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Effect of *Moringa oleifera, Coleus amboinicus, Monoon longifolium,* and *Hibiscus rosa-sinensis* Extracts on Quorum-Sensing-Regulated Production of Virulence Factors in *Chromobacterium violaceum*

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A B S T R A C T

The growing emergence of multidrug-resistant pathogens presents a critical health challenge worldwide, urging the development of novel therapies that can reduce bacterial virulence without promoting resistance. One promising strategy involves targeting quorum sensing, a bacterial cell-to-cell communication system that regulates the expression of virulence factors. This study evaluated the quorum quenching potential of aqueous leaf extracts of *Moringa oleifera*, *Coleus amboinicus*, *Monoon longifolium,* and *Hibiscus rosa-sinensis* against *Chromobacterium violaceum* MTCC 2656. Antibacterial activity was observed at 3.1875 mg/mL for *M. oleifera*, 25.5 mg/mL for *C. amboinicus*, and 8.5 mg/mL for both *H. rosa-sinensis* and *M. longifolium*. *Moringa* showed the most potent antibacterial action. Further, all four aqueous extracts, at sub-MIC concentrations, inhibited biofilm formation and production of violacein, lipase, and protease without affecting bacterial viability. Phytochemical screening revealed the presence of flavonoids, sterols, terpenoids, alkaloids, and phenols in all extracts, with *Moringa* showing higher sterol and phenolic content. Bioautography of *C. amboinicus* extract showed two active bands, which inhibited violacein production in the overlaid *C. violaceum* culture. These results suggest that plant extracts offer a sustainable, natural strategy to combat bacterial infections, including MDR strains, and can also serve to control biofilm formation in the food processing industry.

Introduction

The growing prevalence of multidrug-resistant bacteria undermines the effectiveness of current antibiotics.[1] This crisis demands innovative strategies to focus on reducing bacterial virulence and pathogenicity. One such approach is to target bacterial communication systems, such as quorum sensing, rather than directly inhibiting

bacterial growth.[2] Quorum sensing (QS) is a communication system in bacteria that regulates gene expression, especially that of virulence genes, in response to population density.[3] It uses diffusible signalling molecules called autoinducers to coordinate energy-intensive behaviours like biofilm formation, toxin production, motility, and enzyme secretion.[4] In Gram-negative bacteria, N-acyl homoserine lactones are the main autoinducers, which control key virulence factors.[3] Antivirulence strategies aim to disarm pathogens by disrupting QS pathways, which can be achieved by: (i) using signalling molecule analogs that disrupt communication, (ii) applying antagonists that bind QS receptors without activating them, or (iii) inhibiting downstream QS signal transduction.[5] QS inhibitors (QSIs) do not inhibit bacterial growth, preserving the host microbiome often disrupted by traditional antibiotics.[6] Combining QSIs with antibiotics can enhance efficacy, allow lower doses, and reduce side effects.[7] These approaches offer a promising alternative to conventional antibiotics by reducing virulence without promoting resistance. Bioactive compounds in plant extracts are emerging as promising QSIs, capable of disrupting bacterial signalling and reducing virulence, biofilm formation, and motility.[8,9] Phytochemicals like phenols, flavonoids, terpenoids, and alkaloids target QS pathways.

The organism used in this study, *Chromobacterium violaceum,* is a useful model for QS research because its pigment violacein, controlled by the *vio* operon, acts as a quantifiable indicator of QS activity. Its QS system includes *cviI*, which synthesizes N-decanoyl-L-homoserine lactone, and *cviR*, the receptor and transcriptional regulator.[10,11] QS in *C. violaceum* also regulates virulence factors such as elastase, chitinase, and biofilm formation, all of which contribute to pathogenicity.[12] This study aimed to evaluate the effect of sub-MIC levels of plant extracts on the expression of virulence factors in *C. violaceum* by the quantification of inhibition of violacein, biofilm, protease, and lipase, and also analyse the effect on the growth curve.

Materials and Methods

Bacterial Strain

Chromobacterium violaceum MTCC 2656 (gifted by Prof. S. Rajagopalan) was maintained on nutrient agar slants at 30°C.

Plant Material and Preparation of Extracts

Leaves of *Moringa oleifera*, *Coleus amboinicus* (Indian mint), *Monoon longifolium* (ashoka) and *Hibiscus rosa-sinensis* were collected from local sources in Mumbai, washed, air-dried, and kept in the hot-air oven overnight at 60°C. The dried leaves were ground, sieved, and 4 g of powder was extracted in 40 mL of distilled water (maceration method) with overnight shaking.

The mixture was centrifuged, and the supernatant was filtered. The filtrate was evaporated to dry powder, dissolved in DMSO to make a 85% solution, and stored at -20 °C.

MIC Determination

Minimum inhibitory concentrations (MICs) of the plant extracts against *C. violaceum* were determined using the broth microdilution method.[13] Varying concentrations of the plant extract (from 85 mg/mL stock) were prepared in sterile Luria-Bertani (LB) broth to a final volume of 200 µL in a sterile 96-well microtiter plate. Overnight culture of *C. violaceum* (1×10^8 cfu/mL) was diluted 1:100, and 50 µL of this suspension was added to each well. Extract controls and growth controls were included. After overnight incubation at 25°C, the MIC was recorded as the lowest concentration showing no visible growth, and OD_{600} readings were measured using a plate reader.

Assay for Biofilm Inhibition

The antibiofilm potential of the plant extracts was assessed by the crystal-violet staining method in 96-well sterile microtiter plates.[14] Each well received 180 µL of sterile LB broth (with 0.2% (w/v) glucose) and 20 µL of bacterial culture with plant extract at MIC and sub-MIC concentrations.

After gentle mixing, the plates were incubated on a flat surface at room temperature for 72 h. After incubation, cultures were discarded, and wells were washed with PBS (pH 7) to remove planktonic cells. The biofilms adhering to the walls of the wells were stained with 0.1% crystal violet. Excess dye was washed off with PBS, and the dye bound to the biofilms was solubilised using acetic acid. The absorbance of the extracted dye was then recorded at 595 nm using a plate reader. The percentage reduction in biofilm in the presence of plant extract was calculated using the following equation:

$$\% \text{ biofilm inhibition} = \left(\frac{OD \text{ of untreated control at } 595 \text{ nm} - OD \text{ of treated sample at } 595 \text{ nm}}{OD \text{ of untreated control at } 595 \text{ nm}}\right) \times 100$$

Violacein Inhibition Assay

Violacein inhibition assay was performed using the method by Venkatramanan *et al.,* (2020).[10] *C. violaceum* culture in log phase (OD_{600} = 0.6–0.8) was inoculated in LB broth with and without the plant extract and incubated in a shaker incubator at 30°C for 24 h. From this, 1 mL culture was lysed by adding 100 µL of 10% SDS in 1.5 mL microtubes, vortexed for 30 s, and incubated at room temperature for 5 min (to ensure complete lysis). The lysate was centrifuged at 13,000 rpm for 20 min, and the pellet was resuspended in 200 µL DMSO and vortexed vigorously to extract violacein. After a second centrifugation at 13,000 rpm for 10 min (to pellet the cell debris), the supernatant containing violacein was transferred to a microplate well, and absorbance was measured at 585 nm.

Assay for Inhibition of Proteolytic Activity

The ability of the plant extracts to inhibit the induction of protease in *C. violaceum* was tested using the method by AlShaikh-Mubarak *et al.,* (2023).[15] *C. violaceum culture* was grown in LB broth overnight with sub-MIC concentrations of plant extract (⅔, ½, ⅓, ¼, and ⅛ of MIC). After centrifugation at 4°C, the cell-free supernatant (CFS) was used as the enzyme source for this assay. 0.5 mL of 0.08% (w/v) azocasein, prepared in 0.2 M potassium phosphate buffer (pH 7.0), was mixed with 500 µL CFS and incubated at 37°C for 30 min to allow enzymatic activity.

The reaction was terminated using 1 mL of 10% (w/v) trichloroacetic acid, followed by incubation in an ice bath for 1 h to facilitate protein precipitation. The mixture was centrifuged to remove unreacted azocasein, and equal volumes of the supernatant and 1 N NaOH were mixed. Absorbance was recorded at 440 nm, with a blank sample prepared by replacing the enzyme source with distilled water and processed likewise.

Assay for Inhibition of Lipolytic Activity

Overnight cultures of *C. violaceum* in LB broth with varying (MIC and sub-MIC) concentrations of plant extract were centrifuged at 14,000 rpm for 20 min to obtain CFS, which was added to wells in Tributyrin Agar plates (tributyrin is the lipid substrate). Untreated and vehicle controls were also added to measure baseline lipolytic activity. The plates were incubated at room temperature for 24 h. Lipase inhibition was determined by measuring the diameter of the clear zones (visible against a light source) around the wells.

Bioautography Assay

TLC was performed using a methanol-chloroform (1:1) solvent system,[16,17] and the dried plates were used for bioautography. Pre-inoculum (5 mL) was mixed with 25 mL semi-solid LB agar to achieve a final OD_{600} of 0.1, which was spread uniformly over the dried plates, forming a layer ~3 mm thick. The plates were incubated at room temperature for 24 h to allow bacterial growth and violacein production.

Phytochemical Screening of the Plant Extracts

The analysis was conducted using standard qualitative assays as described by Kancherla *et al.,* (2019) and Harborne (1984) with slight modifications.[18,19] Each extract was tested for the presence of flavonoids, phenols, alkaloids, tannins, terpenoids, and sterols.

Characteristic colour changes or formation of precipitates were recorded as qualitative indicators of each phytochemical class.

Results and Discussion

Antibacterial Potential of the Plant Extracts

The plant extracts were screened for antibacterial activity against *C. violaceum* by the agar well diffusion method. Aqueous leaf extracts of *Moringa oleifera*, *Coleus amboinicus* (Indian mint), *Monoon longifolium* (ashoka), and *Hibiscus rosa-sinensis* showed zones of inhibition, with mint exhibiting the highest antibacterial activity (Fig. 1.B) with the largest zone of inhibition (zone diameter: 20 mm), whereas ashoka (12 mm), moringa (13 mm), and hibiscus (14 mm) showed moderate zones of inhibition (Fig. 1.A).

Determination of MIC

The MICs of the plant extracts against *C. violaceum* were determined by the broth microdilution method and found

to be 3.1875 mg/mL for *M. oleifera*, 25.5 mg/mL for *C. amboinicus*, 8.5 mg/mL for *H. rosa-sinensi*s, and 8.5 mg/mL for *M. longifolium* (Figure 2).

Inhibition of Biofilm Formation

The antibiofilm potential of the plant extracts was assessed by the crystal violet staining method in 96-well microtiter plates. All four extracts inhibited biofilm formation to a certain extent at sub-MIC concentrations. Moringa showed the highest inhibition across a wide sub-MIC range. *M. longifolium* and hibiscus demonstrated moderate antibiofilm effects, with a gradual decrease in biofilm biomass; however, complete inhibition was achieved only at MIC (Figure 3). Hibiscus showed stronger sub-MIC antibiofilm activity compared to *M. longifolium*. Overall, moringa displayed the most pronounced antibiofilm activity at non-lethal concentrations, while Indian mint showed relatively moderate efficacy, followed by hibiscus and *M. longifolium* (Figure 3). These findings highlight *Moringa oleifera* as the most promising candidate for biofilm inhibition at sub-MIC concentrations.

Inhibition of Violacein Production in *C. violaceum*

Qualitative Assessment of Violacein Inhibition

Violacein inhibition was observed on gradient MHA plates supplemented with various concentrations of extract. *C. violaceum* showed normal growth with violacein inhibition at sub-MIC levels, indicating suppression of virulence without affecting viability. In sub-MIC plates, the region with the highest extract concentration showed bacterial growth with reduced pigment, while in MIC plates, no growth was observed in that region (Figures 4 & 5).

Quantitative Assessment of Violacein Inhibition

Among all plant extracts tested, *C. amboinicu*s (Indian mint) showed the strongest violacein inhibition across the widest range of sub-MIC concentrations, with over 95% inhibition at 2/3 MIC. *M. oleifera* also showed strong activity, with over 93% inhibition at 2/3 MIC (Figure 6). Hibiscus showed moderate inhibition of 82.11% at half MIC, while *M. longifolium* showed the least inhibition. The extract concentrations required for 80% and 100% inhibition of violacein are mentioned in Table 1.

Inhibition of Lipase Production

C. amboinicus and *M. oleifera* showed the strongest lipase inhibition, achieving complete inhibition (no clear zone) at sub-MIC concentrations. In *M. oleifera*, lipase activity was fully inhibited at 1.595 mg/mL, well below its MIC (3.1875 mg/mL). Similarly, *C. amboinicus* achieved complete inhibition at 8.5 mg/mL, one-third of its MIC (25.5 mg/mL). In contrast, *M. longifolium* and *H. rosa-sinensis* showed complete inhibition only at MIC levels, with slight zone reduction compared to the control (Figures 7 & 8).

Inhibition of Protease Production in *C. violaceum*

Protease cleaves azocasein (substrate), releasing an azo dye measured at 440 nm. *M. oleifera* showed complete inhibition at sub-MIC concentrations above 2.125 mg/mL and moderate inhibition above 1.0625 mg/mL. *M. longifolium* showed minimal inhibition below 3.1875 mg/mL, with a sharp decrease in proteolytic activity from 4.25 mg/mL onwards (Figure 9). *C. amboinicus* also showed negligible inhibition below 12.75 mg/mL (Figure 9), with significant inhibition above 17 mg/mL (sub-MIC).

Growth Curve Analysis

The growth curves of *C. violaceum* with varying sub-MIC concentrations of aqueous leaf extracts of *C. amboinicus, M. oleifera*, *H. rosa-sinensis,* and *M. longifolium* showed distinct patterns. *C. amboinicus* exhibited clear dose-dependent inhibition (Figure 10), with significant growth reduction at 8.5, 12.75, and 17 mg/mL, before reaching its MIC (25.5 mg/mL). *M. longifolium* showed weaker inhibition, with noticeable reduction only near its MIC (8.5 mg/mL). *M. oleifera* showed intermediate inhibition (Figure 10), with mild to moderate reduction at 1.0625, 1.595, and 2.125 mg/mL, and stronger inhibition near its MIC (3.1875 mg/mL). Overall, *C. amboinicus* showed the strongest inhibition at sub-MIC, while *H. rosa-sinensis* was the weakes. All extracts caused complete inhibition at MIC, though kinetics and inhibition levels varied at sub-MIC concentrations. Sterile controls remained flat, confirming bacterial origin of growth and not contamination. As this study focuses on anti-virulence and anti-QS strategies to combat antimicrobial resistance (AMR), the risk each plant extract poses in resistance development must be considered. *C. amboinicus* poses the highest risk due to

its potent, dose-dependent antimicrobial activity at sub-MIC, which could drive resistance with prolonged exposure. *M. longifolium* and *H. rosa-sinensis* pose lower risks due to weaker effects, while *M. oleifera* presents a moderate risk. Despite these differences, *M. oleifera* can be considered the most promising candidate for anti-QS studies, showing superior inhibition of virulence factors compared to *M. longifolium* and *H. rosa-sinensis*. While *Moringa* shows moderate growth inhibition, its primary mechanism of action, affecting bacterial virulence, has the potential to reduce pathogenicity without rapidly promoting AMR.

Qualitative Analysis of the Phytochemicals in the Plant Extracts

Aqueous leaf extract of *M. oleifera* contains flavonoids, alkaloids, terpenoids, sterols, tannins, and phenols; *C. amboinicus* contains flavonoids, alkaloids, terpenoids, sterols, and phenols; *M. longifolium* contains flavonoids, sterols, terpenoids, and alkaloids; *H. rosa-sinensis* contains flavonoids, alkaloids, terpenoids, sterols, and phenols. Chemical analysis of aqueous *M. oleifera* seed extract by Silva *et al.,* (2024) identified moringin, coumarin, benzaldehyde, and quinic acid as key antibiofilm compounds. GC-MS analysis of aqueous and ethanolic *M. oleifera* leaf extracts by Enerijifofi *et al.,* (2021) showed the highest flavonoid concentration (20.76 mg/100 g) compared to alkaloids, saponins, and tannins, detecting eleven compounds, with 1,2-epoxyhexadecane and 2-octenoic acid at higher levels in aqueous than ethanolic extracts.[24] The phytochemicals detected in *H. rosa-sinensis* align with Vastrad & Byadgi (2018), who reported alkaloids, flavonoids, and tannins in its aqueous leaf extract.[25]

Bioautography

Bioautography was performed with Quercetin as the positive control (Rf 0.91). A single clear band indicating antibacterial activity was observed for *M. longifolium* (Rf 0.89), hibiscus (Rf 0.95), and moringa (Rf 0.91). *C. amboinicus* (mint) showed two bands (Rf 0.86 and 0.97), suggesting the presence of two distinct bioactive compounds inhibiting bacterial growth (Figure 11).

This study evaluated the antibacterial, anti-QS, and anti-virulence potential of aqueous leaf extracts of *Moringa oleifera, Coleus amboinicus* (Indian mint), *Monoon longifolium,* and *Hibiscus rosa-sinensis* against *Chromobacterium violaceum.* Moringa and mint showed strong anti-QS effects at sub-MIC concentrations. Moringa showed the highest inhibition (more than 93% at ⅔ MIC) in biofilm formation, while mint showed the highest inhibition (more than 95% at ⅔ MIC) of violacein production. Complete inhibition of lipase activity was seen at 1.595 mg/mL (½ MIC) for moringa and 8.5 mg/mL (⅓ MIC) for mint. Growth curve analysis showed that moringa moderately inhibited growth at sub-MIC (suggesting a lower risk of resistance), whereas mint showed the highest inhibition. Phytochemical analysis revealed flavonoids, sterols, terpenoids, alkaloids, and phenols in all extracts. Bioautography helped enumerate the compounds with anti-QS effects. Mint showed two distinct bands, suggesting the presence of multiple bioactive compounds.

Suhartono *et al.,* (2019) reported an MIC of 10 mg/mL for ethanolic leaf extracts of *Moringa oleifera* against *C. violaceum*.[20] In the present study, MIC values are much lower for aqueous extracts. Ankwai *et al.,* (2023) found MICs of 100, 200, and 200 mg/mL for aqueous extracts of *Monoon longifolium* against *E. coli, P. aeruginosa,* and *S. aureus*, respectively.[21] Considering that *C. violaceum* is also Gram-negative, like *E. coli* and *P. aeruginosa*, the MIC values with aqueous extracts are 1/10th of those with ethanolic extracts. This is the first study of the effects of aqueous extracts of *Hibiscus rosa-sinensis* and *Coleus amboinicus* on *C. violaceum*, as no literature is available on this yet.

M. oleifera has been studied for its antibiofilm activity, mostly using aqueous and ethanolic seed extracts. Silva *et al.,* (2024) reported significant biofilm inhibition of *P. aeruginosa* and *S. aureus* by aqueous extracts of Moringa seeds.[22] Savu *et al.,* (2022) found that the methanolic leaf extract of *M. longifolium (Polyalthia longifolia)* showed the highest inhibition (99.5%) of *S. aureus* biofilm at 0.039 mg/mL, with activity even at one-fourth of the MIC.[23] However, no studies investigating the antibiofilm activity of *M. longifolium* aqueous leaf extract on *C. violaceum* could be found. Similarly, the quorum-quenching effects of *Hibiscus rosa-sinensi*s and *Coleus amboinicus* leaf extracts on *C. violaceum* remain unstudied. In the food industry, biofilms form on plastic, glass, rubber, stainless steel surfaces, and even on food. This study evaluated the inhibitory effect of plant extracts on biofilm forxmation on plastic (microtiter plate) and borosilicate glass (test tubes) surfaces.

Figure.1 Determination of antibacterial potential of various plant extracts against C. violaceum by agar well diffusion method on MHA plates. (A) Aqueous leaf extracts of Hibiscus rosa-sinensis, Monoon longifolium (ashoka), Moringa oleifera, and vehicle control (15% DMSO). (B) Aqueous leaf extract of Coleus amboinicus (Indian mint).

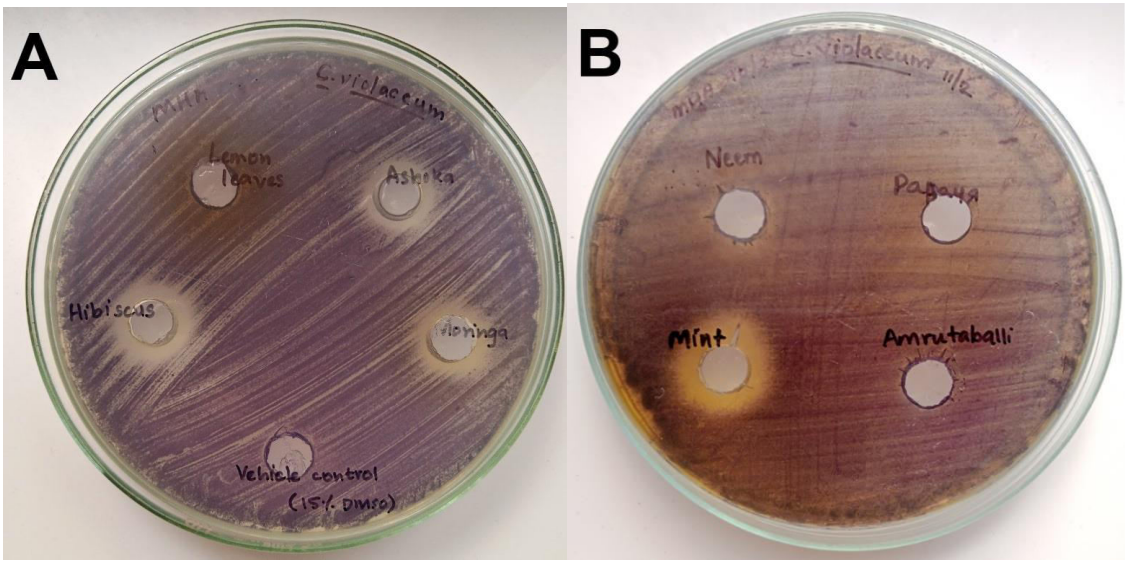

Figure.2 Determination of MIC of Moringa oleifera, Hibiscus rosa-sinensis, Monoon longifolium, and Coleus amboinicus against Chromobacterium violaceum.

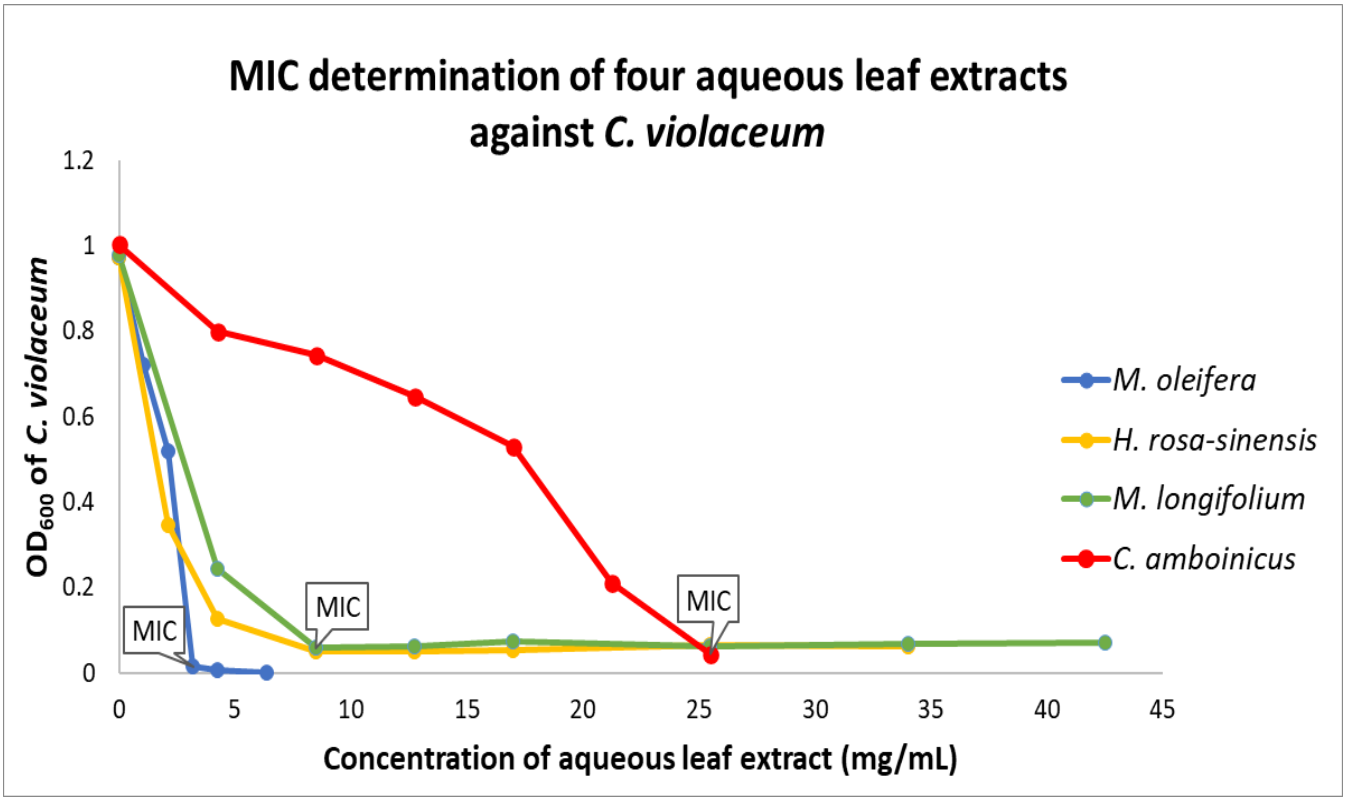

Figure.3 Biofilm inhibition in *C. violaceum* with varying concentrations of aqueous leaf extracts of *M. oleifera, C. amboinicus, M. longifolium,* and *H. rosa-sinensis.*

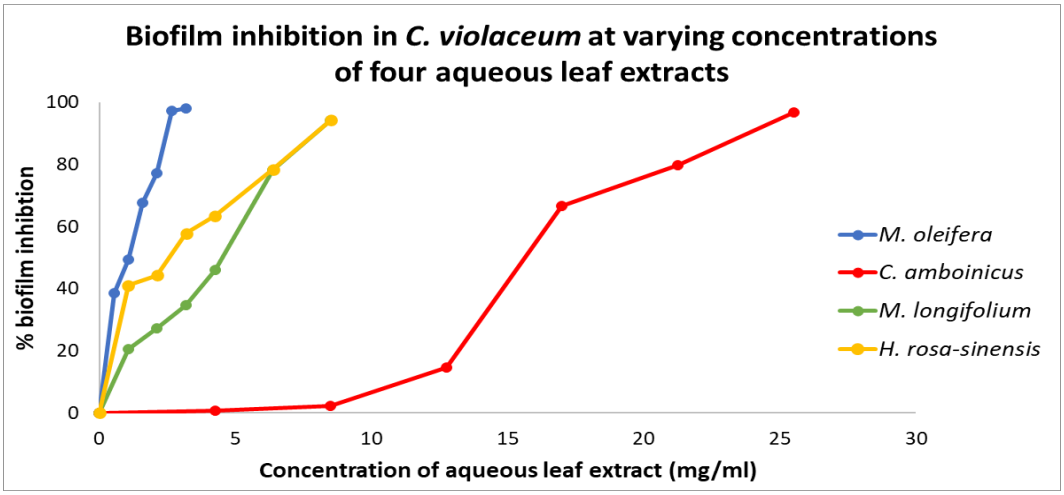

Figure.4 *C. violaceum* streaked on gradient MHA plates with various concentrations of *C. amboinicus* aqueous leaf extract. (A) 8.5 mg/mL, (B) 17 mg/mL showing pigment inhibition, (C) 25.5 mg/mL showing inhibition of growth.

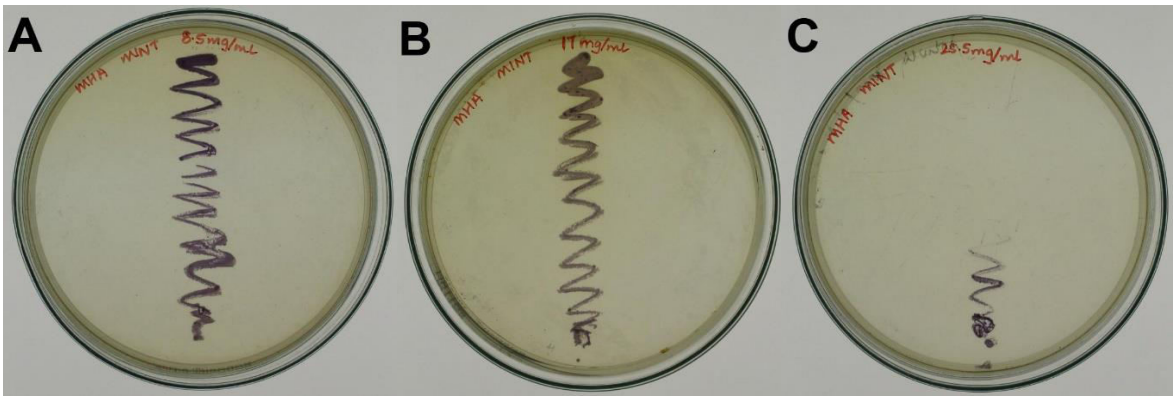

Figure.5 *C. violaceum* streaked on gradient MHA plates with various concentrations of aqueous leaf extracts of (A) *Monoon longifolium,* (B) *Moringa oleifera, and* (C) *Hibiscus rosa-sinensis.*

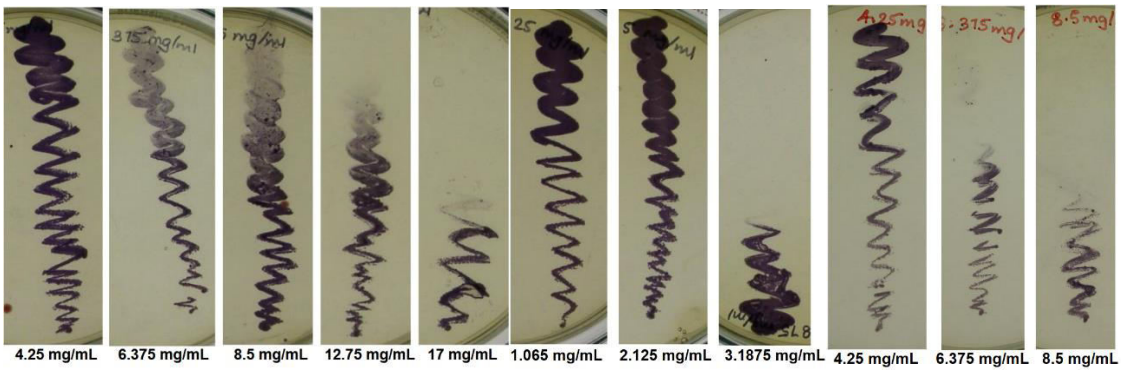

Figure.6 Violacein inhibition in *C. violaceum* with varying concentrations of aqueous leaf extracts of *M. oleifera, C. amboinicus, M. longifolium,* and *H. rosa-sinensis.*

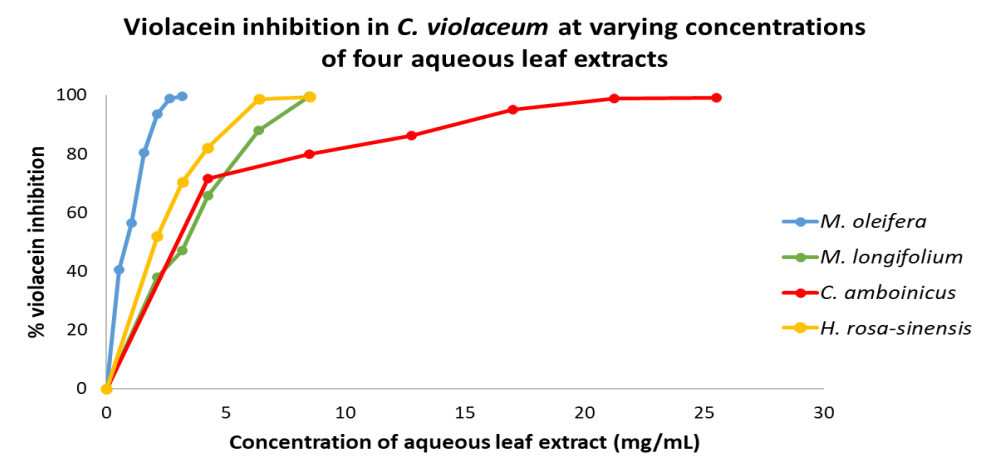

Figure.7 Lipid hydrolysis test (using tributyrin agar). Clear zones indicate lipase activity. *C. violaceum* treated with various concentrations of aqueous leaf extracts of (A) *M. oleifera,* (B) *C. amboinicus,* (C) *H. rosa-sinensis, and* (D) *M. longifolium*.

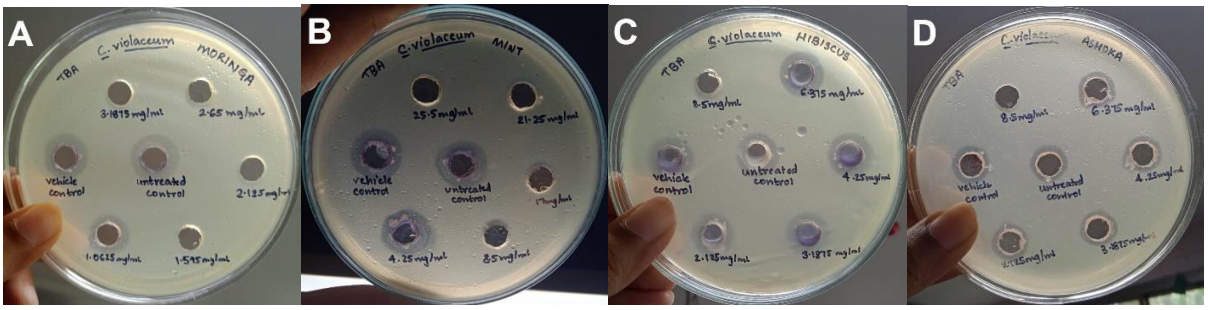

Figure.8 Lipase inhibition in *C. violaceum* with varying concentrations of aqueous leaf extracts of *M. oleifera, C. amboinicus, M. longifolium,* and *H. rosa-sinensis.*

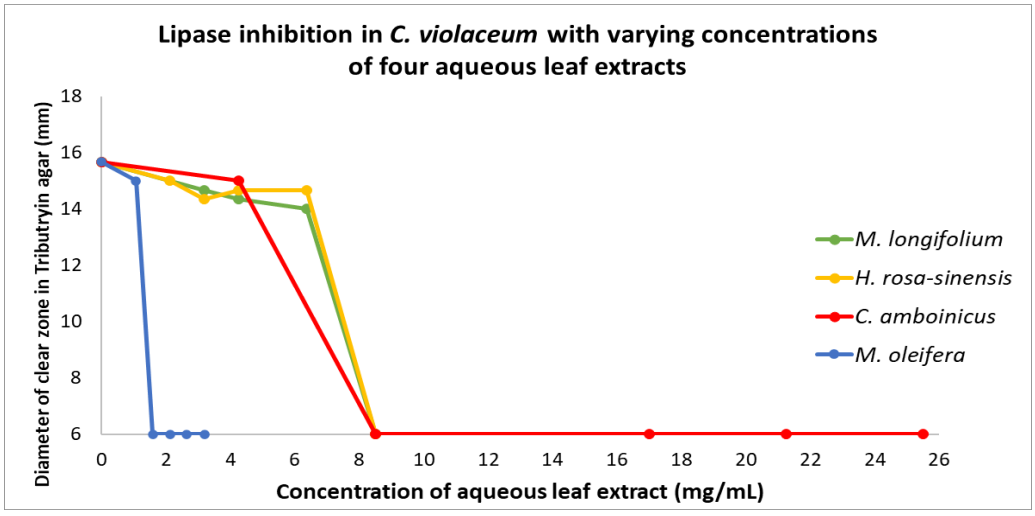

Figure.9 Protease inhibition in *C. violaceum* with varying concentrations of aqueous leaf extracts of *M. oleifera, C. amboinicus,* and *M. longifolium.*

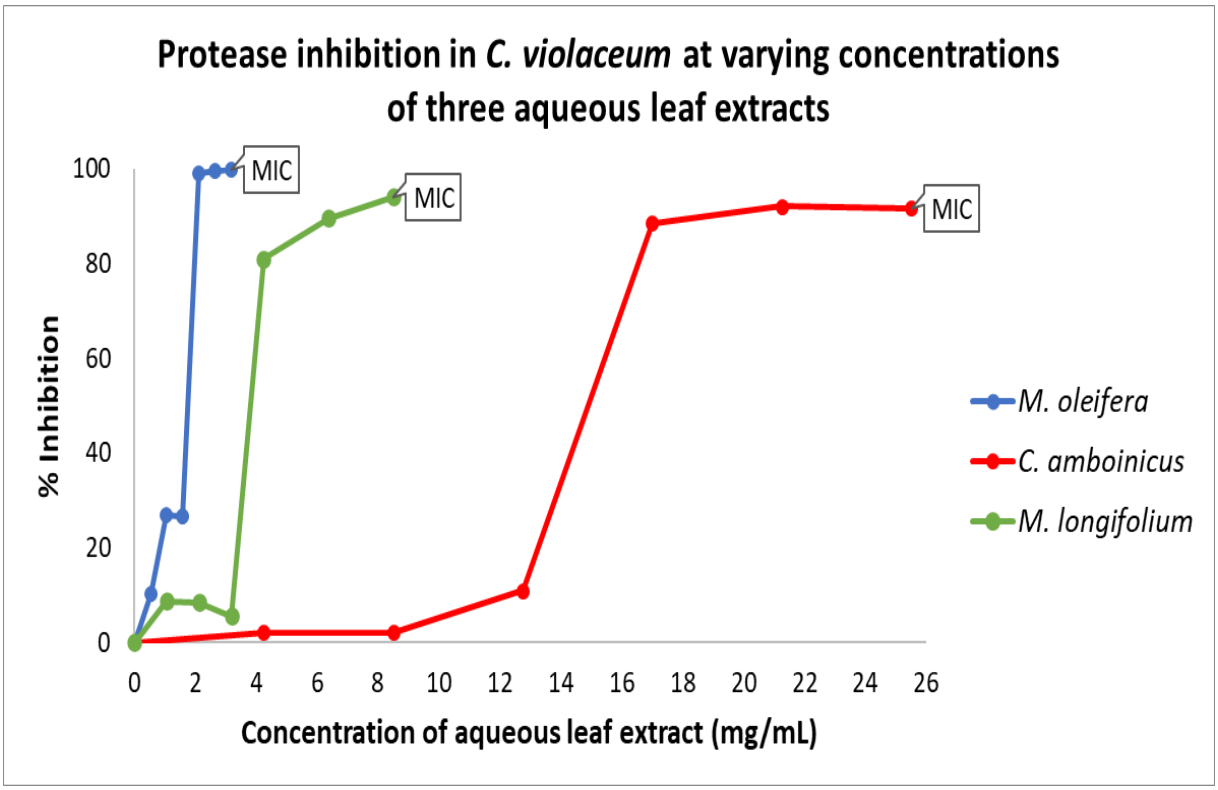

Figure.10 Growth curve of *C. violaceum* in the presence of varying concentrations plant extracts.

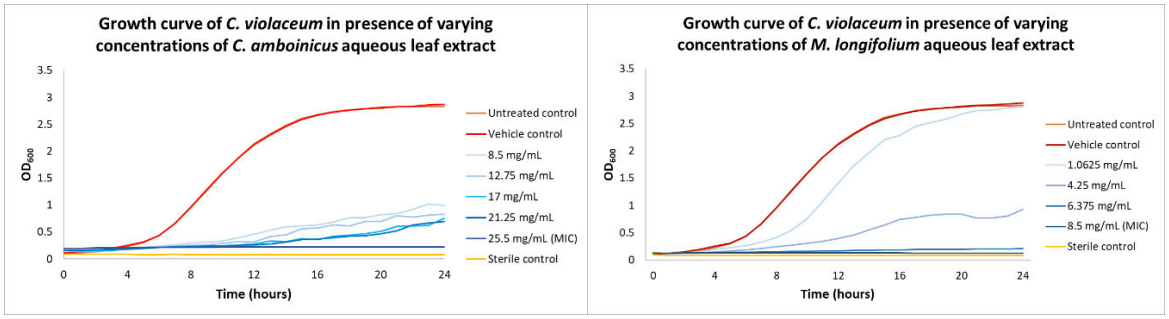

Figure.11 Bioautography of the plant extracts with C. violaceum by the agar-overlay method. (A) Image of the bioautograph. (B) Bands observed are labelled.

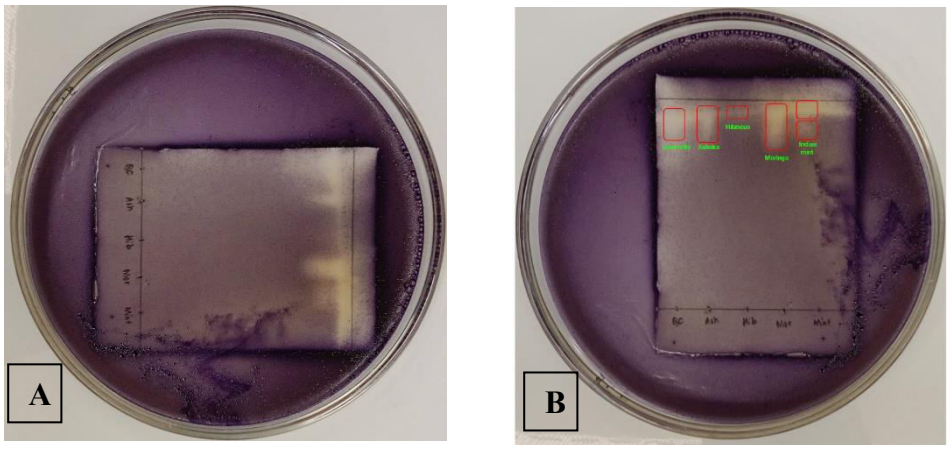

Table.1 Extract concentrations required for 80% and 100% inhibition of violacein.

Plant extracts	100% violacein inhibition	80% violacein inhibition
M. oleifera	3.1875 mg/mL (MIC)	1.595 mg/mL
C. amboinicus	25.5 mg/mL (MIC)	8.5 mg/mL
M. longifolium	8.5 mg/mL (MIC)	6.375 mg/mL
H. rosa-sinensis	8.5 mg/mL (MIC)	4.25 mg/mL

Further testing of these extracts against clinical and resistant pathogen strains is needed to determine their true potential. The prospects for quorum quenching using plant extracts remain highly promising.

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Author Contributions

Phileo Abraham: Investigation, formal analysis, writing—original draft. Sangeeta Shetty: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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