Unlocking the antibiofilm and anti-virulence potential of *Pithecellobium dulce* against *Chromobacterium violaceum* CV12472

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Abstract. *Chromobacterium violaceum* (*C. violaceum*) is a Gram-negative bacterium commonly found in tropical and subtropical environments, such as soil and water. It is known for producing a distinctive violet pigment known as violacein, which is regulated by its quorum sensing (QS) system. The present study examined the compound, *Pithecellobium dulce* (*P. dulce*), and its inhibitory effects on the quantity of violacein pigment generated by *C. violaceum* CV12472. *P. dulce* is recognized as a potent natural compound with significant inhibitory effects against various pathogens. The present study explored the antimicrobial and antibiofilm properties of *P. dulce* (seed and fruit) against *C. violaceum* CV12472 through a series of *in vitro* experiments. These experiments included minimum inhibitory concentration (MIC) tests, biofilm inhibition assays, violacein pigment assay and growth curve analysis, providing valuable insight into the potential of *P. dulce* as a natural therapeutic agent in combating microbial infections. MIC assay revealed that both the seed and fruit extracts effectively inhibited bacterial growth at 20 mg/ml. Furthermore, biofilm inhibition was observed, with *P. dulce* seed extract significantly reducing biofilm formation by 58.91 and 29.68% at 10 and 5 mg/ml, respectively, without affecting planktonic growth. Additionally, the present study demonstrated that *P. dulce* seed extract inhibited violacein production by 80.66% at 10 mg/ml, confirming its anti-QS properties. However, the fruit extract did not exhibit any notable effect on biofilm or pigment production. These findings suggest that *P. dulce* seed extract disrupts key bacterial survival mechanisms, thus suggesting its potential for use as s natural alternative for managing biofilm-associated infections caused by antibiotic-resistant pathogens. To the best of our knowledge, the present study is the first report of *P. dulce* seed extract inhibiting QS-regulated virulence factors in *C. violaceum*. The findings of the present study highlight the potential use of plant-based compounds in the fight against antibiotic resistance and bacterial virulence.

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Introduction

Chromobacterium violaceum (*C. violaceum)* is a Gram-negative coccobacillus and an environmental bacterium commonly found in soil and water, particularly in tropical and subtropical regions. Infections with *C. violaceum* are often linked to skin injuries, trauma, or water exposure, which provide a route for the bacterium to enter the body (1,2). A distinctive characteristic of *C. violaceum* is its production of violacein, a violet pigment regulated through a quorum sensing (QS) system (3,4). *C. violaceum*, as with numerous other opportunistic pathogens, forms biofilms with structured communities of bacterial cells encased in a protective matrix (5). This biofilm formation enhances its ability to produce various exotoxins, including hemolysins, which can lyse red blood cells (RBCs) and other host cells (1,2). Additionally, its outer membrane contains lipopolysaccharides (LPS), potent endotoxins that are recognized by the host immune system as danger signals, triggering an inflammatory response. In systemic infections, the excessive release of LPS can lead to septic shock a severe and life-threatening condition marked by widespread inflammation and multi-organ failure (1,6).

C. violaceum possesses both type III and type VI secretion systems (T3SS and T6SS), needle-like structures that inject virulence proteins directly into host cells. These systems are crucial for delivering effector proteins that manipulate host cell functions, such as inhibiting immune responses or inducing apoptosis, enhancing the ability of the bacterium to survive and multiply within host tissues (7). Additional virulence factors include motility via flagella, siderophore production, antioxidant enzymes and proteases, all of which contribute to rapid invasion, tissue destruction and resistance to immune

defenses, complicating treatment and increasing the severity of infections (8).

One of the most alarming characteristics of *C. violaceum* is its unexpected resistance to a number of commonly used antibiotics, largely attributed to its QS mechanisms (9). The link between QS and pathogenesis underscores the urgent need for the development of innovative strategies to combat infections and mitigate their harmful effects on human health (10). In light of these challenges, early treatment of the pathogen, particularly through the use of natural compounds, is vital for improving patient outcomes (11). Given that plant-based medicines are often reported to be reliable, effective and relatively safe, they continue to be widely used in traditional medicine worldwide (12). Furthermore, as they are derived from natural sources, plant-based treatments are considered to cause fewer side-effects than modern synthetic drugs. A recent study demonstrated that natural compounds, particularly plant-derived flavonoids, have greater potential to combat dental bacterial biofilms; these compounds exhibit promising antibiofilm properties, rendering them effective alternatives for preventing and managing dental infections (13).

Pithecellobium dulce (*P. dulce*), a fruit of American origin from the *Fabaceae* family, is native to tropical America and widely grown in India and the Andaman Islands (14). Commonly referred to as 'Jungal Jalebi' or 'Black Bead Tree' in English, 'Vilayati Babul' in Hindi and 'Kodukkapuli' in Tamil, *P. dulce* is an evergreen, medium-sized, spiny tree (15). Various parts of the plant have notable medicinal uses, with the root extracts exhibiting estrogenic activity (16). Traditionally, different plant parts have been employed to treat earaches, leprosy, peptic ulcers, toothaches and venereal diseases, and serve as emollients, abortifacients, anodynes and larvicides (17). The bark of *P. dulce* is used as an astringent for dysentery and febrifuge, as well as to treat dermatitis and eye inflammation. Polyphenols in the bark have demonstrated anti-venomous properties (18). Additionally, ethanolic extracts from the pod pulp of *P. dulce* have been shown to exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria, including *Bacillus subtilis* and *Klebsiella pneumoniae*, with secondary metabolites, such as flavonoids and saponins contributing to this antibacterial effect (19).

To the best of our knowledge, the present study is the first study to date aiming to investigate the effects of *P. dulce* isolates on *C. violaceum*. The anti-QS functions of *P. dulce* in relation to *C. violaceum* have not yet been thoroughly investigated.

Materials and methods

Bacterial strains and growth conditions. C. violaceum (CV12472) was generously provided by Dr Busi Siddhartha from Pondicherry University, Puducherry, Tamil Nadu, India.. The strain was cultured under aerobic conditions at 30˚C in Luria-Bertani (LB) (HiMedia Laboratories, LLC) broth to support optimal growth. For experimental analysis, the bacterial culture was sub-cultured to ensure optimal growth conditions. The identity of *C. violaceum* was verified through the automated VITEK 2 system, as previously detailed by David H. Pincus (BioMérieux, Inc.)., delivering precise and reliable bacterial classification (20). Additionally, the initial identification of the *C. violaceum* CV12472 was carried out using standard microbiological methods, focusing on its characteristic growth patterns on LB agar. These unique characteristics facilitated its identification, aligning with observations reported by August *et al* (21).

Collection of samples. Fruits and seeds from *P. dulce* were collected for the present study from the Neelakudi Campus of the Central University of Tamil Nadu, Thiruvarur, Tamil Nadu, India. The collected plant parts were authenticated at the Indian Medical Practitioner Co-operative Society (IMCOPS) herbarium in Chennai, India.

Preliminary screening of herbal derivatives. The *P. dulce* (fruits and seeds) were collected, and following three rounds of washing with distilled water, they were allowed to soak for 2 min in 70% ethanol (v/v). The plant sections were then surface sterilized by immersing them in 0.1% mercury chloride for 1 min and rinsing them three times with sterile distilled water. The fruits were air-dried in the shade after being sterilized. Subsequently, a mechanical grinder was used to grind the dried fruits into a coarse powder.

A total of 10 g of coarsely ground fruit powder and 10 g of coarsely ground seed powder were immersed in 100 ml ethanol and methanol (Rankem Laboratories, LLC), respectively, to carry out the extraction process. These combinations were incubated in a shaking incubator at 150 revolutions per minute (rpm) and 37˚C for 48-72 h. All extracts were filtered through Whatman (HiMedia Laboratories, LLC) after 48 h.

Determination of minimum inhibitory concentration (MIC). The MIC values of *P. dulce* (fruits and seeds) against *C. violaceum* CV12472 were determined using previously established protocols (22). Briefly, 20 μl overnight cultures were added to the LB broth with extracts at 20 to 0.039 mg/ml of both seed and fruit extract of *P. dulce* (2-fold serial dilution) and without extracts (control). The tubes containing *C. violaceum* CV12472 culture were incubated at 30˚C and the MIC values were observed and recorded.

Biofilm assay. A previously described protocol for the crystal violet staining assay was followed, with slight modifications made to suit the specific requirements of the experiment (9). In addition to a control without *P. dulce, C. violaceum* CV12472 was cultured with *P. dulce* fruit and seed extract at sub-MIC of 10 to 0.019 mg/ml. The cultures were incubated in a microtiter plate at 30˚C for 48 h to observe the effects. The planktonic cells were read at 600 nm using optical density (OD) and were disposed of after 24 h without causing any disturbance to the biofilm. Following the addition of 200 μl crystal violet (HiMedia Laboratories, LLC) to each well, the plate was incubated for 15 min at room temperature to allow staining. The unbound stain was eliminated from the wells containing the crystal violet after 15 min of gentle washing with sterile distilled water. At 520 nm, the absorbance was determined using spectrophotometer (JASCO UV/Vis, India) after the adherent biofilm-bound crystal violet was eluted in 70% ethanol.

Quantification of violacein production in C. violaceum (CV12472). The quantitative analysis of violacein production was previously described by Venkatramanan *et al* (9), at a sub-lethal concentration of *P. dulce* seed extract at 10 to 0.019 mg/ml, alongside a control without *P. dulce*. Serial 2-fold dilutions of *P. dulce* seed extract were loaded into test tubes containing LB broth, facilitating a gradient of concentrations for further analysis. Following the inoculation of 10 µl *C. violaceum* CV12472 overnight cultures into each test tube, the tubes were cultured for 18 h at 30˚C. The negative control, sterility control and positive control *(C. violaceum* CV12472) were also maintained throughout the assay. After incubation at 30˚C for 24 h, all tubes were centrifuged at 5,724 x g for 10 min at 4˚C.. Once the culture supernatant was disposed of, 200 µl DMSO (SRL Chemicals, Mumbai, India) were added to the pellets and well mixed until the pigment was extracted. The tubes were then centrifuged at 4,832 x g for 15 min at 4˚C. A 200-µl sample of the extracted violacein was added to the microtiter plate and measured at 520 nm using spectrophotometer (JASCO UV/Vis, India) By comparing the OD at 600 nm between the treated strain and the untreated control, the percentage growth of each was determined.

Bacterial growth curve. C. violaceum CV12472 growth curve was examined both simultaneously with and without *P. dulce* seed extract. Briefly, an overnight culture of *C. violaceum* CV12472 was incubated into LB broth with seed extract of *P. dulce* at 10 mg/ml and without seed extracts (control) separately. The OD at 600 nm was measured every hour while the culture setup was incubated at 37˚C for up to 24 h.

Statistical analysis. All experiments, including the biofilm assay, violacein pigment assay, and growth curve analysis, were performed in triplicate to ensure accuracy and reproducibility of the results. Statistical significance was determined using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test, performed using GraphPad Prism 10.1.0 software (Dotmatics). A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Identification of C. violaceum. The bacterial morphology was confirmed using the VITEK 2 automated system. When cultured on LB agar, the isolate formed colonies displaying a characteristic violet pigmentation, as depicted in Fig. 1. This distinctive chromogenic trait is a hallmark of *C. violaceum*, aiding in its identification.

MIC evaluation. The *P. dulce* seed and fruit extract were found to inhibit the growth of *C. violaceum* CV12472 at 20 mg/ml. The crude extracts anti-QS and antibiofilm activities were then investigated at concentrations lower than their MIC values (Table I).

Effect of P. dulce extract on biofilm inhibition in C. violaceum CV12472. The inhibition of biofilm formation in *C. violaceum* (CV12472) was evaluated using the microtiter plate method with 0.1% crystal violet staining. Compared with the untreated controls, treatment with *P. dulce* seed extract markedly reduced the biofilm-forming ability of *C. violaceum* CV12472 (Fig. 2A). Spectrophotometric analysis revealed maximum biofilm inhibition of 58.91 and 29.68% at concentrations of 10 and 5 mg/ml, respectively (Fig. 2B). By contrast, the *P. dulce* fruit extract had no notable effect on biofilm formation (Fig. 2C). Notably, the seed extract did not interfere with planktonic cell growth, indicating biofilm inhibition was achieved at sub-MIC levels.

Quantification of violacein in C. violaceum. C. violaceum CV12472 is commonly used for the detection of QS signals. C6-HSL is a signaling molecule involved in the production of violet color pigment of *C. violaceum*. Thus, any disturbances occurring in C6-HSL molecule will affect the ability of the organism to produce pigment (10). In the present study, *C. violaceum* CV12472 was used as a control strain for the qualitative and quantification of violacein pigment production.

Table I. Minimum inhibitory concentration.

S. no	2-fold dilution concentration (mg/ml)	Growth measured[a]; *P. dulce* (seed and fruit)
1	20	-
2	10	+
3	5	+
4	2.5	+
5	1.25	+
6	0.62	+
7	0.31	+
8	0.15	+
9	0.078	+
10	0.039	+

At a minimum inhibitory concentration of 20 mg/ml, *P. dulce* (seed and fruit) inhibited growth of *C. violaceum* CV12472. [a]The growth measured refers to the presence (+) or absence (-) of visible growth in the microbial culture after exposure to the respective 2-fold dilution concentrations (mg/ml) of *P. dulce* (seed and fruit).

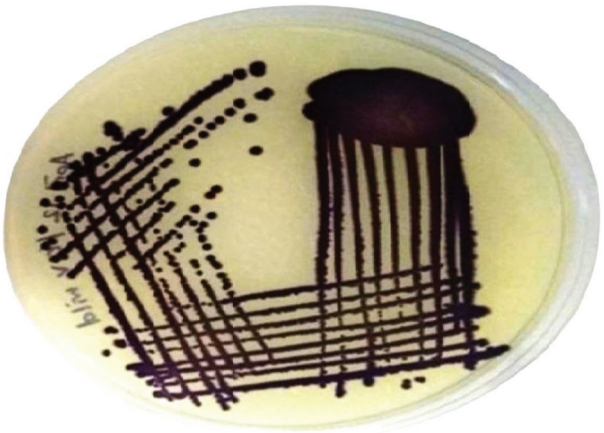

Figure 1. Illustration of the growth and pigmentation of *Chromobacterium violaceum* CV12472 on Luria-Bertani agar.

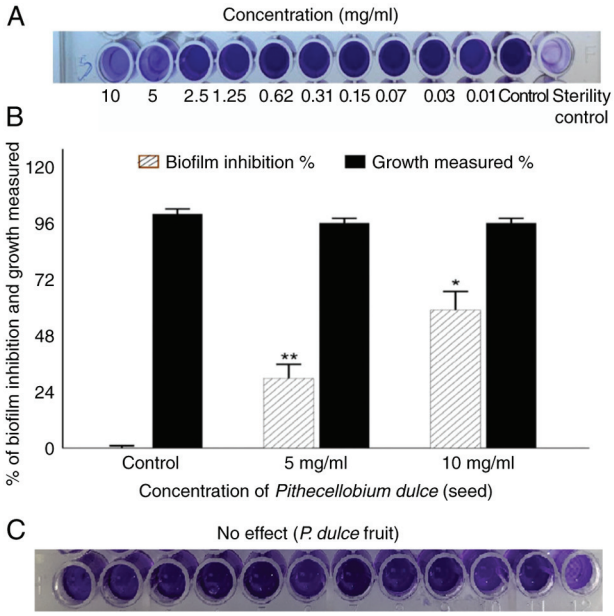

Figure 2. (A) Crystal violet biofilm inhibition assay. *P. dulce* seed extract inhibited *C. violaceum* CV12472 biofilm at sub-inhibitory concentration of 10 and 5 mg/ml. (B) Graphical representation; 58.91 and 29.68% of biofilm inhibition in *C. violaceum* CV12472 when treated with 10 and 5 mg/ml *P. dulce* seed extract, respectively. Statistical analysis was conducted using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test, with comparisons made between the untreated and groups treated with *P. dulce* seed extract at concentrations of 5 and 10 mg/ml. *P<0.05 and **P<0.01, significant difference compared to the untreated control. The x value refers to the concentration of *P. dulce* seed extract in mg/ml (presented on the x-axis), and the y value refers to the % of biofilm inhibition and growth measured (presented on the y-axis). (C) *P. dulce* fruit extract on *C. violaceum* CV12472 revealed no significant effect on biofilm formation. *P. dulce, Pithecellobium dulce*; *C. violaceum, Chromobacterium violaceum*.

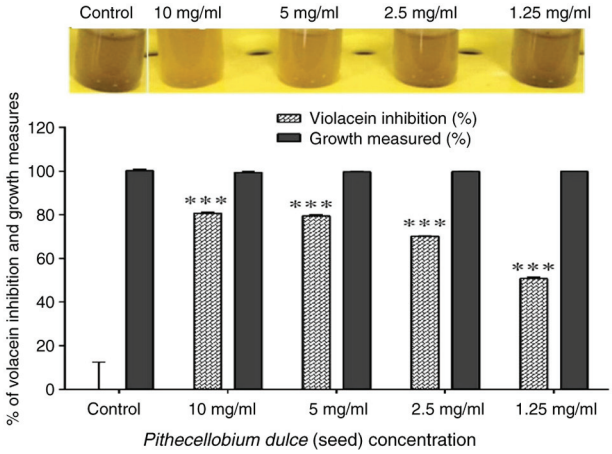

Figure 3. Inhibition of violacein pigment formation in *C. violaceum* CV12472 by *P. dulce* (seed) extract in a concentration-dependent manner. Violacein production is reduced at a concentrations of *P. dulce* (seed) extract 10 and 5 mg/ml to a level of 80.66 and 79.5%, respectively. (*** indicates P<0.001, denoting statistically significant differences compared to the untreated control.) Statistical analysis was performed using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test,, with comparisons made between the untreated and groups treated with *P. dulce* seed extract at concentrations of 10, 5, 2.5 and 1.25 mg/ml. The x value refers to the concentration of *P. dulce* seed extract in mg/ml (presented on the x-axis), and the y value refers to the % of violacein inhibition and growth measured (presented on the y-axis). *P. dulce, Pithecellobium dulce*; *C. violaceum, Chromobacterium violaceum*.

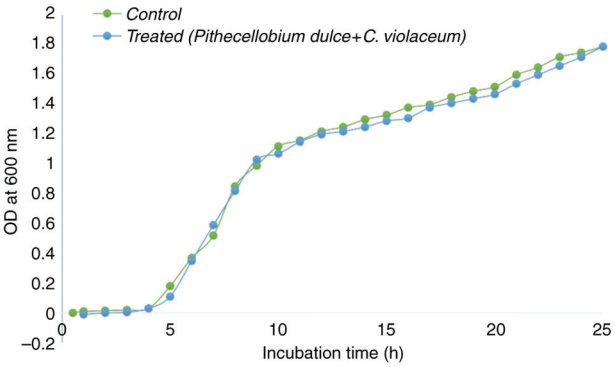

Figure 4. Growth curve analysis. *C. violaceum* CV12472 grown without (control) and in the presence of *P. dulce* (seed) extract at a concentration of 10 mg/ml. *P. dulce, Pithecellobium dulce*; *C. violaceum, Chromobacterium violaceum*.

Violacein pigment formation against *C. violaceum* CV12472 was found to be inhibited by *P. dulce* (seed) extract in a concentration-dependent manner and through qualitative analysis. Only *P. dulce* (seed) exhibited a substantial reduction in violacein production in *C. violaceum* CV12472 to the level of 80.66 and 79.5% when treated with *P. dulce* at 10 and 5 mg/ml, respectively (Fig. 3).

Bacterial growth curve analysis. In order to determine the growth inhibitory activity, *C. violaceum* CV12472 was grown both with and without *P. dulce* seed extract. As illustrated in Fig. 4, at a concentration of 10 mg/ml, *P. dulce* seed extract did not inhibit planktonic growth. This emphasizes that the extract specifically targets biofilm formation rather than exhibiting general antibacterial activity.

Discussion

C. violaceum is an opportunistic pathogen that is usually linked to serious infections that occur after skin injuries or water contamination exposure (23). Treatment is complex, and the risk of systemic infections, such as septicemia and meningitis are increased due to its capacity to produce virulence factors such as violacein and different exotoxins, as well as its ability to form biofilms. The growing resistance of bacteria to widely used antibiotics has made treating infections increasingly challenging, pushing the need for alternative solutions (12,24). In this context, natural remedies are gaining recognition as a promising approach for the future. As antibiotic resistance escalates, natural compounds known for their diverse bioactive properties offer the potential for safer and more effective treatments. This marks a pivotal shift in the management of infections, paving the way for innovative strategies in the coming era.

The present study highlights the potent antibacterial and antibiofilm properties of *P. dulce*, demonstrating its effectiveness against *C. violaceum* CV12472. The extracts from both the seeds and fruits exhibited significant growth inhibition at concentrations as low as 20 mg/ml, indicating that *P. dulce* may serve as a viable natural alternative to conventional antibiotics. This is particularly relevant given the increasing prevalence of

antibiotic resistance among pathogenic bacteria. Studies have demonstrated that *P. dulce* exhibits significant antibacterial effects against *Streptococcus mutans* at concentrations of 25, 50 and 100 μl (25). These findings add to the growing body of evidence supporting the potential of natural substances as alternatives to conventional antibiotics. Additionally, *P. dulce* has been shown to exhibit bactericidal activity against *Acinetobacter baumannii* at a concentration of 233 mg/ml, as well as against *Staphylococcus aureus* and *Escherichia coli* at 300 mg/ml. These results highlight the promising role of *P. dulce* as a natural antimicrobial agent with broad-spectrum activity (26).

Additionally, the investigation into the anti-QS properties of *P. dulce* is a novel aspect of the present study, as QS plays a critical role in the virulence of many bacteria, including *C. violaceum* CV12472. By inhibiting QS mechanisms, *P. dulce* may disrupt biofilm formation and reduce virulence factor production, thereby enhancing treatment outcomes. Recent studies have highlighted a range of natural compounds with notable anti-biofilm and QS inhibitory activities (9,22,25). One such compound is epigallocatechin gallate (EGCG), a polyphenol derived from green tea. EGCG has demonstrated notable efficacy in disrupting biofilms, achieving up to 95% inhibition in certain bacterial strains, particularly when used in combination with antibiotics. This underscores its potential as a valuable adjunct in antimicrobial therapies aimed at overcoming biofilm-associated infections (27). This synergistic approach not only enhances the efficacy of existing antibiotics, but also addresses the challenge posed by biofilm-associated infections. The observed inhibition of biofilm formation in the present study by *P. dulce* (seed) at sub-MIC level of 10 mg/ml, with a reduction of up to 58.91%, along with an impressive 80.66% decrease in violacein production in *C. violaceum* CV12472, underscores its strong potential to disrupt key survival mechanisms of the pathogen. By contrast, the *P. dulce* fruit extract exhibited limited effects on biofilm formation. This comparative insight suggests that the bioactive compounds in the seed extract may target key pathways in biofilm-related infections more effectively than those in the fruit extract. These results position *P. dulce* (seed) as a promising candidate for combating biofilm-related infections and QS-mediated virulence. Similarly, research on QS inhibitors (QSIs) has shown significant reductions in biofilm biomass when combined with antibiotics. For instance, Brackman *et al* (28) reported that the co-administration of QSIs alongside antibiotics resulted in a 68-90% reduction in viable bacteria within biofilms. This demonstrates the effectiveness of combination therapies in combating resistant strains, such as *P. aeruginosa* and *S. aureus*, offering a promising strategy to overcome biofilm-associated infections (29). Additionally, a previous study revealed that the synthesis and testing of phytochemical tannic acid-mediated gold nanoparticles effectively inhibited the biofilm of *Streptococcus mutans* at lowest concentration range of 16 μg/ml (30). Furthermore, recent studies have reported that the methanol extract of *Actinidia deliciosa* (kiwi fruit) exhibits significant antibiofilm activity at a concentration of 2.5 mg/ml (31). These results are consistent with those obtained with *P. dulce*, which likewise functions as an antibacterial agent and an anti-QS compound. Furthermore, the present study (Fig. 4) demonstrated that *P. dulce* seed extract did not inhibit planktonic growth at a concentration of 10 mg/ml, underscoring its specific effect on biofilm formation rather than broad antibacterial activity.

The findings of the present study indicated that both seed and fruit extracts of *Pithecellobium dulce* inhibited the growth of *C. violaceum* CV12472 at 20 mg/ml. However, only the seed extract significantly reduced biofilm formation and violacein production at a sub-MIC concentration of 10 mg/ml, suggesting the presence of bioactive compounds such as flavonoids, anthocyanin, tannins, coumarin, triterpenoids, saponins, alkaloids, sterols and fatty acids that likely target bacterial adhesion and QS pathways essential for biofilm development (32). By contrast, the fruit extract demonstrated limited antibiofilm and anti-QS effects, with no change in violacein production, potentially due to the absence or lower concentrations of these specific compounds.

Integrating *P. dulce* seed extract into existing treatment regimens presents a promising strategy for managing biofilm-associated infections, particularly as an adjunct to conventional antibiotics. Its selective antibiofilm properties highlight its potential as a natural agent in the fight against biofilm formation and pathogen virulence, addressing the urgent challenge of rising antibiotic resistance.

However, the present study focused solely on *C. violaceum* CV12472; thus, while the results are promising, they may not extend to other biofilm-forming bacteria. Furthermore, these findings are based on *in vitro* assays, which may produce different results *in vivo*, where complex host factors can influence bioactivity. Variability in compound concentrations across different *P. dulce* sources may also affect the consistency of therapeutic effects.

In order to validate the efficacy and safety of *P. dulce* seed compounds, *in vivo* studies are essential. Animal models could provide insight into pharmacokinetics, bioavailability and therapeutic potency in physiological conditions, where host factors may modulate the effects. Such studies would help determine optimal dosing strategies and assess potential synergy when combined with conventional antibiotics. Additionally, *in vivo* research could reveal any anti-inflammatory or immunomodulatory effects of *P. dulce*, further supporting its therapeutic potential for biofilm-associated infections.

Future research is required to focus on elucidating the precise mechanisms behind the antibiofilm and anti-QS activities of key compounds in *P. dulce* seeds. Expanding studies to include a broader range of pathogenic strains would also help confirm the broader applicability of *P. dulce* seed extract as a therapeutic agent in managing biofilm-associated infections.

Taken together, the findings of the present study indicate that integrating plant-based extracts into treatment regimens could provide a dual advantage: Combating antibiotic resistance, while simultaneously targeting bacterial virulence mechanisms. This approach aligns with the increasing interest in phytotherapy and the use of natural compounds as adjuncts or alternatives to traditional antibiotics.

In conclusion, the present study made an attempt towards screening edible fruits and seeds that inhibit the QS regulated development of biofilms and virulence factors in antibiotic resistant *C. violaceum*. Notably, to the best of our knowledge, the present study is the first to demonstrate that *P. dulce* seed extract effectively inhibits biofilm formation of *C. violaceum* at a sub-MIC of 10 mg/ml, without affecting planktonic cell

growth. This highlights its targeted effect on biofilm inhibition rather than exerting broad-spectrum antibacterial activity. These findings suggest that *P. dulce* seed extracts may serve as potent anti-QS agents, offering potential for managing *C. violaceum* infections. Therefore, the extracts, either alone or in combination with existing antibiotics, could be effectively utilized as anti-infective agents to help manage stubborn infections caused by *C. violaceum*.

The exploration of the anti-QS properties of *P. dulce* not only adds depth to its antimicrobial profile, but also aligns with emerging strategies that leverage natural compounds to combat antibiotic resistance and enhance treatment outcomes against biofilm-associated infections. As research continues to unveil the complexities of bacterial behavior and resistance mechanisms, plant-derived compounds such as *P. dulce* may play an integral role in future therapeutic developments.

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Availability of data and materials

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

Authors' contributions

SFPM collected and managed the data and participated in the writing of the manuscript. SFPM and NNP participated in writing the proposal (objectives, methodology and scope of the research project), performing data collection and in the writing of the manuscript. GRV and PSG were involved in data curation, data analysis and in revising the manuscript. GRV and PSG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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