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EVALUATION OF THE POTENTIAL OF FIVE MEDICINAL PLANTS TO INHIBIT ACYL HOMOSERINE LACTONE BASED QUORUM SENSING IN *PSEUDOMONAS AERUGINOSA* AND *ACINETOBACTER BAUMANNII*

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ABSTRACT

Pseudomonas aeruginosa and *Acinetobacter baumannii* are common nosocomial pathogens, well recognized for their multidrug resistance. Acyl-homoserine lactone (Acyl-HSL) based Quorum sensing (QS) is known to regulate virulence and biofilm maturation in these organisms; hence, inhibition of QS will help to control their pathogenicity. In the study, 5 plants, *Rubia cordifolia*, *Tinospora cordifolia*, *Picrorhiza kurroa*, *Cassia fistula* and *Bauhinia variegata* were screened for QS inhibition. A comparison of different extracts from the above plants, for short and long acyl-HSL inhibition in both pathogens, was carried out using reporter strains, *Chromobacterium violaceum* ATCC12472 and *Escherichia coli* MG4/pKDT17 respectively. Ethyl acetate extracts of *T. cordifolia* inhibited short as well as long acyl-HSLs with about 78% & 71% inhibition of long acyl-HSLs in *P. aeruginosa* and *A. baumannii* respectively; higher than the positive control, garlic extract (29% and 33% respectively). Further, absence of antibacterial activity of the plant extract confirmed its QS inhibitory potential.

KEYWORDS: Acyl homoserine lactone, Quorum sensing, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Tinospora cordifolia*

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INTRODUCTION

Pseudomonas aeruginosa and *Acinetobacter baumannii* are gram negative bacteria, commonly recognized as problematic nosocomial pathogens throughout the world. They cause ventilator associated pneumonia, urinary tract infections, wound infections and blood stream infections in immunosupressed patients or severely ill patients under hospital care[1, 2, 3]. Worldwide clinical studies have not only alarmed against increasing infection cases but also indicated increasing multidrug resistance developing in the hospital strains of these pathogens[4, 5]. Hence, it has become necessary to develop new anti-infective drugs, especially with low chance of resistance development. Quorum sensing (QS) is a mechanism in bacteria to monitor cell density by producing certain signal molecules and further regulate the expression of secondary characteristics such as bioluminescence, DNA competence, biofilm formation, virulence, etc.[6]. Among gram negative bacteria, N-acyl homoserine lactones (Acyl-HSLs) are the most common signaling molecules produced. *P. aeruginosa* contains two QS systems, LasIR and RhlIR. The Las system produces long acyl chain HSL, 3-oxo-C_{12}-HSL while the Rhl system produces short acyl chain HSL, C_4-HSL. Both systems regulate the production of virulence factors such as adhesins, exotoxin A, rhamnolipid, proteases, pyocyanin and biofilm maturation[3]. The understanding of virulence in case of *A. baumannii* is still at a preliminary stage; but it is known that it commonly occurs as biofilms on invasive devices as well as in the clinical setting. In *A. baumannii*, a synthase protein AbaI, catalyzes the production of 3-hydroxy-C_{12}-HSL, which further regulates biofilm development[7]. Thus, control and inhibition of QS in both these organisms will reduce their pathogenicity and drug resistance due to biofilms. Herbal based products have recently gained much importance in the field of medicine owing to presence of complex bioactive molecules able to treat various ailments. In the present study, 5 medicinal plants were selected to screen for the presence of anti-QS compounds. The plant, *Rubia cordifolia* Linn., family Rubiaceae, commonly known as Indian madder, has been used for skin care and treatment, and internally in urinary disorders[8]. *Tinospora cordifolia* (Willd.) Miers., family Menispermaceae, is a perennial climber; its stem is used in fevers, diabetes, urinary problems, skin diseases, etc[9,10]. *Picrorhiza kurroa* Royle ex Benth., family Scrophulariaceae, is a perennial herb, commonly used to treat jaundice, dyspepsia, anorexia and fevers[11]. *Cassia fistula* Linn., family Fabaceae, is a semi-wild Indian laburnum that has been used in Ayurveda against leucoderma, diabetes, etc. Studies have reported the presence of antioxidant, hepatoprotective and hypoglycemic potentials in this plant[12]. *Bauhinia variegata* Linn., family Caesalpiniaceae, is a deciduous tree, reported to be useful for treating leucoderma, leprosy, asthma, wounds and ulcers[13]. Few studies until recent have indicated the potential of plant based compounds to inhibit QS in *P. aeruginosa* and *Chromobacterium violaceum*[14-17]. More compounds are required to be added to the library of QS inhibitors (QSIs) and find the best amongst them for drug development. The plants under study have never been evaluated for the presence of anti-QS compounds and there has been no study until recent aimed to inhibit QS in *A. baumannii*. Thus, for the first time, the above given plants were screened for their ability to inhibit acyl-HSL based QS in *P. aeruginosa* and *A. baumannii*.

MATERIALS AND METHODS

Luria Bertani medium and agar powder was purchased from Himedia Laboratories, Mumbai, India. ONPG was purchased from Sisco Research Laboratories (SRL), Mumbai, India. All other chemicals were purchased from Qualigens Fine Chemicals, Mumbai, India. *Pseudomonas aeruginosa* PAO1 and *Acinetobacter baumannii* MTCC1425 were

used as reference strains. LB medium was used for bacterial growth. *Escherichia coli* MG4/pKDT17 and *Chromobacterium violaceum* ATCC12472 were used as reporter strains. *E. coli* MG4/pKDT17 was grown in LB medium containing 100µg ampicillin. For growth, all the bacterial strains were incubated overnight at 37°C.

(i) Plant collection

Dried plant material (stem or stem bark) was purchased from local vendor, authenticated and certified by Agharkar Research Institute, Pune, Maharashtra, India. Garlic bulbs were purchased from local market. Garlic extract was used as positive control for QS inhibition.

(ii) Phytochemical extraction

Dried plant material was powdered and then the phytochemicals extracted by hot and cold extraction methods using different solvents, water, methanol, ethyl acetate and n-hexane. In cold extraction method, plant powder was macerated in the solvent with intermittent shaking for 2 days. In hot extraction method, soxhlation was done for 6 hours. The extracts were filtered, dried at 50°C and stored at 4°C. For garlic extract, 150g of garlic cloves was shredded with 300 ml toluene in blender and kept for overnight extraction. The suspension was filtered, 150 ml of sterile distilled water was added to it and stirred for 24 hrs after which 2 phases were allowed to form. The organic phase was separated and used as garlic extract[14].

(iii)Anti-QS activity tests

The plant extracts were reconstituted in appropriate solvents at the specified concentration and screened for short acyl-HSL and long acyl-HSL inhibition as below.

a. C. violaceum pigment production assay

Agar well diffusion assay was performed to evaluate inhibition of short acyl-HSL by plant extracts[18]. *C. violaceum* produces short acyl-HSL (C6-HSL) which regulates production of dark purple pigment, violacein. Inhibition of short acyl-HSL in *C. violaceum* is indicated by inhibition of violacein production. Overnight grown culture of *C. violaceum* ATCC12472 was seeded in 5 ml of semisolid LB agar (0.5%) up to a density of 10^{8} cells/ml. This was overlaid in Petri dish containing 15 ml LB agar (1.5%). 100µl of the appropriate plant extract with 2 mg/ml concentration was added in the agar well. Solvents without extracts were used as negative control. 2% v/v garlic extract was used as positive control. Inhibition of pigment production was checked after 18-24 hrs incubation.

b. Long acyl-HSL production assay

The ability of plant extracts to inhibit long acyl-HSLs in *P. aeruginosa* and *A. baumannii* was tested using *E. coli* MG4/pKDT17[19]. This strain is used to detect 3-oxo- or -hydroxy-substituted or unsubstituted C8-C14 long acyl-HSLs wherein the long acyl-HSL induces the expression of β-galactosidase gene. Decrease in β-galactosidase expression is indicative of long chain acyl-HSL inhibition. A modification of the procedure given by Kirwan *et al*[18] was used. The bacterial culture was grown along with plant extract (final concentration - 2 mg/ml) or only the solvent (negative control) or garlic extract (2%) for 24 hrs. Acyl-HSL obtained in the culture supernatant was then extracted with ethyl acetate, twice; ethyl acetate layers were pooled and evaporated to dryness under nitrogen. The dried extract was reconstituted in LB broth and added to 2.0 ml of overnight grown reporter strain culture and incubated for 5 hours at 37°C for induction of β-galactosidase. Further, the enzyme activity was determined as described by Miller using ONPG as substrate[20].

(iv) Antibacterial activity

Standard disc diffusion assay was performed to study bactericidal activity of the plant extract wherein Whatmann filter paper 1 discs (diameter 6 mm) were impregnated with 6 µl of extract (concentration range - 2 - 20 mg per disc) and the disc was placed on LB agar plate previously seeded with o/n culture of *P. aeruginosa* and *A. baumannii*. Antibacterial activity was indicated by zone of inhibition around the discs after overnight incubation.

(v) Statistical analysis

All data are presented as mean ± S.D. Statistical significance was evaluated using Mann-Whitney-Wilcoxon's test using SPSS Statistics 17.0. P ≤ 0.05 level of significance was considered.

RESULTS

1. Extraction yields

In the study, 5 plants, *Rubia cordifolia*, *Tinospora cordifolia*, *Picrorhiza kurroa*, *Cassia fistula* and *Bauhinia variegata* were explored for their potential to inhibit QS in the pathogens, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Phytochemical extraction was done by cold extraction and hot extraction method and using 4 solvents, viz., water, methanol, ethyl acetate and n-Hexane Extraction yield obtained in different solvents and cold and hot method varied for each plant, which can be attributed to the amount and type of phytochemicals extracted based on their polarity and temperature stability. Garlic extract is known to inhibit QS in *Pseudomonas aeruginosa*, hence was used as positive control[14]. Toluene extract of garlic was prepared as described so as to separate the antibacterial component, allicin from the extract.

Table 1
Phytochemical extraction yields obtained for each of the plant

Type of extract/ Plant	CAE	HAE	CME	HME	CEE	HEE	CHE	HHE
R. cordifolia	20.69	15.27	14.6	34.59	3.46	1.66	0.29	3.48
T. cordifolia	6.56	4.15	2.53	6.85	2.51	6.06	0.55	5.14
P. kurroa	9.63	9.27	14.88	11.73	2.29	7.7	0.63	3.14
C. fistula	15.52	18.62	14.97	30.64	4.06	3.34	0.5	0.55
B. variegata	10.42	7.28	8.3	8.88	0.87	1.12	0.73	0.87

Extraction yields expressed in % w/w

2. Screening of plant extracts for short acyl-HSL inhibition

Inhibition of short acyl-HSLs was indicated by the presence of colorless zone against a purple lawn of the culture of *C. violaceum* (Figure 1). Maximum zone diameter was observed in CEE of *T. cordifolia*, followed by its HEE; and their zone diameter was also found to be larger than garlic extract, the positive control (Table 2).

Figure 1
C. violaceum pigment production assay

A & B - Zone of inhibition of short acyl-HSL for CEE of T. cordifolia, C & D - CAE of T. cordifolia

Table 2
Results of diameter of zone of inhibition in mm obtained in the C. violaceum pigment production assay

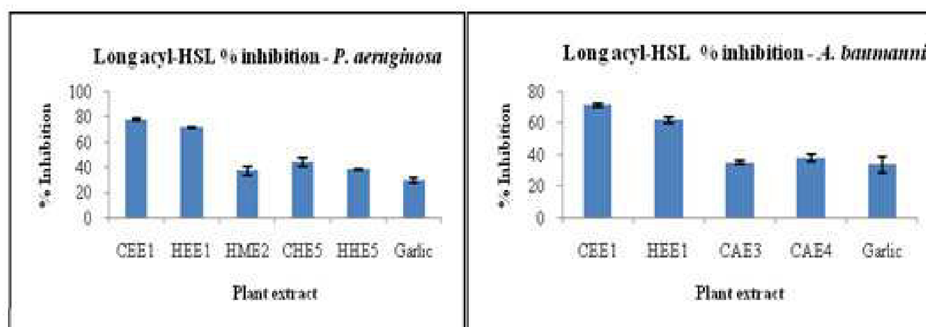
	Diameter of the zone of QS inhibition in mm (Mean + S.D., n = 9)							
Control	Water		Methanol		Ethyl Acetate		n-Hexane	
	9.04 + 0.11		12.74 + 0.57		13.17 + 0.71		11.41 + 0.94	
Type of extract/ Plant	CAE	HAE	CME	HME	CEE	HEE	CHE	HHE
R. cordifolia	9.48 + 0.71	9.55 + 0.73 *	14.59 + 1.63 *	13.30 + 0.54 *	15.81 + 0.69 *	15.44 + 1.10 *	12.52 + 0.44 *	12.29 + 0.42
T. cordifolia	9.0 + 0.0	9.48 + 0.71	13.89 + 0.74*	13.15 + 0.5	21.5 + 1.3*	19.54 + 1.23*	10.7 + 0.69	11.57 + 1.43
P. kurroa	9.11 + 0.33	10.41 + 1.0*	13.81 + 1.43	14.39 + 1.14*	15.55 + 1.01*	14.81 + 1.14*	11.5 + 1.37	12.26 + 1.01
C. fistula	10.85 + 1.0*	10.89 + 0.55*	13.41 + 0.43*	12.74 + 1.14	13.63 + 0.77	15.91 + 0.89*	13.72 + 0.97 *	13.17 + 1.37 *
B. variegata	10.15 + 1.09	10.26 + 1.04*	13.74 + 1.24	13.67 + 1.3	15.48 + 1.08*	14.81 + 1.14*	12.05 + 1.18	13.04 + 1.73
Toluene	14.99 + 0.35							
Garlic extract	18.85 + 0.48*							

***** Statistically significant at P < 0.05***

3. Screening of plant extracts for long acyl-HSL inhibition

Inhibition of long acyl-HSL was indicated by inhibition of β-galactosidase activity. Enzyme activity obtained for control and plant extracts were compared and inhibition in the enzyme activity in presence of plant extracts was calculated by considering negative control as 100% enzyme activity (Graph 1). Garlic extract, the positive control, showed about 29.35% decrease and 33.51% decrease in case of *P. aeruginosa* and *A. baumannii*, respectively. Maximum inhibition was obtained with CEE of *T. cordifolia*, about 77.84% & 71.23% decrease of long acyl-HSL production by *P. aeruginosa* and *A. baumannii* respectively, followed by its HEE that gave 71.41% and 61.75% decrease respectively. The results of the plant extracts that showed statistically significance and similar or higher inhibition than that of garlic extract are represented in Graph 1. Some other plant extracts also showed significant inhibition but less than that of the positive control as depicted in Table 3.

Graph 1
Inhibition of long acyl-HSLs using different extracts of T. cordifolia, R. cordifolia, P. kurroa, B. variegata and C. fistula



A comparison of the statistically significant (P < 0.05) results of % inhibition of long acyl-HSL production in P. aeruginosa and A. baumannii obtained with some of the plant extracts under study with toluene extract of garlic as positive control. CEE1 & HEE1: Cold and hot ethyl acetate extracts of T. cordifolia respectively; HME2: hot methanolic extract of R. cordifolia; CAE3: Cold aqueous extract of P. kurroa; CAE4: Cold aqueous extract of B. variegata; CHE5 and HHE5: Cold and hot n-hexane extracts of C. fistula respectively.

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Table 3
Inhibition of long acyl HSL production

Organism	Plant	Type of Extract	% Inhibition of β - galactosidase activity
P. aeruginosa	*T. cordifolia*	CAE	27.47 + 7.23
		HAE	25.10 + 9.37
		HME	24.53 + 2.74
	R. cordifolia	CAE	22.62 + 8.77
		HEE	15.56 + 3.14
		HHE	27.78 + 4.03
	P. kurroa	HEE	15.81 + 5.91
		CHE	18.29 + 0.68
		HHE	26.39 + 1.45
	B. variegata	CEE	20.43 + 6.94
		HEE	16.67 + 7.01
	C. fistula	CME	22.21 + 9.15
A. baumannii	*R. cordifolia*	CAE	27.65 + 6.92
		HME	24.29 + 0.83
	P. kurroa	HAE	29.24 + 3.41
	B. variegata	HAE	31.85 + 0.95
		CME	15.88 + 0.88
		CHE	18.88 + 2.42
	C. fistula	CAE	30.10 + 2.82
		HAE	29.47 + 2.82
		CME	25.69 + 1.98
		HME	23.84 + 1.85
		HHE	15.99 + 2.43

The values (mean + S.D.) of percent inhibition of β-galactosidase activity obtained for plant extracts with statistically significant results ($P \leq 0.05$) but showing less inhibition than positive control.

DISCUSSION

Five medicinal plants, *Rubia cordifolia*, *Tinospora cordifolia*, *Picrorhiza kurroa*, *Cassia fistula* and *Bauhinia variegata* were screened for the presence of anti-QS activity w. r. t. short and long acyl-HSLs. *P. aeruginosa* produces 2 types of acyl-HSLs, short chain - C_4-HSL (rhl system) and a long chain - 3-oxo-C_{12}-HSL (las system), both of which regulate virulence in the bacterium. In this organism; the las system controls the rhl system; it may be possible that by inhibiting las system, the rhl system will consecutively remain suppressed. However, in a study by Delden *et al*[21], LasR deficient strains were grown in high stress conditions and after growth, certain spontaneous mutants were isolated with restored production of virulence factors such as rhamnolipid and elastase. This was likely due to the over expression of rhlI as compared to the parent. But when strains in which both las and rhl systems were non-functional, were subjected to stress conditions, no mutants capable of producing virulence factors developed. Hence, it is necessary to target both las and rhl (long & short acyl-HSL systems, respectively) for effectively inhibiting virulence in *P. aeruginosa*. Initially the plant extracts under study were assessed for their ability to inhibit short acyl-HSL regulated pigment production in *C. violaceum* (Table 2). CEE & HEE of *T. cordifolia*, showed larger zone of inhibition than that of positive control, when 2 mg/ml concentration was used. Ethanolic

extracts of *Laurus nobilis* and *Sonchus oleraceus* were shown to produce a zone diameter of 24 ± 0.9 and 18 ± 0.5 at a concentration of 3 mg/disc, however, at the same concentration, the plant extracts were also shown to possess antibacterial activity[22]. Hence, there can be chances of resistance development with these plant extracts. Significant inhibitory activity was reported in commonly used fruits, herbs and spices at sub-lethal concentration against short acyl-HSLs regulated violacein production in *C. violaceum* and QS regulated swarming motility in *P. aeruginosa*[15]. The possible mechanism of action was hypothesized as competition with acyl-HSL molecules for binding to regulator proteins and further inhibition of QS, however, this was not confirmed. A similar study exhibited the presence of short acyl-inhibitory potential in the extracts of certain Indian medicinal plants, *Hemidesmus indicus* (L.) Schult, *Holarrhena antidysenterica* (Roth)A. DC., *Mangifera indica* L. and few others[23]. However, it is not known whether the plant extracts could inhibit long acyl-HSLs. The plant extracts under study were then tested for inhibition of long acyl-HSL production in *P. aeruginosa* and *A. baumannii* using *E. coli* MG4/pKDT17. This strain contains a *lasB-lacZ* detection system and a *lasR* gene under control of the *lac* operon promoter. The *lasB* and *lasR* genes are from *P. aeruginosa* and encode the quorum-sensing-regulated elastase and LuxR-type regulator LasR, respectively. Acyl-HSLs, externally added will bind to LasR regulator, the complex will then activate transcription of *lasB* gene and downstream gene *lacZ* which encodes for β-galactosidase. Amount of β-galactosidase activity corresponded to amount of long acyl-HSLs produced by bacteria in presence or absence of plant extracts. It was found that ethyl acetate extracts of *T. cordifolia* showed notable inhibition of long acyl-HSLs, about 78% & 71% in *P. aeruginosa* and *A. baumannii* respectively (Graph 1) while that of garlic extract was about 29% and 33% respectively. Among other plant extracts, higher values of inhibition were obtained with HME of *R. cordifolia* (37% inhibition in *P. aeruginosa* & 24% in *A. baumannii*) that was able to inhibit long acyl-HSLs produced by both pathogens. CHE & HHE of *C. fistula* showed higher value of inhibition in *P. aeruginosa* but quite low inhibition in *A. baumannii*; a possible reason could be specificity in activity against QS of *P. aeruginosa*. It is important to understand that decrease in long acyl-HSL production could be either due to anti-QS activity (specifically targeting long acyl-HSL QS system) or due to antibacterial activity (wherein due to reduction in cell density, smaller amount of acyl-HSL will be produced) of plant extracts. To verify this, antibacterial activity of plant extracts of *T. cordifolia* was studied at the concentration of 2 mg – 20 mg per disc, against *P. aeruginosa* and *A. baumannii*. There was no detectable antibacterial activity.Few studies have been focused to screen plant extracts for inhibition of both short and long acyl-HSLs. About 50 medicinal plants of South Florida were screened for anti-QS activity, out of which six plants, *Conocarpus erectus*, *Bucida buceras*, *Callistemon viminalis*, *Tetrazygia bicolor* and *Chamaesyce hypericifolia* were found to possess anti-QS activity against both short and long acyl-HSL systems, highest activity obtained with *C. erectus* and *B. buceras*[16]. Further these plant extracts were also shown to reduce the production of virulence factors and inhibit biofilm formation controlled in *P. aeruginosa*. Another study suggested that toluene extract of garlic and 4-nitro-pyridine-N-oxide possessed high activity against QS systems and related virulence in *P. aeruginosa*[14]. Also, garlic extract was shown to induce inflammation and enhance bacterial clearance in mice[24]. However, effect of garlic extract or extracts of South Florida plants on QS systems of other organisms have not been studied.

CONCLUSION

The present study explored 5 plants which were never tested earlier for inhibition of short and long acyl-HSL systems in gram negative

bacteria. Among all, ethyl acetate extracts of *T. cordifolia*, for first time, has been reported here to possess statistically significant anti-QS activity against *P. aeruginosa* and *A. baumannii*. Since the extracts not only showed short acyl-HSL inhibition but also inhibited long acyl-HSLs in *P. aeruginosa* as well as *A. baumannii*, it can be assumed that they may have multiple effects on the QS systems. It is known that QS is responsible for production of virulence factors and biofilm development in *P. aeruginosa* and *A. baumannii*. So, further aim is to test extract of *T. cordifolia* for inhibition of virulence and biofilms in these organisms. Also, the exact mechanism of action and identification of active phytochemicals remains to be established.

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ABBREVIATIONS

Acyl-HSL - Acyl homoserine lactone, QS - Quorum sensing, QSI – Quorum sensing inhibitor, ONPG - O-Nitrophenyl-β-D-galactopyranoside, 3-hydroxy-C_{12}-HSL - N-(3-hydroxydodecanoyl)-L-homoserine lactone, 3-oxo-C_{12}-HSL - N-(3-oxododecanoyl)-L-homoserine lactone, C_4-HSL - N-butanoyl-L-homoserine lactone, C6-HSL - N-hexanoyl-L-homoserine lactone, LB - Luria Bertani, MTCC - Microbial type culture collection (Chandigarh, India), ATCC - American type culture collection, CAE - Cold aqueous extract, HAE - Hot aqueous extract, CME - Cold methanolic extract, HME - Hot methanolic extract, CEE - Cold ethyl acetate extract, HEE - Hot ethyl acetate extract, CHE - Cold n-hexane extract, HHE - Hot n-hexane extract, β-gal - β-galactosidase

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