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ISOLATION AND CHARACTERIZATION OF *CHROMOBACTERIUM* VIOLACEUM AND ANTIBACTERIAL ACTIVITIES OF ITS METABOLITE VIOLACEIN

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Key words: Violacein, pigment, *Chromobacterium violaceum*, antibacterial

Abstract

Violacein is a naturally-occurring bacterial secondary metabolite which is known to demonstrate a wide range of biological properties including antibacterial, antifungal, antiviral as well as anticancer and antitumor activities. Bacteria belonging to the genus *Chromobacterium* are the most studied microorganisms for violacein production. In this study, indigenous violacein-producing *Chromobacterium violaceum* was isolated from water and sediment samples of Bijaypur white clay hill lake, Netrokona, Bangladesh, and identified by biochemical and molecular characterization. After confirming the presence of the *vioB* gene in a polymerase chain reaction, crude violacein was purified from *C. violaceum* CV1 by ethanol extraction followed by filtration through 0.22 μm syringe filters. The partially-purified pigment exhibited significant antibacterial activity against three Gram-positive pathogens; *Staphylococcus aureus*, *Streptococcus* sp, and *Listeria monocytogenes*, with the later demonstrating the highest sensitivity. The bactericidal activity was more pronounced when combined with an antibiotic, thereby producing a drug-metabolite synergistic antibacterial activity.

Introduction

Chromobacterium violaceum is a free-living facultative anaerobic Gram-negative saprophyte bacilli found in tropical and subtropical climatic conditions[1]. They are found to be a sessile bacterium in their natural habitat[2]. As sessile bacteria are more prone to predation, secondary metabolite products work as natural defense mechanisms, thereby giving them a competitive advantage in the habitat[2,3]. *C. violaceum* is a prolific producer of a violet-colored secondary metabolite, violacein[4]. This indole-pyrrole compound can also be produced by other bacterial species like *Pseudoalteromonas, Duganella,* and *Janthinobacterium,* frequently found in soils, rivers, and marine environments[5].

Bangladesh falls in the tropical monsoon region and 80% of its land is a part of the 'Ganges–Brahmaputra floodplain'. The enormous diversity in the landscape of the country is expected to produce diverse microbial flora, some of them may possess commercially and

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therapeutically useful molecules. Understanding our indigenous microbial biodiversity and their metabolites are therefore an important area to explore. In the present century, antimicrobial resistance is a looming crisis and many established drugs are failing to act[6], hence it is necessary to find out potential producers of effective medicinal products to work not only against the pathogenic microbial community but also to make existing treatments more effective. Given the multifaceted therapeutic potential of violacein[5], research on this bioactive compound and its bacterial source is of particular interest.

Violacein((3*E*)-3-[5-(5-hydroxy-1*H*-indol-3-yl)-2-oxo-1,2-dihydro-3*H*-pyrrol-3-ylidene]- 1,3-dihydro-2*H*-indol-2-one) is produced through a set of metabolic reactions by five genes (*vioA* to *vioE*) present in the *vio* operon[7,8]. Among many naturally occurring compounds, violacein has caught attention owing to its diverse biological and pharmacological properties namely, antibacterial, antitumor, antiviral, antioxidant, antileishmanial, antiplasmodial, immunomodulatory, analgesic, antipyretic, and antidiarrheal activities[9–16] In addition, its use in the fields of cosmetics, textiles and insecticides has emerged as prospective areas of application[17–20]. Along with violacein, isolates of *Chromobacterium* are considered as sources of many other industrially valuable enzymes. Many of them have usage in several biotechnological applications, including plant diseases and pest control which makes this organism and its metabolites lucrative for study to the researchers[21].

The present study highlighted the isolation followed by morphological and genomic identification of violacein-producing microorganisms from indigenous sources of Bangladesh, thereafter extracting the metabolite for exploring its antibacterial activities against commonly known pathogenic bacteria.

Materials and Methods

Collection of samples: Subsurface (10 to 15 cm) samples of water were collected in sterile falcon tubes from the Lake Birishiri, Netrokona, Bangladesh. Sediment samples were also obtained from the same sampling site. The geographical coordinates of the sample collection site are from 25°9'29"N, 90°38'36"E to 25°9'31"N, 90°38'19"E. All the samples were carried in sterilized 50 ml falcon tubes at 4°C and processed within 24h of collection.

Isolation and identification of bacteria: Collected samples were serially diluted[22] through 1:100, and 0.1-ml portions were spread over LB agar plates with sterile glass spreaders followed by incubation (New Brunswick Scientific shaker flask incubator, Redline, Blinder, Germany) for 24 h at 37°C. The violet colonies on the medium were primarily selected as the violacein-producers and the genus was later identified following morphological and biochemical characterization as described in Bergey's Manual of Systematic Bacteriology[23]. For molecular identification, the 16S rDNA gene sequence was amplified in Polymerase Chain Reaction (PCR) using universal primer pair, 27F (5'-GAGTTGATCCTGGCTCAG-3') and 1492R (5'-GTTACCTTGTTACGACTT-3')[24], later purifying the PCR product and sequencing the gene with Sanger Sequence technique. The violacein-producing ability of

the isolate was verified by detecting the presence of *vioB* gene, one of the five genes in *vio* operon that is only found in violacein-producer microorganisms[7].

Primer designing for vioB: For PCR amplification of the *vioB* gene, a primer set (Vio-F and Vio-R) was designed in this study according to published *vio* operon sequence[17] and synthesized by a commercial biotech company (Macrogen, Korea). A 918bp conserved region in *vioB* was found to be the most conserved sequence in the operon that belonged to the same gene and also had a short amplicon size. FastPCR (PrimerDigital), PerlPrimer v1.1.21, OligoAnalyzer3.1 (Integrated DNA Technologies), and NCBI primer BLAST were used to design the primers and to assess primer quality (Specificity, primer dimer, PCR quality and linguistic complexity:Table 1).

Table 1. List of primers

Primer ID	Primer Sequence	Nucleotide number	%GC	T_m	Linguistic Complexity	Efficiency	Amplicon size
Vio-F	AAAGACCATCCG CACTTCCTGT	22	50	62.1	92	92	918bp
Vio-R	CAGCTCGTAATA GGCCATCACGT	23	52.2	64.7	90	94	

Production and extraction of Violacein: Crude violacein was extracted from the liquid culture of the isolates following the ethanolic extraction method with modifications[25]. For pigment production, bacteria were grown in 150 ml LB (Luria Bertani) broth at 37°C for 48 hours. Aliquots of 20 ml LB were taken out in sterile falcon tubes and centrifuged at 8000Xg for 10 min. The supernatant was discarded and the pellet was mixed with 10 ml ethanol (Merck, USA) followed by sonication of the solution at 40W for 10 minutes with a 10-second pulse. The sonicated mixture was centrifuged at 12000Xg for 10 minutes to separate the ethanol-violacein portion from the cell biomass. Violacein dissolved in ethanol in the supernatant was collected, dried under vacuum using a Rota-vapor (Buchi R-200, USA) at 40°C, re-dissolved in ethanol and filtered through 0.22 μm syringe filter (Millipore corporation, Germany) for use in experiments.

UV-Vis Spectrophotometry and quantification of violacein: UV–Vis spectroscopy (Hitachi U-2900 Double Beam Spectrophotometer, Japan) of violacein was performed in between wavelengths of 300 nm and 700 nm, with maximum absorbance recorded at 575 nm, a characteristic feature of *this* pigment[25]. The peak absorbance value was put into Beer-Lambert Law ($A = \varepsilon cl$) for quantification, where 'A' is the absorbance at 575 nm, 'c' is the molar concentration, and 'ε' is the molar extinction coefficient of violacein at 575 nm (0.05601 ml μg^{-1} cm^{-1})[26].

Antimicrobial Activity of Violacein: Violacein was tested for antibacterial activity against three different Gram-positive pathogens; *Staphylococcus aureus*, *Streptococcus* sp, and *Listeria monocytogenes*, following the Kirby-Bauer agar disc diffusion technique[27–29]. Whatman filter paper grade 4 (Merck, USA) was used to prepare the violacein-containing paper discs (6 mm in diameter). The discs were sterilized prior to soaking with a concentration of 40 μg/ml of violacein. Appropriately incubated bacterial inoculum of turbidity similar to 0.5

McFarland standard or 1.5×10^8 CFU/ml was spread on Mueller Hinton Agar (OXOID, UK) plate to produce bacterial lawn. Violacein discs prepared earlier were placed on the inoculated plate along with a negative control disc containing only the diluent of violacein, hereafter ethanol.

Synergistic activity of violacein with antibiotics: The synergistic potential of the pigment was tested by combining violacein with tetracycline against *Listeria monocytogenes*, *Staphylococcus aureus* and *Streptococcus* sp. following the Agar Well Diffusion technique as described by Valgas *et al*. (2007)[30]. In each case, 2.4 µg/ml of either tetracycline (Wako Chemicals, USA), or violacein, or tetracycline-violacein combination solution were introduced to separate wells. After an overnight incubation at 37ºC, the diameters of the inhibition zones were measured and compared for synergy between violacein and the antibiotic.

Results and Discussion

Isolation of the violacein-producing bacteria: Sediment and water samples collected from Bijaypur white clay hill lake, Birisiri, Netrokona (Fig. 1A) were the sources of our violacein-producing bacteria. Initially five isolates, CV1, CV2, CV3, CV4 and CV5, with smooth, round and deep purple-colored colonies on LB agar medium (Fig. 1B) were short-listed as violacein-producers and subjected to biochemical characterization. The isolates were found to be motile, Gram-negative, glucose-consuming bacteria with morphology and biochemistry aligned with *Chromobacterium* sp. The biochemical properties corresponded to *Chromobacterium violceum* isolated by Parajuli *et al.* (2016)[31] and Guo *et al.* (2017)[32], also by Mazumder *et al.* (2020)[33] in Bangladesh. All the isolates displayed similar results in our biochemical characterization, except some variations in their sugar fermentation and citrate reduction tests (Table 2), findings supported by a study conducted by Dall'Agnol *et al.* (2008)[34].

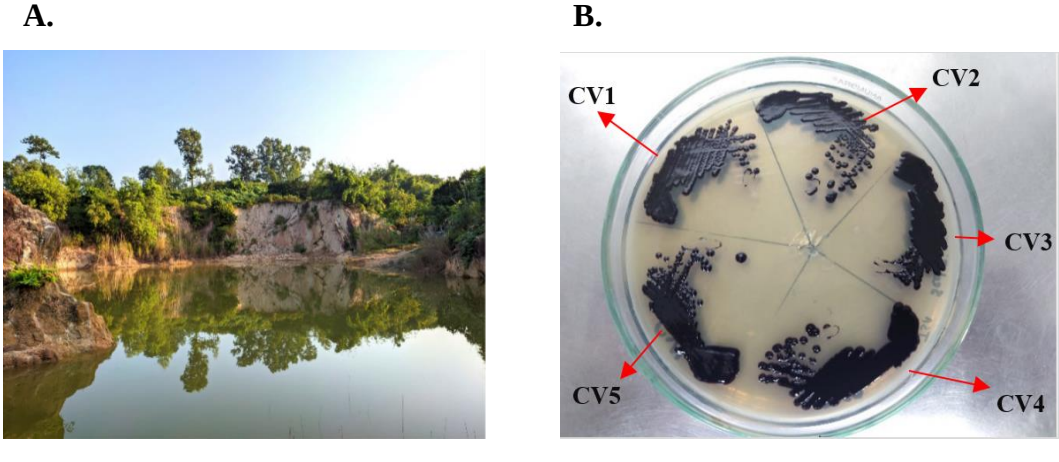


Fig. 1. A. Sampling site; Bijaypur white clay hill lake, Netrokona, Bangladesh, B. Growth of Chromobacterium sp. on LB agar media

Table 2. Biochemical characteristics of the isolates

Characteristics	CV1	CV2	CV3	CV4	CV5
Lactose	-	-	-	-	-
Glucose	+	+	+	+	+
sucrose	-	+	+	-	-
Gas	-	-	-	-	-
H_2S production	-	-	-	-	-
Indole	-	-	-	-	-
MR	-	-	-	-	-
VP	+	+	+	+	+
Mot	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Citrate	-	+	+	-	-
Urease	-	-	-	-	-

Production and quantification of Violacein: All the five isolates were observed to produce violacein in LB broth medium. The UV-Vis spectrum of violacein yielded a characteristic strong absorption at the visible region at 575 nm (Fig. 2A). The isolates demonstrated variable pigment production ability where CV1 appeared as the most potent strain yielding a concentration of 22.5 μg/ml (Fig. 2B).

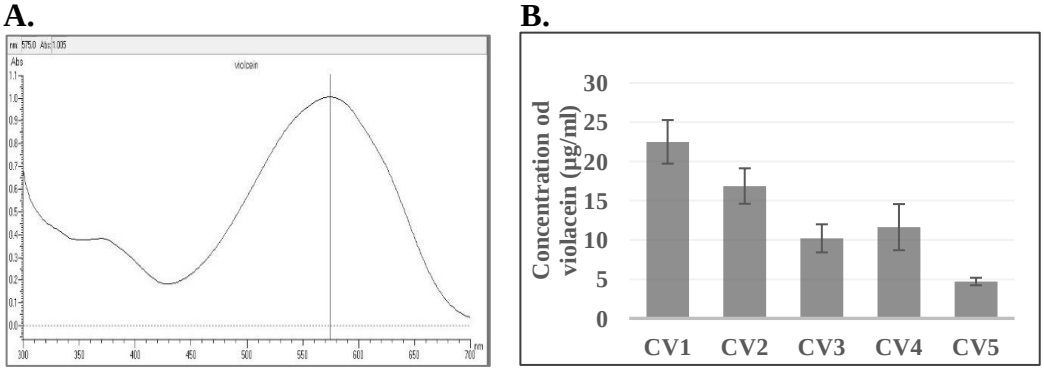
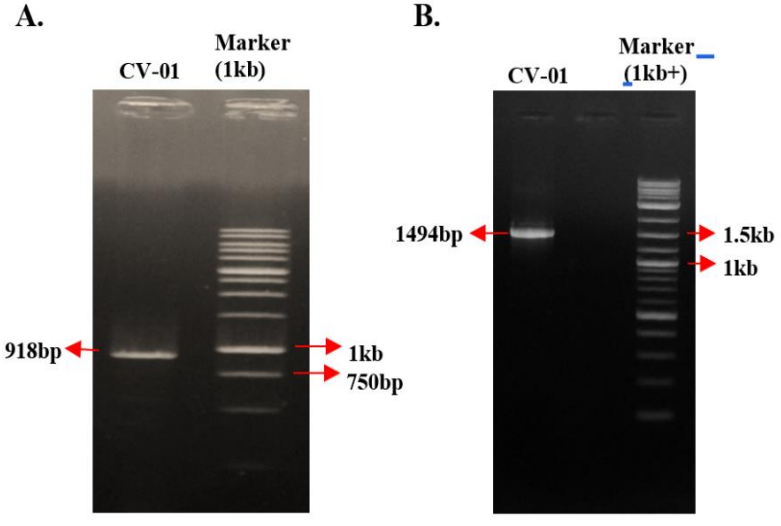


Fig. 2. Production and quantification of violacein. A. UV-Vis Spectrophotometry of violacein with peak absorbance at 575 nm. B. Varied concentrations of violacein production of isolates were averaged from three observations.

Identification of bacteria: Isolate CV1, being the highest violacein-producer, was selected for its further characterization. The violacein-producing ability of the isolate was verified by detecting the *vioB* gene, a signature gene of the biosynthetic cluster of violacein-producing operon in the organism. The gel electrophoretogram showed a distinct band at the expected size of 918 base pairs, confirming the successful amplification of the target

sequence by the designed primer-pair (Fig. 3A). Bacterial species was identified by 16S ribotyping where blast analysis of the sequence data showed 98.80% identity and 100% query coverage with '*Chromobacterium violaceum* strain 08022018 16S ribosomal DNA gene partial sequence' identifying CV1 as *Chromobacterium violaceum* (Fig. 3C). The sequence data is deposited in NCBI GenBank repository (https://www.ncbi.nlm.nih.gov/genbank/) under the accession number 'ON629610', designated as 'CV-DU-Micro-215-001'. The Maximum Likelihood Phylogenetic tree constructed in MEGA X (https://www.megasoftware.net/) using the 16S rDNA partial sequence data demonstrated close evolutionary relationship of CV1 with different *Chromobacterium* species, also an ally to other violacein-producing strains of *Duganella* and *Janthinobacterium* (Fig. 3D). Here, the bacterium, *Thermus thermophilus* M1 served as an out-group in the analyses.



C.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
Chromobacterium violaceum **strain CV-DU-Micro-215-001 16S ribosomal RNA gene, partial sequence**	*Chromobacterium violaceum*	2285	2285	100%	0	100	1241	ON629610.1
Chromobacterium violaceum **strain 08022018 16S ribosomal RNA gene, partial sequence**	*Chromobacterium violaceum*	2217	2217	100%	0	98.8	1471	MH790126.1
Chromobacterium violaceum **strain 726249W 16S ribosomal RNA gene, partial sequence**	*Chromobacterium violaceum*	2217	2217	100%	0	98.8	1472	MG938493.1
Chromobacterium violaceum **strain 726249P 16S ribosomal RNA gene, partial sequence**	*Chromobacterium violaceum*	2217	2217	100%	0	98.8	1497	MG938492.1

***Chromobacterium violaceum* strain BF-R1 16S ribosomal RNA gene, partial sequence**	*Chromobacterium violaceum*	2217	2217	100%	0	98.8	1494	KY292417.1
***Chromobacterium violaceum* strain FDAARGOS_1273 chromosome, complete genome**	*Chromobacterium violaceum*	2217	17720	100%	0	98.8	4758933	CP069587.1
***Chromobacterium violaceum* strain FDAARGOS_1274 chromosome, complete genome**	*Chromobacterium violaceum*	2217	17720	100%	0	98.8	4758933	CP069442.1

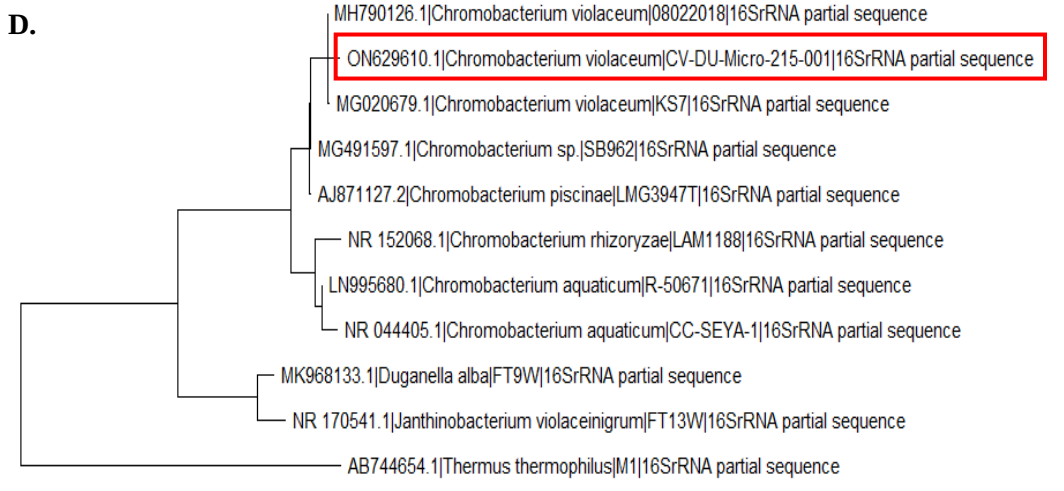


Fig. 3. Identification of the isolate CV1. A. Amplification and detection of the *vioB* gene, B. Amplification and detection of the 16S rDNA gene C. Nucleotide blast analysis of the 16S rDNA sequence in NCBI database, D. Phylogenetic tree showing the isolate, CV-DU-Micro-215-001 positioned with *C. violaceum* clade.

Characterization of antibacterial; activity of violacein: In the agar disc diffusion assay, violacein has been observed to inhibit microbial growth of *Streptococcus* sp., *Listeria monocytogenes* and *Staphylococcus aureus* (Fig. 4). Here, only the Gram-positive bacteria were tested to demonstrate antibacterial potential of violacein, based on the pigment's superior antibacterial efficacy against Gram-positive bacteria than that of Gram-negatives[11,35].

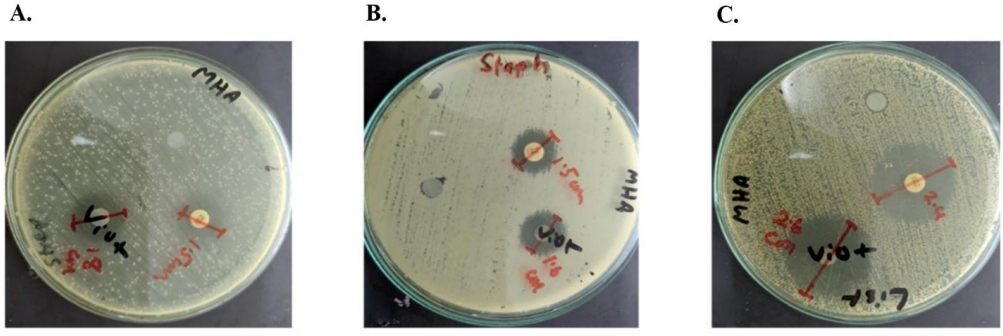


Fig. 4. Antibacterial activity of violacein to different bacteria by agar disc diffusion technique. A. *Streptococcus* sp., B. *Staphylococcus aureus,* and C. *Listeria monocytogenes*.

Clear inhibition zones around the discs are indications of antibacterial activity of the pigment. The negative control disc treated with ethanol, the solvent for violacein extraction, had no zone of inhibition showing that the inhibition of bacterial growth is solely credited to violacein, not the solvent. As shown in a similar work by Periz *et al.* in 2020[36], violacein demonstrated excellent anti-bacterial activity against *S. aureus* (Fig. 4B), as well as against *L. monocytogenes* (Fig. 4C) that appeared as the most sensitive bacterium in this study.

Synergistic activity of violacein with tetracycline: To observe whether violacein displays positive interactions with other antimicrobials, synergistic activity between violacein and a broad-spectrum antibiotic drug, tetracycline, for example, was observed on MHA (Muller Hinton Agar) plates against Gram-positive pathogens: *Streptococcus* sp., *Staphylococcus aureus* and *Listeria monocytogenes.* For all three pathogens, tetracycline-violacein combined solution produced larger zones of inhibition i.e. improved antibacterial activity than that of the antimicrobials alone (Fig. 5), which established violacein's functional synergistic ability with the drug.

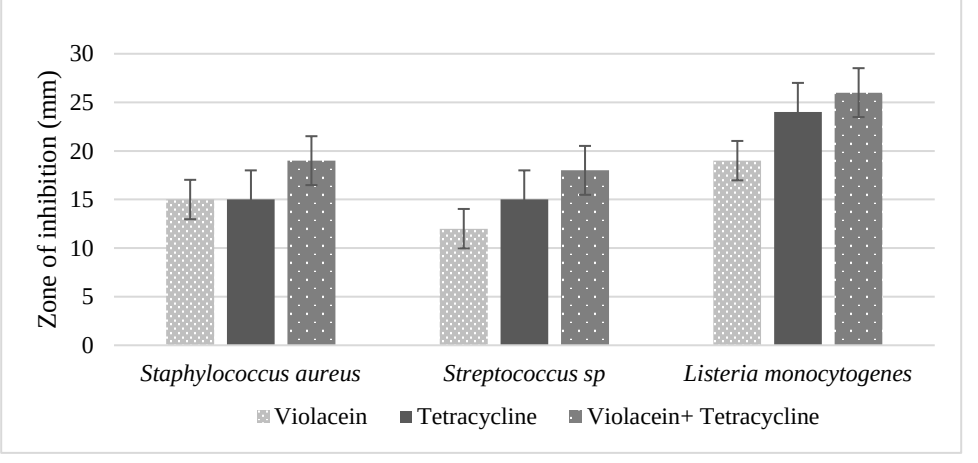


Fig. 5. Zone of inhibitions of violacein, tetracycline, and tetracycline plus violacein in well diffusion assay (n = 3).

Thus, violacein showed its prospect as a future antibacterial drug, both individually and in combination, for treatment of *S. aureus* and *Listeria* infections. These bacteria are known to cause common food poisoning[37] and wound infections[38] that can quickly escalate to a life-threatening condition if not managed properly. The rising antibiotic resistance makes it harder to treat the diseases with the drugs available, which is why, it is necessary to search for new and better antimicrobial agents like violacein.

Violacein has previously also been observed to have positive association with other antibiotics against several common microbial pathogens like *S. aureus*, *Pseudomonas* and *Salmonella*[21,39,40]. In order to fully harness the therapeutic potential of violacein to be an antibiotic drug, future works should address the molecular mechanism of action of the pigment, including a thorough quantitative analysis of its antimicrobial activity.

Conclusion

Bacteria has always been a major source of bioactive, potential therapeutic compounds for their easy availability, higher effectiveness and low production cost[41]. Our work was aimed to bring out a potent bacterial metabolite violacein of *Chromobacterium violaceum* isolated from indigenous environment of Bangladesh and explore its implications in the healthcare industry. We were successful in presenting the antibacterial activity of violacein which has provided a strong foundation for future research to explore other bioactive properties of violacein in Bangladesh. There are still much to learn and understand about the production parameters and efficacy of this compound as a future therapeutic agent. The pigment extracted is yet to be studied and exploited in clinical and industrial microbiology. Additionally, the isolated *C. violaceum* strain in this study will be useful to further study the genomic and phenotypic characteristics of the genus *Chromobacterium*. Taking the findings of this work into account, researches on the therapeutic potential of violacein in Bangladesh deserves special attention, and this study, the first of its kind here in Bangladesh definitely holds a promising start.

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