup et al., 2004), and its enhanced antibiofilm activity against cystic fibrosis isolates. Earlier studies (Geethangili et al., 2008; Rajagopal et al., 2003; Smith et al., 2006; Yoopan et al., 2007) also revealed the richness of *A. paniculata* bioactive phytoconstituents and their varied modes of action and drug properties against various human ailments. In fact, a number of traditional medicinal plants with antibacterial activity were shown to be ''QS mimics'' capable of controlling bacterial QS (Sohaibani and Murugan, 2012; Vattem et al., 2007). As the herbal medicine proponents believe, the enhanced antibiofilm efficacy of *A. paniculata* methanol extract may be due to the multitude of phytochemicals and the synergistic interactions among them. Therefore, molecular docking and virtual screening based studies were performed. The involvement of bacterial QS in various pathologically relevant events makes QS inhibitors prospective therapeutic agents. The *in silico* molecular interactions of the *A. paniculata* phytoconstituents with the biofilm implicated QS and other virulence factor proteins indicate a multitude of actions for targets with different roles. However, when designing bacterial QSI as therapeutic agents, several factors, including the normal ''drug-like'' and ADME (absorption, distribution, metabolism, and excretion) issues, must be taken into consideration (Ni et al., 2009). ADME, in addition to safety issues, are key factors that lead to drug failure. Therefore, they must be determined early during the drug discovery process to reduce late-stage attrition (Tambunan and Wulandari, 2010). In this study, the ADME parameters were calculated according to Lipinski's "Rule of Five." This rule has been used as a filter for substances that may have drug lead properties or may be added to drug design programs. This rule follows several principles because compounds with more than five H-bonds donors, H-bond

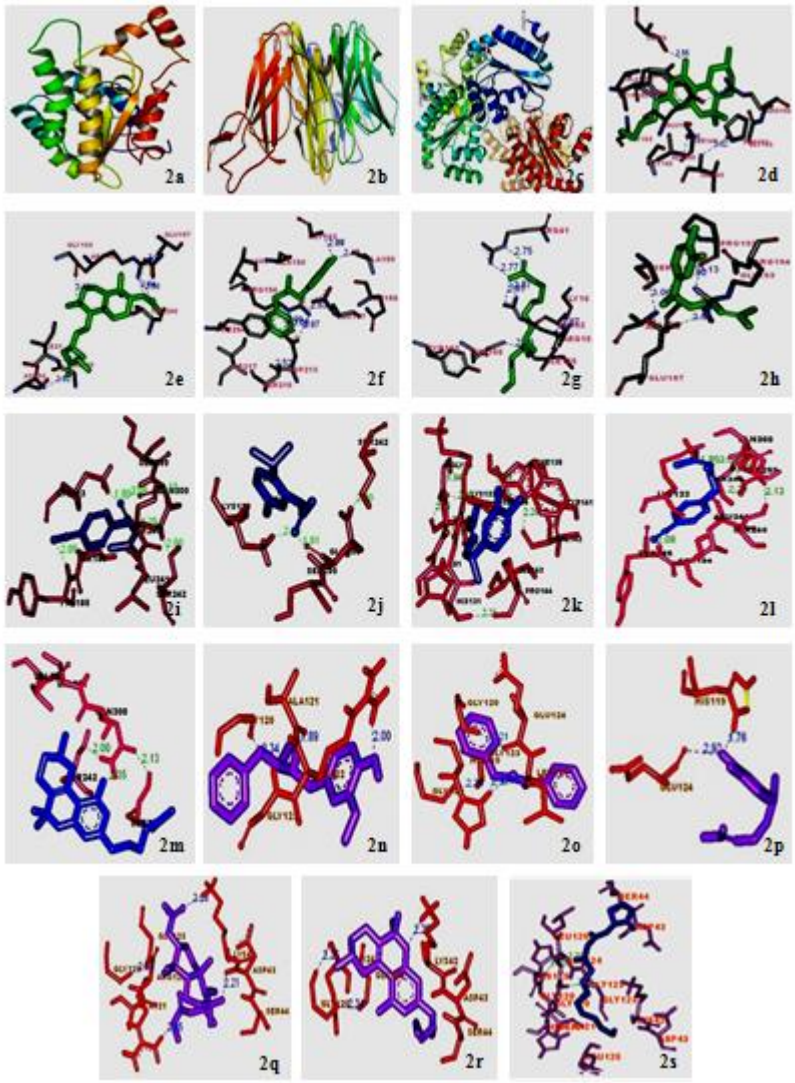

Fig 2. Docking results of the *Andrographis paniculata* phytochemical constituents' natural ligands with *Pseudomonas aeruginosa* quorum sensing regulators. (2a) Crystal structure of the *P.aeruginosa*RhlG, beta-KetoacylReductase (PDBID: 2B4Q) and its binding (2d-2h) with *A. paniculata* phytochemicals (Table 1compounds 1-5 respectively). (2b) *P.aeruginosa* sialidase (PDBID 2w38) regulator docking structure (2i-2m) with *A. paniculata* phytochemicals (Table 1 compounds 6,7,5, 2 &1 respy). (2c) *P. aeruginosa* transcriptional activator protein LasR (PDBID: 3JPU) proposed binding mode (2o-2r) with the phytochemicals (Table 1 compounds 3,9,5,2,& 1) respectively. (2s) The autoinducer3-oxo-C12HS docking with 3JPU.

acceptors, a molecular weight (MW) greater than 500, and a calculated log P value greater than five may show poor absorption or permeation. Violation of more than one of these rules may lead to bioavailability problems (Gonçalves et al., 2012). It was noted that *A. paniculata* compounds do not violate Lipinski's rules, rendering them promising agents for biofilm targeting for CF therapy. The interaction of *A. paniculata* phytochemicals with ion channel modulators and kinase inhibitors may lead to pharmaceutical agents that act via CFTR modulation. This warrants further investigation. Collectively, our results show that the *A. paniculata* has biofilm specific QS inhibitory activity and activity against other virulence factors, which may lead to the development of drugs that can be administered orally.

Materials and Methods

Organisms

The clinical isolates *Pseudomonas aeruginosa* KMS P03 and *P. aeruginosa* KMS P05 are associated with cystic fibrosis and produce biofilms. They were obtained from the culture collections of the Department of Microbiology, K.S.R College of Arts and Science, Tiruchengode, India. The slime forming ability of the isolates was confirmed using the tube adherence method and graded as described previously (Murugan et al., 2010). The Congo Red Agar (CRA) method (Freeman et al., 1989) was used to confirm the presence of extracellular polysaccharide substance and slime producing ability of the isolates. The isolates were maintained on blood agar plates and were sub-cultured at weekly intervals.

Plant collection, bioactive compound extraction and GC-MS analysis

The entire *A. paniculata* plant was collected from selected locations in and around the Erode district, which lies between the geo-coordinates 10° 36' – 11° 58' N; 76° 49' – 77° 58' E, Tamil Nadu, India. Fresh leaves were collected in polythene bags, washed thrice with sterile distilled water, shadow-dried

and powdered. Voucher specimens of the plants were also collected and identified using standard taxonomic procedures (BS1/SC/5/23/08-09/tech-1594). Bioactive phytochemicals were extracted using 25 g ground leaves in 250 ml methanol. The extract was concentrated to constant weight under reduced pressure and stored at 4°C until further use. The GC-MS analyses of the extracts were performed using a PerkinElmer Clarus 500 Mass Spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, USA). The interface temperature was 280°C and the MS scan range was 40-450 atomic mass units (AMU). For chromatography, a capillary column Elite-5ms was used. The stepped up temperature program was as follows: 70°C for 10 min, followed by a temperature increase from 70 to 150°C at a rate of 10°C/min (5 min), and then to 280°C at 8°C/min. Helium was used as the carrier gas injected at 1 ml/min. The injection volume was 1 µl. The individual peaks were referred to the Wiley NIST reference mass spectral database for compound identification.

Evaluation of antibiofilm and quorum sensing inhibitory activity of the extracts

The biofilm inhibitory potential of the extracts was determined using microtitre plate analysis (Murugan et al., 2011). *P. aeruginosa* isolates were grown in plant extract incorporated trypticase soy broth for 18 hours and allowed to form a biofilm. Water and tobramycin were the negative and positive controls, respectively. After incubation at 37°C, the biofilms were stained with crystal violet (0.1%) and quantified using microtitre analysis (OD 490 nm). The antibiofilm activity of the extract on the exopolysaccharide (EPS) was determined using a modified gradient plate technique with Congo red medium. A continuous gradient of plant extract on CRA medium was prepared, and the biofilm forming isolates were inoculated into the centre of the plate as a single streak. The organisms were again streaked zigzag perpendicular to the original streak crossing it each time. The colour and nature of the developed colonies along the streaked line were observed. The quorum sensing inhibitory activity of the extract was determined using the soft-agar overlay method as described previously (Murugan et al., 2013b). The influence of the extracts on the growth and pigment production by the indicator organism *C. violaceum* ATCC 12472 on LB agar supplemented with N-hexanoyl homoserine lactone (10 µmol ml^{-1}, Sigma- Aldrich, India) was described previously (Bosgelmez-Tinaz et al. 2007).

Molecular docking

The crystal structure of *P. aeruginosa* 3JPU (*P. aeruginosa* LasR, transcriptional regulator of Las QS system), 2B4Q (RhlG, beta-ketoacyl reductase, QS regulated virulence factor rhamnolipid synthesis enzyme) and 2W38 (*P. aeruginosa* sialidase, *in vivo* biofilm formation enzyme) were retrieved from the NCBI-PubChem database. Protein–compound docking simulation was performed using AutoDock 4.0 (Sohaibani and Murugan, 2012). The ADME molecular properties and bioactivity scores of the drug targets were calculated using Molinspiration according to Lipinski's rule for all analysed ligands (Ertl, 2012).

Conclusions

Our results are valuable for identifying plant derived natural QS inhibitors as well as other biofilm targeting drug leads that inhibit *in vivo* biofilm establishment. Moreover, our results provide a scaffold for the development of novel biofilm specific therapeutic agents against CF *P. aeruginosa* and other infective biofilm forming pathogens. Furthermore, the bioactivity guided fractionation of individual quorum quenching compounds and their isolation and identification is of interest because they are antipathogenic and less likely to lead to resistance development among pathogens such as *P. aeruginosa.*

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