



## A Simple Characterisation of Violacein Compound Derived from *Chromobacterium* sp. strain Dyh27s2016 and its Antimicrobial Activity Against *Pseudomonas aeruginosa*

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### KEYWORDS

*Chromobacterium* spp.  
Minimum inhibitory  
Concentration  
*Pseudomonas aeruginosa*  
Violacein pigment

### ABSTRACT

*Chromobacterium* sp. strain Dyh27s2016 was isolated from the lake at Manipal International University. Its purple pigmented violacein is hypothesised for a broad spectrum of intriguing biological properties, specifically as an antibacterial agent against Gram-negative bacteria. Hence, the current study aimed to isolate, characterise, and review the antimicrobial property of violacein from the Dyh27s2016 strain against *P. aeruginosa*. The bacterial strain was cultured in nutrient broth with L-tryptophan (0.1 mg/ml); after 24 h, the bacteria were lysed with methanol (3:1 ratio) and mixed with ethyl acetate (4:1 ratio). The top layer was then separated to obtain a concentrated purple pigment. The pH was changed at varying ranges and measured with UV/Vis to characterize this pigment. Then the antimicrobial test was performed against *P. aeruginosa* using the microdilution method and gauged its minimal inhibitory concentration (MIC). Violacein pigment derived from *Chromobacterium violacein* was used as a control in all experiments. For the result, crude violacein from *Chromobacterium* sp. strain Dyh27s2016 was obtained, and the purple pigment in different pH was observed in varying colours; the compound was particularly decolourised at a highly alkaline solution. The pigment was also able to significantly inhibit the growth of *P. aeruginosa* from 200 µg/mL - 800 µg/mL. Tests show that this pigment has a maximum absorption of light wavelength at approximately 600 nm and antibiotic test results show that the pigment can be considered a potential antimicrobial drug against *P. aeruginosa*.

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### 1. INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic Gram-negative bacterium commonly associated with hospital-acquired infections and implicates a significant public health concern worldwide [1]. The bacteria is invasive, especially to children and immunocompromised individuals, thus succumbing to nosocomial infections, urinary tract infections, bacteremia, and pneumonia [2]–[5]. It was reported that the emergence of multi-drug resistance among *P. aeruginosa* had caused an estimated 32 600 infections among hospitalised patients in the U.S and 2 700 deaths in 2017, leading to a staggering medical cost of USD 767 million [6]. Furthermore, the isolated strains were primarily resistant to clinically important drugs, including the carbapenems commonly used as the last line of defense against multi-drug resistant bacteria [7]. The lack of therapeutic alternatives and the widespread of

carbapenem-resistant *P. aeruginosa* had forced the World Health Organization (WHO) to declare such strains as a “critical” group, consequently necessitating a search for new antibiotics [8]. Moreover, the antibiotics employed today were mainly discovered between 1940 – 1960, and the emergence of new antibiotic-resistant strains with multiple acquired modifications makes the existing drugs no longer relevant for the current clinical application. Therefore, there is an utmost urgency to develop novel antimicrobials with a specific mechanism against *P. aeruginosa* [8].

Alternatively, violacein is a violet-coloured pigment of indole derivative, a natural metabolite synthesised by several bacterial genera via the condensation of tryptophan molecule [9]. The compound was first reported in *Chromobacterium violaceum*, owing to wide-ranging biological activities, including anti-tumoral, anti-cancer, and anti-viral properties

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[10]–[12]. However, violacein's bacteriostatic and antibiotic potential against a broad range of bacteria has developed a ubiquitous interest. As a result, the compound has been extensively studied against Gram-positive bacteria such as *Staphylococcus aureus*, either as a stand-alone drug or synergistic with other antimicrobial drugs [13]. The efficiency of violacein against Gram-positive bacteria was reported as very promising; thus, the exploration against Gram-negative bacteria was proposed. On top of that, violacein is commonly used in synergism with most clinical drugs; hence, its stand-alone antimicrobial mechanism against Gram-negative bacteria was least explored [14]. Therefore, the present study aims to investigate violacein's antimicrobial properties against the Gram-negative bacteria *P. aeruginosa* and determine its minimal inhibitory concentration via a microdilution method. This study is a preliminary investigation to assess the prospect of violacein as a novel antibiotic against *P. aeruginosa*. Thus, the outcome of this study will significantly benefit the scientific community to further investigate violacein as an alternative drug for Gram-negative bacteria like *P. aeruginosa*.

## 2. METHODOLOGY

### 2.1 Bacterial culture

The *Chromobacterium* sp. strain Dyh27s2016 (glycerol stock culture obtained from the laboratory at MIU), *Chromobacterium violaceum* Bergonzini (ATCC: 12472<sup>TM</sup>), and *Pseudomonas aeruginosa* (ATCC: 27853<sup>TM</sup>) were cultured on the nutrient agar (37 °C, 24 h). These strains were maintained as pure culture in aseptic conditions and utilised throughout the experiments.

### 2.2 Violacein extraction

The overnight cultured *Chromobacterium* sp. strain Dyh27s2016 and *C. violaceum* (control) were inoculated into the nutrient broth (250 mL) (Chem Soln, India) and added 0.1 mg/mL of tryptophan (Chem Soln, India), respectively. The cultures were incubated at 30°C for 24 h. Following the overnight incubation, 30 mL of the cultures were mixed with 10 mL of methanol (1:3 ratio) (Sigma, U.S.) and incubated for 15 min (120 rpm). The methanol lysate was mixed well with 10 mL of ethyl acetate (1:4 ratio) (Sigma, U.S.). The top ethyl acetate layer containing a violet pigment was then separated using a separatory funnel. The extract was concentrated using a rotary evaporator (50°C) (Witeg, Germany), and the remaining solvent was further evaporated in the oven at 60°C [15].

### 2.3 Violacein characterisation

The extracted violet pigment was characterised based on visual inspection subjected to varied pH ranges and subsequent UV-Vis spectrometry (Cecil, UK) analysis at different wavelengths. Pigment extracted from *Chromobacterium* sp. and *C. violaceum* were prepared at pH 0.54 to 13.00 (0.54, 0.61, 3.00, 7.00, 9.00, 13.00) using 1M NaOH and 1M HCl, adjusting to appropriate pH values. The visual inspections were made for the colour changes at discrete pH, and graphs were plotted based on the light absorption for the prepared pigment at various wavelengths from 400 to 800 nm [16].

### 2.4 Antimicrobial test

The antimicrobial test was performed against *Pseudomonas aeruginosa* using the microdilution method and the minimum inhibitory concentration (MIC). *Pseudomonas aeruginosa* was inoculated into Muller-Hinton broth (MHB)

(Oxoid, UK) to reach  $1 \times 10^8$  CFU/mL. The culture (200 µL) was then mixed with 19.8 mL MHB to obtain  $1 \times 10^6$  CFU/mL. In the microdilution plate, 50 µL of the prepared *P. aeruginosa* was added to 50 µL of violacein (1600 µg/mL) in triplicate. At this point, the concentration of violacein became 800 µg/mL, and the bacterial concentration was approximately  $5 \times 10^5$  CFU/mL at each well. A serially diluted violacein was also prepared, and *P. aeruginosa* (final bacterial concentration in each well =  $5 \times 10^5$  CFU/mL) was added up to 100 µg/mL in triplicate wells. Violacein was extracted from *C. violaceum*, and 2.5% NaOCl served as control. *Pseudomonas aeruginosa* with MHB alone served as negative control. The cultures were then incubated at 37°C (24 h) and analysed using a microplate reader (Tecan, Infinite F50, US) (OD<sub>660</sub>) [17]. All experiments were repeated at least three times.

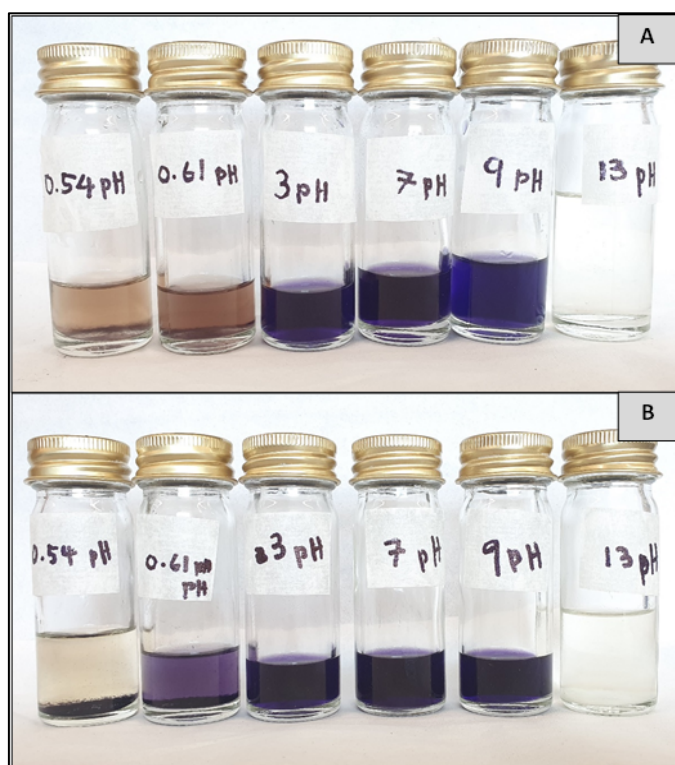
### 2.5 Statistical analysis

Statistical analysis was performed using Microsoft Excel, and Student T-test was used to gauge the significance of the results. P-value <.05 was considered significant.

## 3. RESULT AND DISCUSSION

The outcome of the current study provided substantial input for violacein pigment derived from *Chromobacterium* sp. strain Dyh27s2016 and its ability to inhibit *Pseudomonas aeruginosa* (ATCC: 27853<sup>TM</sup>). Simple techniques such as pH stability tests and light wavelength absorption analysis can be employed to characterise violacein [18], [19]; thus, the test for pH stability on the pigment derived from *Chromobacterium* sp. strain Dyh27s2016 yielded a light beige colour at pH 0.54, beige at pH 0.61, darker violet at pH 3.00 and pH 7.00, and crystal violet at pH 9.00. It turned colourless in a highly alkaline solution (pH 13.00). Similarly, the pigment from *C. violaceum* observed a yellowish/ grey at pH 0.54, pale crystal violet at pH 0.61, darker violet at pH 3.00 and pH 7.00, crystal violet at pH 9.00, and colourless at pH 13.00 (Fig. 1). When the pigments were characterised for light absorption at various wavelengths, it was observed that violacein extracted from both *Chromobacterium* sp. strain Dyh27s2016 and *C. violaceum* showed a maximum peak at approximately 600 nm (Fig. 2). A similar finding was previously reported by Ahmad et al. (2012). When violacein from *C. violaceum* strain S1a was subjected to a pH stability test, it was observed that at low pH, the purple pigment turned greenish-blue and appeared dark blue at pH 3.00 – 9.00. The pigmentation was, however, almost decolourised in a highly alkaline solution [18]. pH plays a significant effect in the deconstruction of the electron system in the violacein compound [20]. Hence, the decolourisation of violacein in a highly alkaline solution is due to the deprotonation of nitrogen by NaOH at the conjugated ring of violacein, resulting in electron conjugation in the structure, which enhances its stability [21]. On the other hand, Ahmad et al. (2012) also reported that the maximum light wavelength absorption by the pigment was observed at 623.67 nm when the pH was 0.54, 576.61 nm at pH 0.61, 589.81 nm at pH 0.64, and 573.17 nm at pH 3.00, 7.00, 9.00 and 13.00 [18]; similar to current findings. The pigment has stronger absorption at longer light wavelengths due to the electron conjugation effect [18].

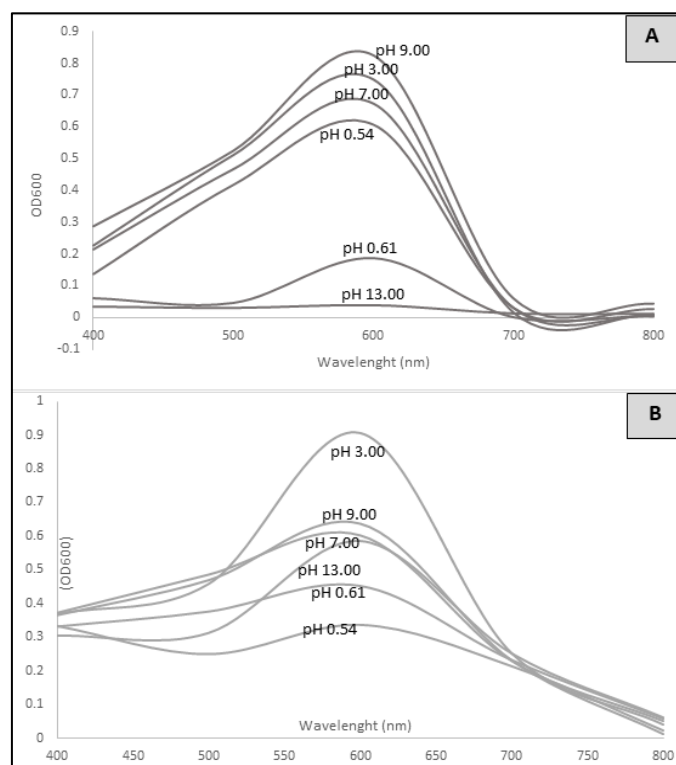
Besides, the crude violacein extracted from *Chromobacterium* sp. strain Dyh27s2016 has demonstrated a significant *P. aeruginosa* inhibition at a concentration ranging from 200 µg/mL - 800 µg/mL. However, at a 100 µg/mL concentration, the compound was insufficient to inhibit them.



**Fig. 1: Visual characterisation of violacein pigments at various pH ranges.** (A) Violacein pigment extracted from *Chromobacterium* sp. strain Dhy27s2016. (B) Violacein pigment extracted from *Chromobacterium violaceum* Bergonzini (ATCC: 12472<sup>TM</sup>). The colour changes were visually observed when violacein pigments were tested at various pH ranges (0.54, 0.61, 3.00, 7.00, 9.00, and 13.00). The experiment was repeated at least three times.

Conversely, the pigment derived from *C. violaceum* inhibited *P. aeruginosa* at 800 µg/mL but not at a lower concentration (Fig. 3). The antimicrobial activity of violacein against Gram-positive bacteria was well documented, and bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis* were demonstrated with significant inhibitory effect [13], [22]. However, the inhibitory effect of this compound against Gram-negative bacteria such as *Flavobacterium balustinum*, *Serratia marcescens*, and *Escherichia coli* was the least effective [23], [24]. The mechanism of violacein exerting its antimicrobial effect against Gram-positive bacteria was not well understood till a recent study indicated that the compound could easily permeabilise into the membrane of Gram-positive bacteria without affecting the cell wall and eliciting a bactericidal effect [25]. Thus, the cytoplasmic membrane is the primary target of violacein. Contrarily, Gram-negative bacteria present a thick outer membrane that may act like a sponge to absorb violacein, consequently protecting its cytoplasmic membrane from the bactericidal effect of violacein.

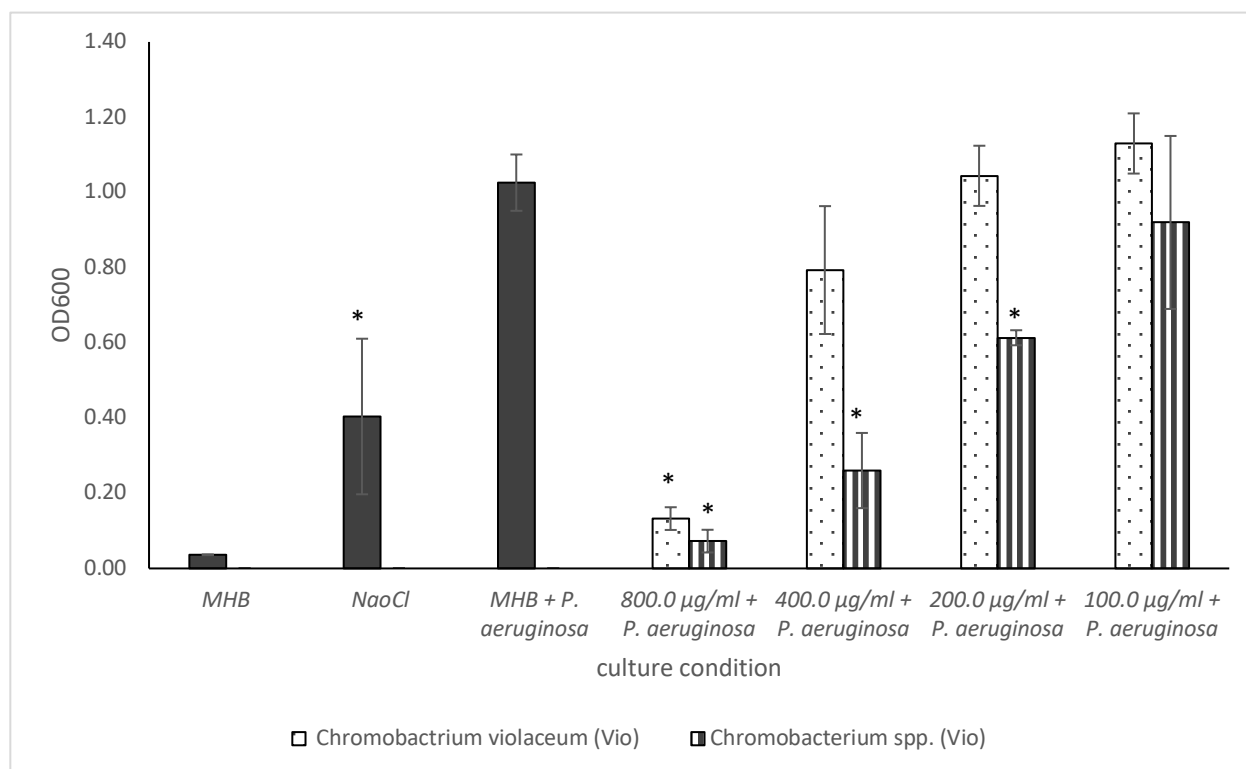
In vitro studies also indicated that violacein was effective against Gram-positive bacteria at lower concentrations (MIC range: 1.6 µM – 25 µM) [26]–[28]. However, the violacein was reported as either least effective (required in higher concentration) or resistant against Gram-negative bacteria such as *P. aeruginosa* [29]. In the current study, violacein derived from *Chromobacterium* sp. strain Dhy27s2016 was comparatively effective against *P. aeruginosa* (200 µg/ml - 800 µg/ml).



**Fig. 2: UV-Vis spectrometry characterisation of violacein pigments at various wavelengths.** (A) Violacein pigment extracted from *Chromobacterium* sp. strain Dhy27s2016. (B) Violacein pigment extracted from *Chromobacterium violaceum* Bergonzini (ATCC: 12472<sup>TM</sup>). The graph was plotted when violacein pigments were gauged at various wavelengths (400 – 800 nm). The experiment was repeated at least three times.

Aruldass et al. (2015) had shown that violacein conferred a MIC on *P. aeruginosa* at 125 µg/ml, which is comparatively closer to the MIC value obtained in the current study [20]. The bacterium *Chromobacterium* sp. strain Dhy27s2016 was isolated from the lake water located at Manipal International University, and its phylogenetic analysis revealed that this strain is closely related to *Chromobacterium amazonense* (98% sequence-homology) [30]. As such, the disparity in the effectiveness of violacein derived from *Chromobacterium* sp. strain Dhy27s2016 against *P. aeruginosa* in this study then the compound extracted from *Chromobacterium violaceum* Bergonzini (ATCC: 12472<sup>TM</sup>) could be attributed to the species variations. Violacein production and its effect depend on several factors, including the microorganism's species and the environmental condition where the microorganisms grow [31].

The antimicrobial effect of violacein is usually investigated in synergism with commercially available antibiotic drugs. For instants, violacein was demonstrated to own a MIC against *S. aureus* and *Salmonella* Typhi at 5.7 µg/ml. Nevertheless, the combined effect of violacein-gentamycin and violacein-cefadroxil was far more effective and showed a MIC of just 1.0 µg/mL on *S. aureus*. Similarly, the synergic effect of violacein-azithromycin and violacein-kanamycin significantly impacted *Salmonella* Typhi, exhibiting a fractional inhibitory concentration (FIC) index of just 0.3 [32]. However, such a synergism study is still lacking for *P. aeruginosa*, which would provide a better understanding of the therapeutic milieu against the pathogenic strains of Gram-negative bacteria.



**Fig. 3: Antimicrobial activity of violacein against *Pseudomonas aeruginosa*.** At 800 µg/mL, violacein from *C. violaceum* and *Chromobacterium* sp. strain Dyh27s2016 significantly inhibited the *P. aeruginosa*. At concentrations of 400 µg/ml and 200 µg/ml, violacein extracted from *Chromobacterium* sp. strain Dyh27s2016 significantly inhibited *P. aeruginosa* but not for *C. violaceum*. At a 100 µg/mL concentration, violacein extracted neither from *C. violaceum* nor *Chromobacterium* sp. strain Dyh27s2016 showed significant *P. aeruginosa* inhibition. The NaOCl (2.5%) served as positive control. The experiment was repeated at least three times. P-value < .05\* is significant.

#### 4. CONCLUSION

Violacein compound was extracted from the *Chromobacterium* sp. strain Dyh27s2016 and a simple characterisation was made using the pH stability tests and light wavelength absorption analysis. It was noticed that the compound appeared pale (beige colour) in the acidic condition, blueish at pH 3.00 – 9.00, and decolourised in a highly acidic solution. The pigment was also observed to absorb light at a maximum wavelength of approximately 600 nm regardless of the pH ranges. This experiment used violacein extracted from *C. violaceum* as a control and showed a similar outcome. On the other hand, violacein derived from *Chromobacterium* sp. strain Dyh27s2016 demonstrated a bacteriostatic effect against *P. aeruginosa* at a concentration ranging from 200 µg/ml - 800 µg/ml, thus owning MIC at 200 µg/ml. The inhibitory effect of violacein isolated from *Chromobacterium* sp. strain Dyh27s2016 can be considered a potential therapeutic drug to treat Gram-negative bacteria like *P. aeruginosa* infections in humans

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest while preparing the manuscript.

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