

Comparative Study on Quorum Modulatory Effect of Selected Medicinal Plants on *Chromobacterium violaceum* ATCC 12472 (MTCC 2656)

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Abstract

Among the main global health concern is the rampant rise in antibiotic resistant bacteria. One of the appealing and promising strategies to combat this menace is to target the adaptive mechanism called quorum sensing (QS) used by bacteria to survive. Exploratory research on anti-QS compounds derived from natural products has been a promising area. The present study investigated methanolic extracts from 26 plants to compare their anti-QS activity using the QS biosensor strain *Chromobacterium violaceum* American Type Culture Collection (ATCC 12472) (Microbial Type Culture Collection MTCC2656). QS-mediated violacein pigment inhibition was carried out using agar well diffusion method with concentrations ranging from 10 mg/ml to 100 mg/ml. Leaf extracts of *Mangifera indica* and *Pimenta dioica* and peel extract of *Punica granatum* were the only three plants found to exhibit violacein inhibitory potential till 10 mg/ml. The result of the minimum inhibitory concentration (MIC) showed 1.6 mg/ml for *M. indica* and *P. dioica* and 6.25 mg/ml for *P. granatum*. Further, violacein inhibitory properties of these extracts at and below MIC were evaluated by well diffusion assay (qualitative) and by flask incubation assay (quantitative). The zone of inhibition (well diffusion assay) was found to be 14.51 ± 0.63 mm to 10.37 ± 0.68 mm for *M. indica*, 15.23 ± 0.57 mm to 9.62 ± 1.29 mm for *P. granatum* and 17.01 ± 0.1 mm to 13.14 ± 0.18 mm for *P. dioica*. The inhibitory effect of the plant extracts via quantitative assay on violacein ranged from 83-49%, 89-81%, and 89-49% for *M. indica*, *P. granatum*, and *P. dioica* respectively. Our findings suggested the potential of *M. indica*, *P. granatum*, and *P. dioica* methanolic extracts as a source of effective inhibitors of QS-mediated violacein production.

Keywords: Antibiotic Resistance, *Chromobacterium violaceum*, Plant Extracts, Quorum Sensing, Violacein, Virulence Factors

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INTRODUCTION

The increased prevalence of multidrug-resistant bacteria is taking the world back to the pre-antibiotic era¹ and has encouraged researchers to focus on alternative approaches.² Antibacterial resistance exhibited by many bacteria is reported to be associated with QS-regulated behaviour. QS is the density-dependent process that allows bacterial interaction for survival and subsequent enhancement in their pathogenicity.³ One of the promising options to deal with pathogenic bacteria is to interfere with the mechanism leading to their virulence factors production. Researchers are trying to fight infections due to pathogenic bacteria by identifying methods/compounds that target particular modulators of QS-mediated virulence factors.⁴ Current studies suggest that various virulence factors produced by bacteria (both Gram-negative and Gram-positive) are related to QS which are facilitated by auto inducers. Auto-inducing peptides (AIP) produced by Gram-positive and acyl homoserine lactones (AHL) by Gram-negative bacteria are not just known to contribute to inter-microbial communication but also directly or indirectly cross-communicate with their host.^{1,5} Novel ways that block or manipulate the QS pathways are evolving as promising antipathogenic or antivirulence therapeutic strategies to combat bacterial colonization and infection.⁶

Despite the multitude of antibacterial agents available, resistance to them is increasing primarily due to their misuse or overuse coupled with poor infection prevention and control. Plant extracts with diverse array of phytoconstituents like alkaloids, flavonoids, phenols, saponins, coumarins and terpenes have been considered effective in inhibiting the multidrug resistance phenotype of pathogens via their counter-action towards diverse factors that promote the emergence of drug resistance.⁷ The antibacterial potential of various phytochemicals as well as their effect on virulence factors production such as swarming motility, pyocyanin and biofilm production have been reported. Inhibitory properties of quercetin, a common phytoconstituent, on virulence factors and biofilm development in *Pseudomonas aeruginosa* PAO1, have been reported.⁸ A study that investigated the effect of selected phenolic secondary metabolites on QS-related virulence

factor production of PAO1, demonstrated the reduction in the pyocyanin and biofilm formation by caffeic and ferulic acids.⁹

Attempts to develop easy and rapid techniques utilizing model organisms to identify new and potent QS inhibitors (QSI) have resulted in an increased interest towards natural products from both microbes and plants.¹ *C. violaceum* has been frequently used for QS investigations as a model organism because of its indicator ability associated with QS-regulated violacein biosynthesis, and its suitability in understanding how molecules interact and affect the QS mechanism of microorganisms. This property of *C. violaceum* has been explored for understanding how disrupting bacterial communication can affect their pathogenicity in qualitative and quantitative terms.

Detailed understanding on QS regulated virulence factor production has helped scientists identify different mechanisms of bacterial pathogenicity. Therefore it is important for scientists to focus on novel ways and products particularly from plants for interrupting QS.¹⁰ The plant extracts which exhibited quorum sensing mediated pigment inhibition in *C. violaceum* appear promising for use as sources of phytochemicals that will reduce bacterial resistance via inhibition of diverse virulence factor production. In this context, the present study was designed to investigate 26 medicinal plant extracts to validate their quorum sensing inhibition potential and relative quorum modulatory efficiency.

MATERIALS AND METHODS

Plant material collection and authentication

Twenty-six plant materials were sourced from Ernakulam city, Kerala, India, Indian Institute of Horticultural Research Bangalore (IIHR) and online ayurvedic product dealers. Out of this, 23 plants were authenticated by Dr. P.E. Rajasekharan, Principal Scientist, Division of Flower and Medicinal Crops, IIHR, Bangalore. The remaining three plants were authenticated by Dr. V. Rama Rao, Research Officer (Botany) at Central Ayurveda Research Institute, Uttarahalli, Bengaluru-560109, with voucher numbers RRCBI-13286 (*Punica granatum*), RRCBI-MUS110 (*Mangifera indica*) and RRCBI-

MUS411 (*Pimenta dioica*). The list of plants screened for their quorum sensing inhibitory activity is given in Table 1.

Preparation of plant extracts

Plant materials consisting of leaves, roots and peels were washed in tap water, shade dried and ground to powder. Two hundred milliliters of absolute methanol was added to 20 grams of the powdered plant materials and kept in the orbital shaker incubator at room temperature. After 48 hours of extraction, filtration was done using filter paper (Whatman No. 1) and was dried at 45°C.³¹

The dried crude extracts were stored at -20°C until further analysis. Stock concentration of 50 mg/ml was made in 1% DMSO from which further assays were performed.⁴¹

Bacterial strain and culture conditions

Lyophilized culture of *C. violaceum* (MTCC 2656) was obtained from MTCC, IMTECH, Chandigarh, India. This bacteria is used as biosensor strain because it produces a purple pigment called violacein that is QS regulated. The strain was routinely cultured in Luria-Bertani (LB) broth (1% NaCl, 1% tryptone, 0.5% yeast extract)

Table 1. List of Indian medicinal plants screened for anti-quorum sensing activity against *C. violaceum* MTCC 2656

No.	Plants	Family	Common name	Parts used	Ref.
1.	<i>Aegle marmelos</i>	Rutaceae	Indian bael	leaves	11,12
2.	<i>Aloe barbadensis</i> Miller	Asphodelaceae (Liliaceae)	aloe	leaves	13
3.	<i>Asparagus recemosus</i>	Asparagaceae	shatawari	root	14,15
4.	<i>Azadirachta indica</i>	Meliaceae	neem	leaves	16
5.	<i>Bacopa monnieri</i>	Plantaginaceae	brahmi	leaves	17,18
6.	<i>Baliospermum montanum</i>	Euphorbiaceae	danti, wild castor	leaves	19
7.	<i>Bryophyllum pinnatum</i>	Crassulaceae	miracle leaf	leaves	20
8.	<i>Calotropis gigantea</i> L.	Apocynaceae	giant milk weed	leaves	21
9.	<i>Cassia fistula</i>	Fabaceae	golden shower	leaves	22
10.	<i>Chromolaena odorata</i>	Asteraceae	siam weed, christmas bush	leaves	23,24
11.	<i>Cissus quadrangularis</i>	Vitaceae	veldt grape, asthisamharaka	stem	25
12.	<i>Citrus reticulata</i> L.var	Rutaceae	mandarin orange.	Pericarp (of fruit)	26
13.	<i>Gmelina arborea</i>	Lamiaceae	gamhar, white teak	leaves	27,28
14.	<i>Glycyrrhiza glabra</i>	Fabaceae	licorice	root	29
15.	<i>Gymnema sylvestre</i>	Apocynaceae	gurmar, Australian cowplant	leaves	30
16.	<i>Hemidesmus indicus</i>	Apocynaceae	anantmool	roots	31
17.	<i>Hemigraphis colorata</i>	Acanthaceae	red ivy	leaves	32
18.	<i>Mangifera indica</i>	Anacardiaceae	mango tree	leaves	33
19.	<i>Moringa oleifera</i>	Moringaceae	drumstick tree	leaves	34
20.	<i>Pimenta dioica</i>	Myrtaceae	Jamaica pepper	leaves	35
21.	<i>Piper betle</i>	Piperaceae	betel	leaves	36
22.	<i>Piper nigrum</i>	Piperaceae	black pepper	Leaves	37
23.	<i>Psidium guajava</i>	Myrtaceae	common guava	leaves	38
24.	<i>Punica granatum</i>	Lythraceae	pomegranate	peel and membrane	39
25.	<i>Syzygium cumini</i>	Myrtaceae	Jamun	leaves	4
26.	<i>Vitex negundo</i>	Lamiaceae	Chinese chaste tree	leaves	40

between 30-37°C.¹ Glycerol stock cultures of the organism were made and stored at -80°C for further use.

Media and chemicals

LB agar (Miller), Muller Hinton Broth (MHB), LB broth (Miller), and glycerol were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd, Mumbai. p-iodonitrotetrazolium violet (INT) was supplied by Himedia Laboratories, Mumbai. Cinnamon oil was purchased from essencia (<https:// essenciaonline.com>). All the chemicals used for the study were of analytical grade.

Assays for anti-QS activity

Screening for violacein inhibition (Qualitative assay)

The agar well diffusion assay was performed to screen the violacein pigment inhibitory activity of 26 selected medicinal plants on *C. violaceum* MTCC 2656 strain. Overnight bacterial cultures in LB broth were standardized to 0.5 McFarland and 0.1 ml of the inoculum was spread onto LB agar plates.⁴² Wells of 8 mm diameter were made on the agar plates, and 100, 75, 50, 25, and 10 mg/ml of the plant extracts were added to the wells and incubated for 24 hrs at 30°C. Cinnamon oil (2 µl/ml) and 1% DMSO served as positive and negative controls, respectively. The quorum inhibitory effect was determined by measuring the diameter (mm) of the zone of violacein inhibition around the wells.¹

Determination of MIC

MIC of the plant extracts, which exhibited violacein inhibition up to 10 mg/ml was performed. A two-fold serial dilution method in 96 well microtiter plate was used for MIC determination. An aliquot of 50 µl MHB was added to the wells of microtiter plate (1-10th well). Methanolic extract (50 mg/ml) was made in 1% DMSO. From the 50 mg/ml stock prepared, 100 µl was added to the first well. A serial dilution of the extracts was made by mixing and taking 50 µl to the subsequent well up to the 10th well. The last 50 µl was discarded from the 10th well. Bacteria, MHB broth and 10 µg/ml of gentamycin (positive control) were added to the 11th well. The media (MHB broth) and bacteria (12th well) was considered as negative control.⁴³

Bacterial cultures were grown overnight in MHB and were adjusted to a turbidity of 0.5 McFarland equivalent to 1.5×10^8 cfu/ml. The inoculum was diluted 100 times with MHB, and 50 µl of this was added into each well (1-12) to arrive at bacterial suspension with a density of 5×10^5 CFU/ml.⁴⁴ The bacteria were exposed to extract concentrations ranging from 50 to 0.1 mg/ml and were incubated for 24 hrs at 37°C. 40 µl of 0.2 mg/ml of INT was added to each well and further incubated for 1 hr. The change in the colour of dye from colourless to pink was due to the live and active bacteria. MIC of the plant extracts were performed in 3 independent trials in triplicates.⁴⁵

Qualitative violacein inhibition assay

The agar-well diffusion assay was performed on the selected plant extracts which exhibited violacein inhibition at and sub-MIC concentration following the method described in the screening for violacein inhibition above.¹

Quantification of violacein pigment (flask incubation assay)

The quantification of violacein pigment was performed using the method described by Choo *et al.*⁴⁶ with slight modifications. *C. violaceum* MTCC 2656 was incubated in LB broth and kept at 30°C for 24 hrs. The bacterial turbidity was later adjusted to 0.5 McFarland using UV-visible spectrophotometer which is equivalent to 1.5×10^8 cfu/ml. One ml of this bacterial suspension was added to conical flask containing LB broth and plant extracts to achieve the following concentrations (6, 5, 4, 3, 2, 1, 0.5 mg/ml) for *P. granatum* peel and (1.6, 1.4, 1.2, 1.0, 0.8, 0.6 mg/ml) for *M. indica* and *P. dioica* leaves. Bacteria and broth only served as negative control, while 2 µl/ml cinnamon oil as positive control. After overnight incubation, 1 ml from each flask was transferred to Eppendorf tubes and centrifuged at 13000 rpm for 10 minutes to precipitate violacein. The pellets obtained were resuspended in 1 ml DMSO. After rigorous mixing for 30 s to solubilize violacein pigment, samples were centrifuged at 13000 rpm for 10 minutes to remove the cells. Two hundred microliters of the suspension were transferred to 96 well microtiter plate and the absorbance was taken at 585 nm spectrophotometrically (Varioskan™ lux multimode plate reader, Thermo Scientific

United States). The experiments were carried out in three independent trials in triplicates. The mean absorbance of the samples were determined and the violacein inhibition was calculated using the equation below:

$$\% \text{ Violacein Inhibition} = \frac{\text{OD}_{(\text{control})} - \text{OD}_{(\text{test})}}{\text{OD}_{(\text{control})}} * 100$$

Where OD_(control) is the average OD of negative control (bacteria and broth). OD_(test) is the average OD of the test for each concentration of the extract.⁴⁷

Statistical analysis

The experiments were carried out in triplicate of 3 replicate analysis and the data were presented as mean ± standard error of the mean (SEM). One way analysis of variance (ANOVA) followed by Tukey's multiple comparison to understand statistical significance between the control and the various concentrations of treatments was used. GraphPad Prism (Version 8.0.1) was used for statistical analysis and p value ≤ 0.05 was considered statistically significant.

RESULTS

Screening of plants for their quorum sensing activity

Results of the twenty-six (26) plant extracts screened for their quorum sensing mediated violacein inhibition in *C. violaceum* MTCC 2656 are presented in Table 2. For initial screening, the plant extracts were tested for the violacein inhibition using concentrations ranging 10-100 mg/ml. Among the plant extracts that exhibited violacein inhibition, *P. betle* (leaf), *P. nigrum* (leaf), *P. guajava* (leaf) and *S. cumini* (leaf) showed inhibition up to 25 mg/ml while *M. indica* (leaf), *P. dioica* (leaf) and *P. granatum* (rind) showed violacein inhibition up to 10 mg/ml. The three plants (*M. indica*, *P. granatum* and *P. dioica*), which gave violacein inhibition at 10 mg/ml and below, were shortlisted for this study.

Minimum inhibitory concentration (MIC)

Three plants (*M. indica*, *P. granatum* and *P. dioica*) that had violacein pigment inhibition

potential till 10 mg/ml were selected to check their minimum inhibitory concentration against *C. violaceum* MTCC 2656. Table 3 presents the results from this experiment.

Qualitative violacein inhibition assay

Agar well diffusion assay was performed for the selected plant extracts using MIC and sub-MIC concentrations (Table 4). Loss of purple colour around the wells in which plant extracts were placed is indicative of their quorum inhibitory activity (Figure 1).

From the above results (Figure 1), it was observed that the quorum inhibitory activity was concentration-dependent. With the decrease in

Table 2. Screening results of plant extracts for their violacein inhibition

No.	Plants	Inhibition of violacein production
1.	<i>Aegle marmelos</i>	negative
2.	<i>Aloe barbadensis miller</i>	negative
3.	<i>Asparagus recemosus</i>	negative
4.	<i>Azadirachta indica</i>	negative
5.	<i>Bacopa monnieri</i>	negative
6.	<i>Baliospermum montanum</i>	negative
7.	<i>Bryophyllum pinnatum</i>	negative
8.	<i>Calotropis gigantea</i> L.	negative
9.	<i>Cassia fistula</i>	negative
10.	<i>Chromolaena odorata</i>	negative
11.	<i>Cissus quadrangularis</i>	negative
12.	<i>Citrus reticulata</i> L.var	negative
13.	<i>Gmelina arborea</i>	negative
14.	<i>Glycyrrhiza glabra</i>	negative
15.	<i>Gymnema sylvestre</i>	negative
16.	<i>Hemidesmus indicus</i>	negative
17.	<i>Hemigraphis colorata</i>	negative
18.	<i>Moringa oleifera</i>	negative
19.	<i>Vitex negundo</i>	negative
20.	<i>Mangifera indica</i>	positive
21.	<i>Pimenta dioica</i>	positive
22.	<i>Piper betle</i>	positive
23.	<i>Piper nigrum</i>	positive
24.	<i>Psidium guajava</i>	positive
25.	<i>Punica granatum</i>	positive
26.	<i>Syzygium cumini</i>	positive

Positive = showing violacein inhibition, Neegative = not showing violacein inhibition.

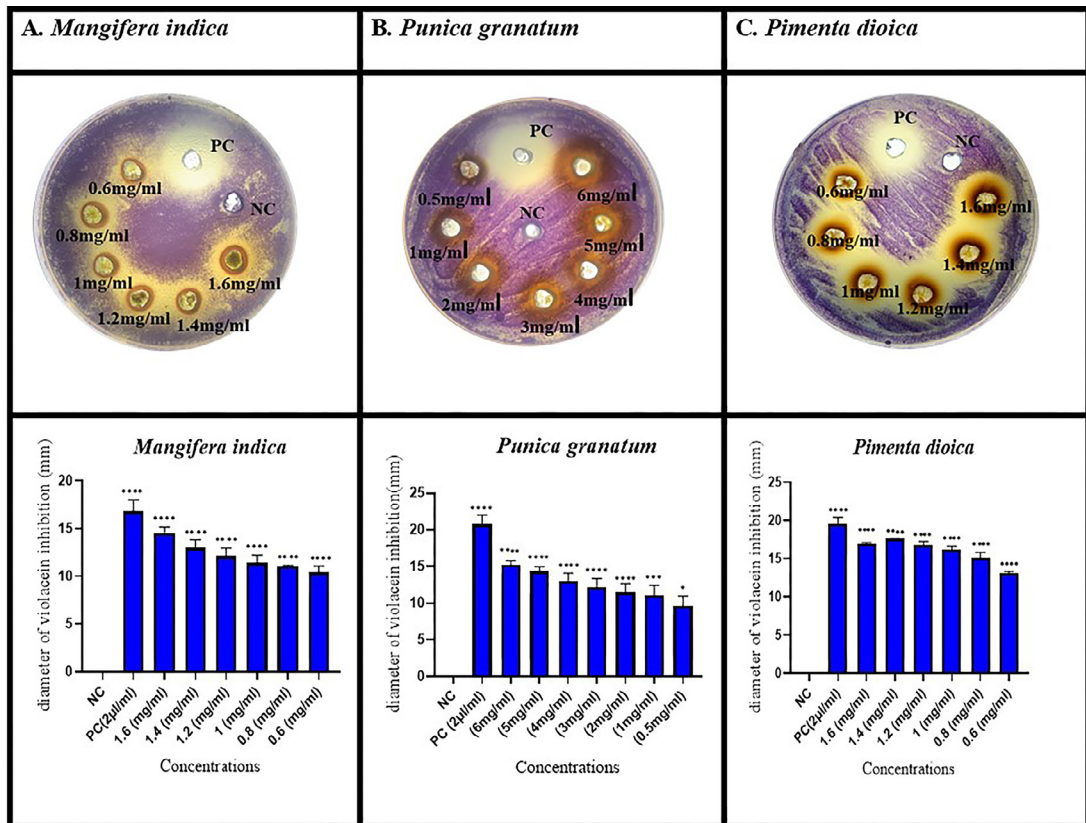


Figure 1. Violacein inhibition by well diffusion assay
Data were considered as mean ± SEM of three independent biological replicates. p values were calculated using one-way ANOVA. Tukey’s post hoc was used for multiple comparisons. p < 0.05 is considered significant. (*p < 0.05, ***p < 0.001, ****p < 0.0001). PC = positive control (cinnamon oil), NC=negative control (1% DMSO). All the treatments were compared with the negative control"

Table 3. MIC of top 3 violacein inhibiting plant candidates

Plant extracts	MIC against <i>C. violaceum</i> 2656
<i>M. indica</i>	1.6 mg/ml
<i>P. granatum</i>	6.25 mg/ml
<i>P. dioica</i>	1.6 mg/ml

Table 4. Plant extract Concentrations used for violacein inhibition assays

Plant extracts	Concen. used (mg/ml)
<i>M. indica</i>	1.6, 1.4, 1.2, 1.0, 0.8, 0.6
<i>P. granatum</i>	6, 5, 4, 3, 2, 1, 0.5
<i>P. dioica</i>	1.6, 1.4, 1.2, 1.0, 0.8, 0.6

the concentration of extract, the diameter of the clear zone of pigment inhibition around the wells also decreased.

Violacein quantification

Violacein pigment was quantitatively determined in response to the above-mentioned concentrations (Figure 2) of the shortlisted plant extracts. Concentration-dependent pigment inhibitory effect was observed. These inhibitory effect of the plant extracts on violacein ranged from 83%, -49%, 89%, -81%, and 89%-49% for *M. indica*, *P. granatum* and *P. dioica*, respectively.

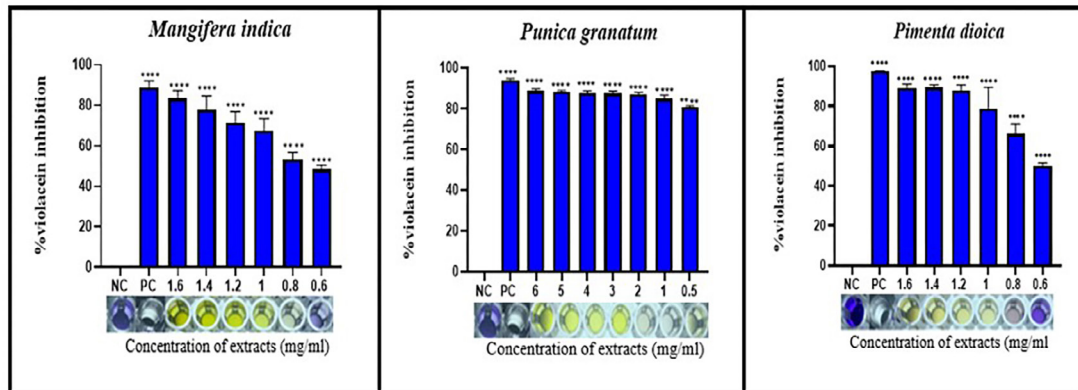


Figure 2. Quantification of violacein pigment in response to treatment

Data were considered as mean \pm SEM of three independent biological replicates. p values were calculated using one-way ANOVA. Tukey's post hoc was used for multiple comparisons. $p < 0.0001$ is considered significant (**** $p < 0.0001$). PC = positive control (2 μ l/ml of cinnamon oil), NC=negative control (LB broth +bacteria only). All treatments were compared with the negative control. Different Concentrations of the 3 plant extracts were expressed in mg/ml

DISCUSSION

Antipathogenic strategies to treat bacterial infections by interrupting quorum sensing regulated virulence factors production have recently drawn interest due to the continuing emergence and spread of multidrug-resistant bacteria.⁴⁸ The dependence on medicinal plants for managing bacterial infections has been in practice for ages despite the availability of modern medicines. The richness of phytochemicals in them that target diverse pathogenicity-associated pathways contribute to either killing or interfering with the growth of the pathogens, offering a range of microbial infection management agents. For this purpose, in the present study, *C. violaceum* (MTCC 2656), a bio-reporter strain known for the production of a purple-coloured pigment violacein which is a quorum sensing system-regulated phenotype¹⁰ was utilised to screen 26 methanolic plant extracts for their quorum-sensing inhibition potential. Seven of the screened plant extracts '*P. betle* (leaf), *P. nigrum* (leaf), *S. cumini* (leaf), *P. guajava* (leaf), *M. indica* (leaf), *P. dioica* (leaf) and *P. granatum* (rind) have shown anti QS activity at a higher concentration of 25-100 mg/ml. Among these, *M. indica* (leaf), *P. dioica* (leaf) and *P. granatum* (rind) were chosen further as they exhibited pigment inhibitory activity at concentrations of 10 mg/ml and below. The MIC of these three selected plant extracts were

performed, and the pigment inhibitory potential at and sub-MIC levels showed significant pigment inhibition ($P < 0.0001$) compared to the negative control.

In our study, *P. granatum* peel gave an opaque zone of violacein inhibition of 15.23 ± 0.57 mm to 9.67 ± 1.3 mm at concentrations ranging from 6 mg/ml to 0.5 mg/ml, respectively. Similar results were reported by Mehta *et al.*⁴⁹ at lower concentrations of 1.5 mg/ml to 0.075 mg/ml, exhibiting pigment inhibitory effect of 17 mm to 8 mm, respectively. The difference in violacein inhibition in the above report could be due to the different extraction procedures used. In another study by Oh *et al.*,⁵⁰ the quorum inhibitory activity of pomegranate peel extract (PPE) using 75% aqueous methanol at 10 mg/ml was 26 mm. The absolute methanol extract of *P. granatum* in our study showed violacein inhibition of 22 mm at a similar concentration. This difference could also be due to differences in the method of extraction.

Husain *et al.*³³ reported that the methanolic subfraction of *M. indica* inhibited violacein by 19 mm and 17 mm at concentrations 800 and 1600 μ g/ml. Crude methanolic extracts used in our study showed pigment inhibition of 14.51 ± 0.63 mm and 11.08 ± 0.08 mm, respectively, at concentrations 1600 and 800 μ g/ml. Despite the differences in the method of extraction, violacein inhibition at 1600 μ g/ml is comparable to the result of Husain *et al.* above.

Vasvi *et al.*³⁵ used 90% ethanol for the extraction of *P. dioica* leaves, and they have reported that the crude plant extract showed weak violacein pigment inhibition at a concentration ranging from 0.5-1 mg/ml. The results obtained from our study using crude extracts showed inhibition at concentrations of 1 mg/ml (16.22 ± 0.41 mm) and 0.6 mg/ml (13.14 ± 0.18 mm).

QS inhibitory property of the three plants were further quantitatively determined using the flask assay.⁴⁹ In our study, *P. granatum* showed a substantial decrease in the percentage of violacein ($80.76 \pm 0.58\%$) at a concentration of 0.5 mg/ml, which is higher than 41.92% at a concentration of 0.6 mg/ml, as reported by Mehta *et al.*⁴⁹ Similarly, a study by Oh *et al.*⁵⁰ showed percentage violacein inhibition of 78.5%, 70%, 45% and 23% by *P. granatum* at concentrations of 2, 1.5, 1 and 0.5 mg/ml, respectively. Compared to these results, our *P. granatum* extracts showed a higher percentage of violacein inhibition of $86.92 \pm 1.02\%$, 84.84 ± 1.70 and $80.76 \pm 0.58\%$ at similar concentrations of 2, 1 and 0.5 mg/ml, respectively.

The study conducted by Yang *et al.*⁵¹ revealed that the tannin-rich fraction from pomegranate rind (TFPR) effectively inhibited violacein production by 49.65% and 29.88% at concentrations of 0.312 and 0.625 mg/ml, respectively, in *C. violaceum* ATCC 12472. Our methanolic extract of *P. granatum* showed a better violacein inhibition of 80.76% at 0.5 mg/ml.

Yet in another study by Husain *et al.*, the methanolic sub-fraction of the petroleum ether extract of *M. indica* decreased violacein production by 83.6%, 55.6% and 32.1% at concentrations of 0.8, 0.4 and 0.2 mg/ml, respectively.³³ The violacein inhibitory effect achieved in our study by crude methanolic mango leaf extract at concentrations of 1 mg/ml (67.35%), 0.8 mg/ml (53.33%), and 0.6 mg/ml (48.5%) did not agree with the above study possibly due to differences in the method of extraction used. Quantitative violacein inhibitory assay using methanolic leaf extract of *P. dioica* in our study showed a percentage inhibition of $78.67 \pm 10.79\%$ at a concentration of 1 mg/ml. These results agree with the finding of Vasavi *et al.*,³⁵ who conducted the quantification assay using the purified ethyl acetate fraction (EAF), and the percentage of violacein inhibition was more than

80% at concentrations of 0.5-1 mg/ml. Alrashidi *et al.*⁵² reported that the *P. dioica* essential oil showed percentage pigment inhibition of ($38.25 \pm 1.8\%$) and ($32.34 \pm 1.3\%$) at concentrations of 1.25 mg/ml and 0.625 mg/ml, respectively, in CV 12472. Our results using the *P. dioica* leaf extracts showed better percentage pigment inhibition of ($87.81 \pm 2.81\%$) and ($49.78 \pm 1.75\%$) at similar concentrations of 1.2 mg/ml and 0.6 mg/ml, respectively.

Al-Hussaini and Mahasneh,⁵³ in their work on QS-inhibiting plant extracts, presented anti-QS activity as mild (8 to 10.5 mm), moderate (13 mm), and strong (18 mm) based on the diameter of the zone of violacein inhibition. The present study also attempted to compare the performance of QS inhibitory properties of the three selected plants via well diffusion assay (Qualitative) and flask assay (quantitative). At 1 mg/ml extract concentration, *P. dioica* appears to have significant ($p < 0.05$) QS inhibition in qualitative assay compared to *M. indica* and *P. granatum*.

Quantitative assay performance of this corresponding concentration (1 mg/ml) did not show significant QS inhibition. But *P. granatum* (0.5 mg/ml) has shown a significant ($p < 0.0001$) QS inhibition compared to the closer concentration (0.6 mg/ml) of *M. indica* and *P. dioica*.

Findings from the present study suggest the effectiveness of the quantitative violacein inhibition assay for a better comprehension of QSI properties by plant extracts. The observed difference could be attributed to the behaviour of the bacteria in the different media used in culture.⁴⁷

The observed differences in the QSI of plant extracts could be due to the different types of phytochemicals present in them.⁵⁴ Prior research has stated that *P. dioica* leaf extracts are rich in glycosides, alkaloids, carbohydrates, flavonoids, tannins, and proteins contributing to its diverse pharmacological properties. The major constituents of the essential oil extracted from this plant include methyl eugenol, eugenol, myrcene, β -caryophyllene, cineole, and limonene. A significant quantity of eugenol, a phenolic compound present in this essential oil, is known for its wide-ranging antibacterial and anti-quorum sensing activities.^{52,55} Accumulated research

demonstrates the components of *P. granatum* exhibiting anti-inflammatory, antibacterial, antiviral, and antifungal properties.³⁹ Research validates that the antibacterial effect of *P. granatum* is attributed to its abundant phenolic components, hydrolysable tannins, and anthocyanins.⁵⁶ Compounds like punicalagin, chlorogenic acid, rutin, epicatechin, gallic acid, caffeic acid, quercetin, catechin, and cinnamic acid are the primary phytochemical compounds that exhibit significant quorum sensing inhibitory potential.^{39,57} *M. indica* leaves have been reported to contain mangiferin, phenolic acids, benzophenones flavonoids, ascorbic acid, carotenoids, xanthenes, tannins, terpenoids and tocopherols, which have antioxidants and antibacterial activities.^{58,59} The high concentration of gallic acid in the methanolic leaf extract of *M. indica* was suggested as a reason for its higher anti-QS activity.³³

Our findings suggested the potential of *M. indica*, *P. granatum*, and *P. dioica* methanolic extracts as a source of effective inhibitors of QS-mediated virulence factors. These results indicate the presence of QS inhibitory compounds that might be interfering with either the synthesis or reception of autoinducers. Thus, they may disrupt the intercellular communication process by suppressing the synthesis of violacein in *C. violaceum* without affecting its growth.

CONCLUSION

Though the discovery of antibiotics has greatly benefited human civilization, a review of the effectiveness of the antimicrobials in use is necessary due to the rising prevalence of multidrug-resistant bacteria. Antibacterial agents that interfere with the QS system in bacteria, affecting their virulence factor production, can be a potential way to reduce the menace of antibiotic resistance. Given that quorum sensing triggers the activation of virulence genes in various pathogens, agents capable of disrupting bacterial communication can be utilized as antipathogenic drugs. Therefore, this further opens up an opportunity to evaluate their bioactive compounds systematically as drugs adjunct to standard antibiotics. An enhanced comprehension of the mechanism of action of these QS inhibitors on microbial cell communication has the potential

for innovative therapeutic applications in combating the pathogen. The findings of this work demonstrate the potential of Indian medicinal plants as a source of anti-QS phytochemicals. Additionally, it emphasizes the significance of assessing the untapped diversity of these plants for their ability to exhibit such activity. Further studies that elucidate the phytochemicals present in these plants, along with their molecular mechanism associated with such appreciable anti-infective activity, are warranted. Such investigations may pave the way for the development of novel drug candidates for quorum sensing inhibition and associated complications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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