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Optimization of quorum quenching mediated bacterial attenuation of *Solanum torvum* root extract by response surface modelling through Box-Behnken approach

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ABSTRACT

The present study was intended to optimize the quorum sensing inhibitory action of *Solanum torvum* root extract against *Chromobacterium violaceum*. Factors such as bacterial density, frequency of administration and concentration of extract were analysed. Plant samples were collected from Thrissur District, Kerala, India. Response surface modelling of factors by Box-Behnken approach was employed for optimizing quorum quenching activity of extract. The adequacy of mathematical model was verified by ANOVA and Cook's distance table. Results revealed that quorum quenching property of *Solanum torvum* root extract is highly influenced by variables studied whereas maximum activity was found during administration of 300 μg/ml extract thrice in a day. It was also understood that extract does not possess any bactericidal activity wherein it only silence its quorum sensing mediated functions. This observations can be further used in quorum quenching studies.

1. Introduction

Quorum sensing is defined as the density dependent mechanism which enables the bacterial community to communicate each other through which the initiation of virulent activities are triggered. This is a mediated by signalling molecules commonly called as autoinducers (AI). Several bacterial mechanisms like pigment production, biofilm formation, swarming motility, toxin production and exozyme synthesis are controlled by quorum sensing [16]. Quorum sensing can be classified into three groups depending upon its mechanism; acyl homoserine lactone (AHL) mediated quorum sensing, which is exclusively observed only in gram negative bacteria, LuxS controlled autoinducer-2 intermediated communication, which is seen in both gram negative and gram positive bacteria, finally peptide induced signalling, which is spotted in gram positive bacteria [13]. Generally quorum sensing system in gram negative bacteria constitutes of two components; an autoinducer synthesizing gene and an autoinducer receptor cum transcriptional activator. Due to peculiar receptor binding sites on receptor gene, bacterial communication is highly intra-species specific that recognize only precise AHLs thus signals produced by one species will not disturb the mechanism of other [10]. It is possible to inhibit bacterial virulence by suppressing bacterial quorum sensing inside a host system thereby assisting host defence mechanism for successive clearance.

Inhibition of quorum sensing is called as quorum quenching wherein, the signalling mechanism is inhibited without effecting the bacterial growth. This disables the pathogen to initiate its virulent properties, as a result host defence mechanism gets enough exposure and time for effective immune clearance [3]. Quorum quenching can also be explained as in vivo attenuation of pathogens where the premature cessation of virulent genes occurs. Signal mediated bacterial communication could be stopped by the employment of different techniques depending upon the mechanism of signalling in organism and the site of reaction. These inhibitory methods are categorized into two groups such as enzymatic inhibition and non-enzymatic inhibition. Enzymatic inhibition includes the usage of enzymes that degrade signalling molecules; and successive signalling [14]. Non-enzymatic bacterial silencing can be attained by approaches like signal synthesis

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inhibition, competitive or allosteric inhibition of signal binding to response gene and through blocking signal reception by response gene through modification and conformational changes of signalling molecule structure [2].

Recently many plant products have been studied for its anti-quorum sensing activity but a clear documentation of factors effecting action is scarce. In present study we used *Solanum torvum* as our plant source of choice. It is an evergreen, spreading slender shrub growing from 2 to 4 m tall. It is commonly distributed in the Southern Asian countries, Tropical Africa and Latin American countries [18]. *Solanum torvum* have been proclaimed for its edible and medicinal properties. The leaves, stem and root of this plant have been used for the treatment of various diseases [7]. In this study we analyse the factors influencing quorum quenching activity in reference reporter strain *Chromobacterium violaceum* by *Solanum torvum* root extract by the employment of response surface modelling through Box-Behnken approach.

2. Materials and methods

2.1. Sample collection and extraction

Solanum torvum was collected from in and around of Thrissur District, Kerala, India during June 2015. Collected roots were washed and chopped into small pieces. Samples were sun dried until all moisture content was removed. Aqueous extracts were obtained by boiling root sample in hot water at 100 °C for three hours and resulting suspension was filtered through Whatman filter paper which was further concentrated and dried under controlled laboratory conditions to get *Solanum torvum* root extract [1].

2.2. Bacterial strains and maintenance

The quorum quenching activity of a compound was screened against the reporter bacterial strains. Type strains *Chromobacterium violaceum* MCC 2290 (wild strain) and *Chromobacterium violaceum CV26* MCC 2216 (mutated non-chromogenic strain) were obtained from NCCS, Pune, India. Study strains were maintained in Luria-Bertani (LB) agar slants in 4 °C with proper subculture.

2.3. Investigation quorum quenching action and MIC

Overnight broth culture of *Chromobacterium violaceum* CV26 was inoculated onto molten Luria-Bertani (LB) agar plates supplemented with N-3-oxohexanoyl-homoserine lactone (0.25 mg/ml). Aqueous root extracts were incorporated onto a sterile disc and was placed over bacterial lawn culture. Disc loaded with distilled water was maintained as control. Quorum quenching ability of samples was screened by inhibition of chromogenesis and formation of halo turbid zone [6]. Minimum concentration of extract required for quorum quenching was determined by administrating the extract in varying concentrations (0–300 μg/ml) in above mentioned bacterial broth culture containing 1.37×10^8 CFU [17].

2.4. Response surface modelling of quorum quenching action of STRE by Box-Behnken approach

The effects of various factors influencing quorum quenching action of STRE (*Solanum torvum* root extract) was analysed by response surface modelling. The Box- Behnken model for three independent variables such as bacterial density (X_1), drug concentration (X_2) and frequency of administration (X_3) was used in the experimental design model. The ranges and levels of independent variables taken in this study are mentioned in Table 1. The experimental design matrix was obtained from the Box-Behnken model and it is displayed in Table 2. The number of experiments was determined by the employment of equation

$$N = k_2 + k + cp$$

whereas k is the factor number and cp is the number of centre point replicates. The coded values of process variables were found out by substituting the equation

$$x_i = (X_i - X_0)/\Delta X, \quad i = 1;\ 2;\ 3\ \ldots;\ k$$

where X_i is the dimensionless value of a process variable, Xi is the real value of an independent variable, X_0 is the value of Xi at the centre point and ΔX is the step change. Each independent variable was changed over three levels viz. factorial points (− and +), axial points (− and +) and centre point leading to quadratic model. A second-order polynomial regression model equation was investigated in order to predict the quorum quenching efficiency of STRE (*Solanum torvum* root extract) [4].

Table 1
Experimental range and levels of variables selected.

Factors effecting quorum quenching	−1	0	1
Bacterial density (CFU)	1.37×10^8	2.74×10^8	4.11×10^8
Drug concentration (μg/ml)	100	200	300
Frequency of administration	1	2	3

Table 2
Box-Behnken design matrix for the optimization.

Run	Factor 1 A: Bacterial density (CFU)	Factor 2 B: Drug concentration (μg/ml)	Factor 3 C: Frequency of administration
1	4.11×10^8	200	3
2	1.37×10^8	300	2
3	1.37×10^8	200	1
4	2.74×10^8	200	2
5	1.37×10^8	100	2
6	2.74×10^8	200	2
7	2.74×10^8	300	1
8	2.74×10^8	100	3
9	4.11×10^8	200	1
10	4.11×10^8	100	2
11	4.11×10^8	300	2
12	2.74×10^8	200	2
13	1.37×10^8	200	3
14	2.74×10^8	200	2
15	2.74×10^8	300	3
16	2.74×10^8	100	1
17	2.74×10^8	200	2

2.5. Statistical analysis

The data obtained from the study was subjected to statistical analysis. The data was subjected to one-way ANOVA (Analysis of Variance) followed by Dunnett's post test using Graph-pad prism Version 5.01 software.

3. Results

3.1. Screening and quantification of quorum quenching activity

Quorum quenching efficacy of the root sample was confirmed through quorum sensing inhibition plate assay through the formation of non-pigmented turbid zone of violacein inhibition at the site of administration. The activity quantification analysis clearly suggested that the activity of extract is in direct proportion to its concentration, which was evident as higher activity with higher concentration is observed. Maximum quorum quenching activity

(88.66%) was found in the concentration of 300 μg/ml however concentration less than 50 μg/ml did not show any quorum quenching activity.

3.2. Evaluation of adequacy of model for quorum quenching action

The Quadratic model was found to be the appropriate fit for the experimental data compared that with other models based on the evaluation of scores obtained from sequential model sum of squares (Table 3). The Higher F value (450.42) and smaller value of p (<0.0001) specified the high significance of the model. The accuracy and significance of model was further analysed by the employment of ANOVA (Table 4) by identifying significant and non-significant terms based on their F and p values. The F and Prob > F values for lack of fit were 0.22 and 0.88 respectively, which indicates the non-significance, hence the model fits well for quorum quenching analysis. It was evident that all the variables and their interactions were significant for quorum quenching activity which was expressed by the following equation in terms of coded factors where quorum quenching activity is denoted by R_1.

$$R_1 = 50.13 - 26.06 * A + 19.06 * B + 7.53 * C + 6.35 * AB + 2.42 * AC - 0.72 * BC + 12.08 * A^2 - 2.90 * B^2 - 3.53 * C^2.$$

Diagnostics of case statics was scrutinized to determine the value of leverage, internally studentized residuals, externally studentized residuals, DFFITS and Cook's distance (Table 5). The results stated that the chosen model is adequate.

Table 3
Sequential Model Sum of Squares and suggested model.

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	Inference
Mean vs Total	47381.66	1	47381.66			
Linear vs Mean	8791.15	3	2930.38	44.04	< 0.0001	
2FI vs Linear	186.58	3	62.19	0.92	0.4675	
Quadratic vs 2FI	675.01	3	225.00	450.42	< 0.0001	Suggested
Cubic vs Quadratic	0.49	3	0.16	0.22	0.8805	Aliased
Residual	3.01	4	0.75			
Total	57037.91	17	3355.17			

Table 4
ANOVA for Response Surface Quadratic model.

ANOVA for Response Surface Quadratic model						
Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	9652.74	9	1072.53	2147.02	<0.0001	Significant
A-Bacterial density	5431.43	1	5431.43	10872.81	<0.0001	
B-Drug concentration	2906.27	1	2906.27	5817.87	<0.0001	
C-Frequency of administration	453.46	1	453.46	907.74	<0.0001	
AB	161.16	1	161.16	322.62	<0.0001	
AC	23.33	1	23.33	46.70	0.0002	
BC	2.09	1	2.09	4.18	0.0802	
A^2	614.73	1	614.73	1230.59	<0.0001	
B^2	35.52	1	35.52	71.11	<0.0001	
C^2	52.38	1	52.38	104.85	<0.0001	
Residual	3.50	7	0.50			
Lack of Fit	0.49	3	0.16	0.22	0.8805	Not significant
Pure Error	3.01	4	0.75			
Cor Total	9656.24	16				

Table 5
Diagnostics case statistics of statistical analysis.

Run order	Actual value	Predicted value	Residual	Leverage	Internally studentized residual	Externally studentized residual	Cook's distance	Influence on fitted value DFFITS
1	42.85	42.58	0.27	0.750	0.771	0.746	0.178	1.293
2	98.38	98.08	0.30	0.750	0.845	0.826	0.214	1.431
3	79.36	79.63	−0.27	0.750	−0.771	−0.746	0.178	−1.293
4	50.17	50.13	0.036	0.200	0.057	0.053	0.000	0.026
5	72.83	72.66	0.17	0.750	0.492	0.463	0.073	0.802
6	49.55	50.13	−0.58	0.200	−0.924	−0.913	0.021	−0.456
7	55.93	55.96	−0.026	0.750	−0.074	−0.069	0.002	−0.119
8	32.92	32.89	0.026	0.750	0.074	0.069	0.002	0.119
9	22.89	22.69	0.20	0.750	0.566	0.536	0.096	0.929
10	7.55	7.85	−0.30	0.750	−0.845	−0.826	0.214	−1.431
11	58.49	58.66	−0.17	0.750	−0.492	−0.463	0.073	−0.802
12	49.01	50.13	−1.12	0.200	−1.778	−2.223	0.079	−1.111
13	89.66	89.86	−0.20	0.750	−0.566	−0.536	0.096	−0.929
14	51.02	50.13	0.89	0.200	1.402	1.530	0.049	0.765
15	69.47	69.57	−0.099	0.750	−0.279	−0.260	0.023	−0.451
16	16.49	16.39	0.099	0.750	0.279	0.260	0.023	0.451
17	50.92	50.13	0.79	0.200	1.243	1.304	0.039	0.652

3.3. Response surface modelling of factors influencing quorum sensing inhibition

The influence of bacterial density and drug concentration on quorum quenching when the drug was administrated twice a day is been revealed in Fig. 1. Maximum activity was obtained when the bacterial density was kept lowest, the increment of drug concentration resulted in an elevation of response which indicated that drug response is in direct proportion to concentration. The effect of bacterial density and frequency of administration where concentration of antagonist was kept constant (200 μg/ml) as presented in Fig. 2. It was understood that the quorum sensing due to high bacterial density can be neutralized by increasing the frequency of administration whereas the degree of response was found to be increasing with number of administration. Effect of drug concentration and frequency of administration at the bacterial density 274×10^8 CFU can be perceived from Fig. 3. Diagram suggested that the administration of single high dosage is more effective than fractionation whereas 55.93% of quorum quenching was obtained when 300 μg/ml drug administrated once in the time span of 24 h, however there was only 32.92% quorum sensing inhibition occurred after administrating 100 μg/ml thrice in the same time period. The comparative effect of individual variables was identified from perturbation plot (Fig. 4), sharp curvature of all variables suggested that quorum sensing inhibition is highly sensitive to these independent variables.

4. Discussion

Solanum torvum is commonly known as Turkey berry, Devils fig, prickly nightshade etc [12]. S. torvum has be proven for its pharmacological activates such as anti-inflammatory, antioxidant, antidiabetic, antihelmintic, anti-hyperlipidemic and nephroprotective activities [15], however this is the first report of quorum

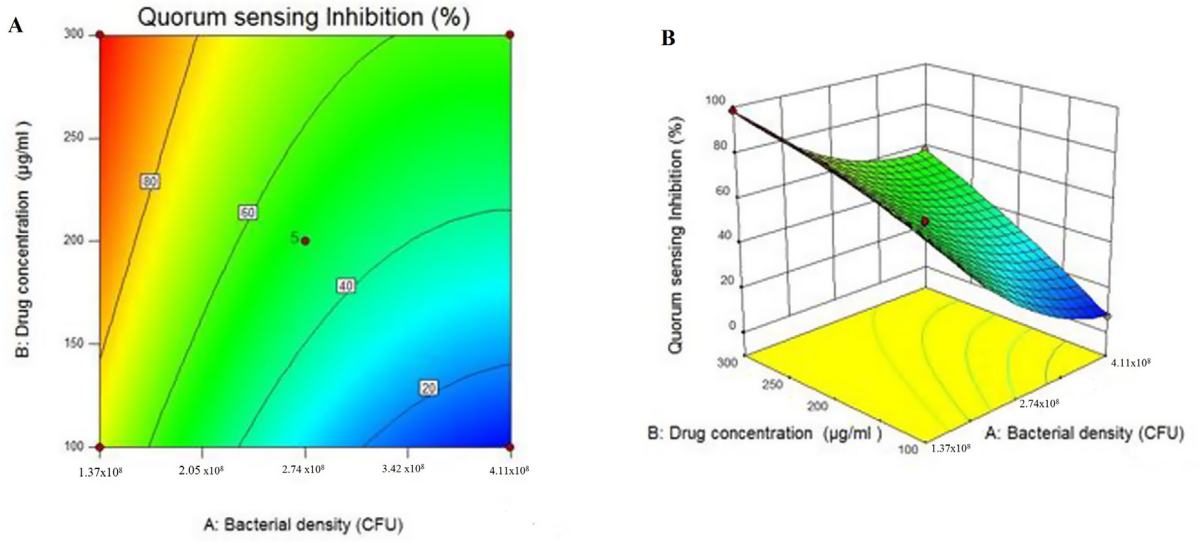

Fig. 1. Effect of bacterial density and drug concentration upon quorum sensing inhibition. (A) Contour plot, (B) 3D surface diagram.

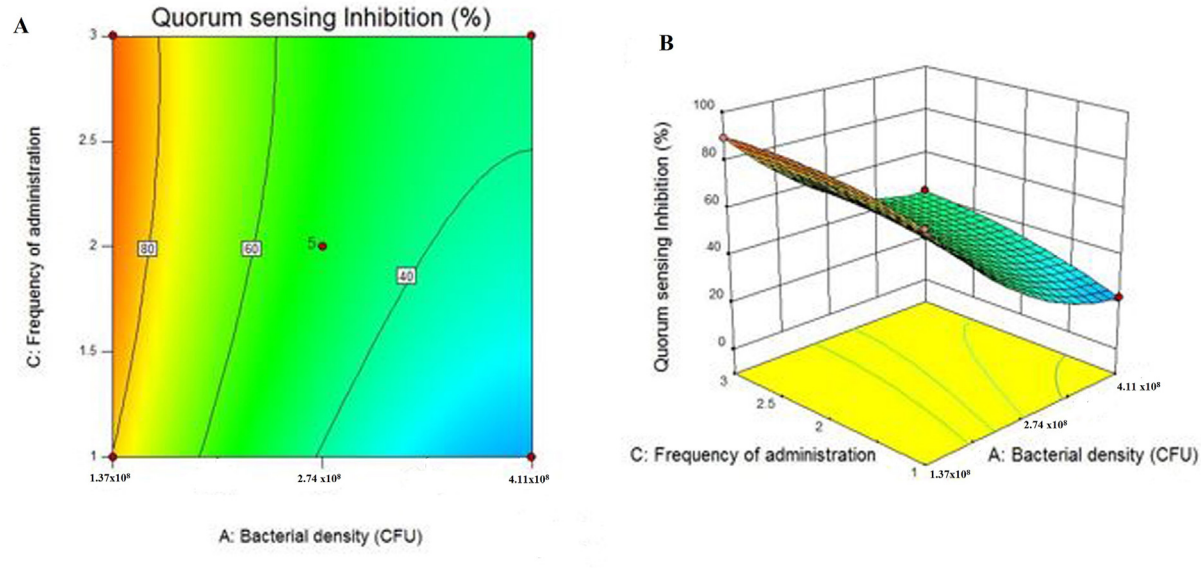

Fig. 2. Effect of bacterial density and frequency of administration upon quorum sensing inhibition. (A) Contour plot, (B) 3D surface diagram.

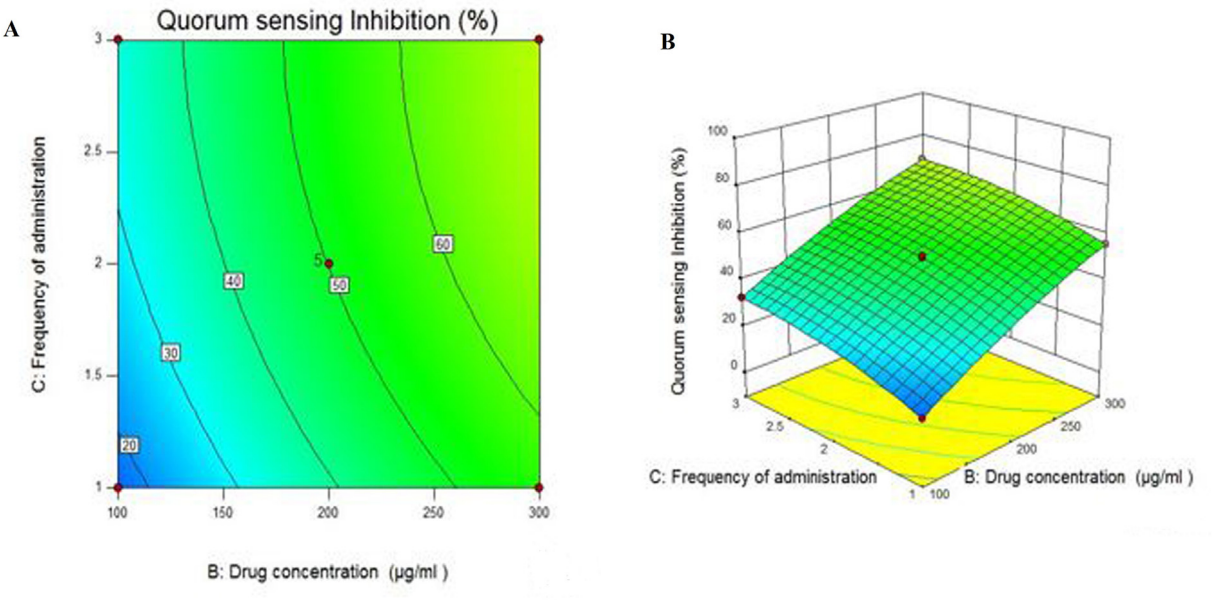

Fig. 3. Effect of drug concentration and frequency of administration upon quorum sensing inhibition. (A) Contour plot, (B) 3D surface diagram.

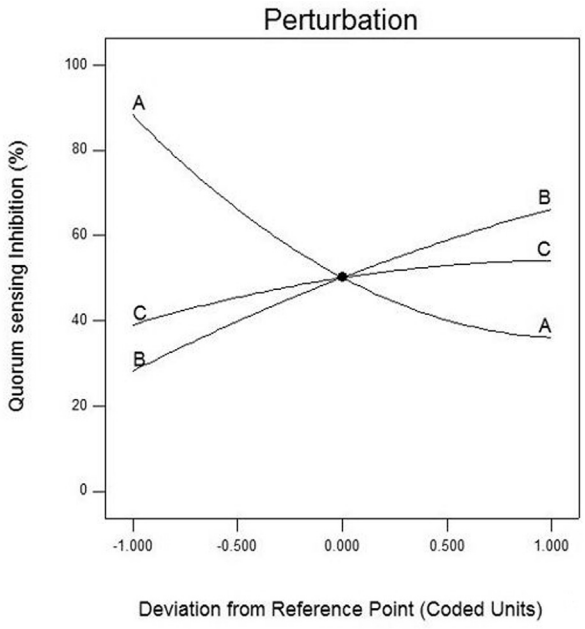

Fig. 4. Perturbation plot showing the combined effect of factors in quorum sensing inhibition.

quenching activity by the same. For analysis roots were chosen as it is in constant contact with rhizosphere microorganisms where high degree of bacterial density mediated quorum sensing is encountered [5], hence it is assumed that roots may contain natural quorum sensing inhibitors in it. It was evident by the quorum quenching activity of root extracts. Similarly antagonistic action of root extract is previously reported by Rajasekharan et al. [11], where the quorum quenching mediated bacterial attenuation was attained by Burdock root extracts against pathogens of urinary tract infection. A minimum 50 µg/ml of extract was essential for inhibiting bacterial quorum sensing in *Chromobacterium violaceum* which suggests that this is a concentration mediated inhibitory mechanism.

Box-Behnken approach to understand the influence of independent variables such as bacterial density, concentration of antagonist and frequency of administrated revealed that the quorum sensing inhibition mechanism is highly sensitive to these factors. It was understood that when bacterial density increased the quorum sensing rate also increases, though there was no linear relationship found. The initial concentration of antagonist played vital role in inhibiting signalling where as in constant time interval a large amount of initial drug administration was more effective than administrating the same concentration as different fractions. Similarly this approach was used for the understanding of factors influencing bisoprolol fumarate matrix tablets for sustained drug release [9]. The reliability of this bio-statistical approach was tested by diagnosing case statistics where the leverage value was within 0–1 and limit of the internally studentized residuals was found to be ±3 sigma through which the number of standard deviation separating actual and predicted values were measured. The influence of the observed value on its predicted value was measured by DFFITS, and its limit lies in between +2 and −2. Cook's distance measures the change in regression when the case is omitted from the analysis, and this must be in the range of ±1 which was fulfilled in our studies. Therefore depending upon the criteria, analysis of diagnostic case statistics of data shows that the model fits well to optimize the independent variables for quorum quenching activity [8].

5. Conclusion

Solanum torvum was found to have anti quorum sensing activity. It was evident that action was strongly influenced by factors such as bacterial density, drug concentration and frequency of administration. Activity varied with respect to variables and the optimum condition was found to be 300 µg/ml extract thrice in a day to get persistent bacterial attenuation.

Conflict of interest

We declare 'no conflict of interest'.

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