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CHARACTERIZATION OF CYANOBACTERIA FROM FRESH WATER FOR POTENTIAL ANTIMICROBIAL ACTIVITY

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Abstract

Multidrug resistance (MDR) bacterial infections are a growing global health concern. As a result, there is a growing need to discover effective antimicrobial agents from non-conventional sources. In recent years, Cyanobacteria have received significant attention in basic and applied research due to their ability to produce wide range of bioactive metabolites with antimicrobial properties. This study aimed to isolate and characterize Cyanobacterial isolates from fresh water streams of Haripur city of Khyber Pakhtunkhwa, Pakistan, and to evaluate their antimicrobial potential. A total of 10 water samples were collected, from which 9 cyanobacterial isolates were cultured using BG-11 medium. Antimicrobial activity was assessed using agar well diffusion method against 4 pathogens, namely *Pseudomonas aeruginosa, Bacillus* spp*., Staphylococcus aureus* and *Escherichia coli*. Among the isolates, 9 exhibited antimicrobial activity against at least one of the tested pathogens. Based on morphological and biochemical characteristics, the isolates were tentatively identified at the generic level as *Oscillatoria* spp. (50%), *Nostoc* spp. (30%), and *Microcystis* spp., (20%). Although species level identification and chemical characterization were not performed, the observed antimicrobial activity suggests that freshwater cyanobacteria may serve as a potential source of bioactive compounds with therapeutic relevance.

Key words: Cyanobacteria; Antibacterial; Antiviral, *Staphylococcus aureus; Escherichia coli, Bacillus* spp*., Pseudomonas aeruginosa*

Introduction

Cyanobacteria, also known as blue-green algae is Gram-negative, autotrophic prokaryotes that exhibit oxygenic photosynthesis making the earth atmosphere suitable for breathing (Mehdizadeh & Peerhossaini, 2022; Singh *et al*., 2011). These organisms are found in a wide range of environment including freshwater, marine habitats, soil, and rocks and even on tree surfaces (Hamouda *et al*., 2017). Cyanobacteria comprising over 150 genera and 2000 species, playing diverse ecological roles in both marine and terrestrial environment (Percival & Williams, 2014; Castenholz *et al*., 2001). They are recognized for producing a broad range of secondary metabolites such as bioactive compounds with antibacterial, antiviral, antifungal, anti-inflammatory and anticancer properties making them valuable in pharmaceutical and agricultural industries (Gupta *et al*., 2013). A significant number of cyanobacteria have been assessed for biomedical applications particularly as antimicrobial agents. Several species are known to produce significant bioactive secondary metabolites, e.g., peptides and alkaloids (Rajabpour *et al*., 2019). They also play important environmental roles through fixing atmospheric nitrogen and solubilizing phosphates (Thajuddin & Subramanian, 2005). The morphology of cyanobacteria ranges from unicellular to filamentous forms and colonies, with some cells specialized for nitrogen fixation or survival in harsh environments, and trichomes that can be either homocystous (*Oscillatoria*) or heterocystous (*Nostoc*). (Singh *et al*., 2011; Token *et al*., 2021). Cyanobacteria are known to produce various compounds, e.g., proteins, enzymes, essential fatty acids, hydrocarbons, phenolic, terpenoids, macrolides, polyketides and pigments (Rojas *et al*., 2020). These compounds exhibit a wide range of structural and functional properties and have been identified as potent antibacterial, antifungal, antiviral and anti-mycobacterial agents (Burja *et al*., 2001; Nowruzi *et al*., 2018; Swain *et al*., 2017). Several genera, including Nostoc spp., *Chroococcus* spp,, *Oscillatoria* spp., and *Stigonema* spp., are known producers of bioactive compounds (Sabat *et al*., 2025). A comprehensive overview showed that approximately 46% of bioactive compounds are derived from members of the order Oscillatoriales, 26% from Nostocales, and 15% from Chroococales (Burja *et al*., 2001; Dittmann *et al*., 2015). Many of these compounds show effectiveness against drug-resistant mycobacterium strains (Dubey *et al*., 2013). For instance, *Anabaena* extract showed strong antibacterial activity against vancomycin-resistant *S. aureus* (MIC: 32–64 mg/mL) while compound Carbamidocyclophanes from *Nostoc* showed moderate activity against *S. aureus* (Bhateja *et al*., 2006). The present research focuses on the isolation and characterization antibiotic producing cyanobacterial species from different freshwater streams located in Haripur, with the objective of evaluating their antimicrobial potential against selected pathogenic microganisms.

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Methodology

Sample collection: This study was conducted as a one-time cross sectional study sample survey. Water samples were collected from stagnant freshwater ponds located in different areas of Haripur city, Khyber Pakhtunkhwa, Pakistan including Haripur city, Khalabat Township, Dobandi, Sikanderpur, Awan colony, Moonan, Talokar, Kangra colony, Panian and Sarai Saleh. Sample was carried out during the month of August- September 2024 morning or afternoon hours. A total of ten sampling stations were selected and all samples were selected within the same sampling period with a time interval of 1-2 days between sites (Fig. 1). The approximate distance between sampling locations ranged from 2-15 KM representing different freshwater habitats within the study area. Sterilized bottles were used for sample collection, and all laboratory analyses were conducted in the Biotechnology Laboratory of the Department of Microbiology, University of Haripur, Khyber Pakhtunkhwa, Pakistan.

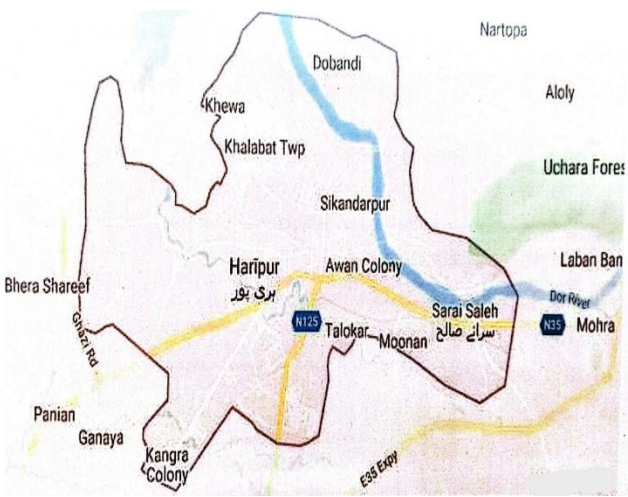

Fig. 1. District Haripur map highlighting areas of sample collection.

Sample preparation and isolation of cyanobacteria: BG-11 medium was prepared according to the manufacturer's protocol and sterilized by autoclaving at 121°C, 15 psi pressure for 15 minutes. After cooling, One milliliter of each water sample was serially diluted (10^{-1} to 10^{-2}) using autoclaved distilled water and inoculated into BG-11 broth. Cultures were incubated for 14 days in a shaker incubator at 30-32°C under the ambient laboratory illumination with natural light-dark cycle (approximately 12 hours light/12h dark). Following enrichment, cultures were streaked onto BG-11 agar plates to obtain discrete colonies. Plates were incubated under the same photoperiod conditions for 7-14 days until visible cyanobaterial growth was observed. Repeated subculturing on BG-11 agar was performed to obtain purified cyanobaterial isolates.

Microscopic and morphological identification of cyanobacteria: Preliminary identification of cyanobacterial isolates was carried out based on morphological characteristics observed under a compound light microscope. Features such as cell shape and size, unicellular or filamentous organization, trichrome structure, pigmentation, presence or absence of mucilaginous sheath, motility, and occurrence of specialized cells including hetrocytes and akinetes were examined. Identification was performed using standard cyanobaterial taxonomic keys (Komárek & Anagnostidis, 2005), and isolates were assigned to their respective genera.

Identification of test bacterial pathogens: The bacterial pathogen used for antimicrobial activity assays (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphlyococcus aureus*, and *Bacillus* spp.) were identified and confirmed using Gram staining and standard biochemical tests. Gram staining and simple staining were performed for morphological observation. Biochemical tests included oxidase, catalase, and nitrogenase assays to identify bacterial strains.

Preparation of cyanobaterial extract from fresh water isolates: The cultured cynobacterial cultures were grown in about 200ml of nutrient broth and tube were placed in shaking incubator for 7days at 37°C. The incubator was set at 160 rpm. After incubation, culture suspension was filtered using filter paper then 100ml of supernatant plus 100 ml of ethyl acetate were added in separating funnel and was shaked for 5 to 10 min. after that layers formed in the funnel then collect the upper ethyl acetate layer was collected in a baker and covered with aluminum foil with holes in it. Collected solution was allowed for evaporation for 24 hours. After that the extract was collected and mixed with 5ml of dimethyl sulfoxide. The collected extract was allowed to dissolve completely and was subsequently used for antimicrobial activity assays.

Antimicrobial activity (Agar well diffusion): An antimicrobial activity of the isolated cyanobacterial strains was evaluated by using agar well diffusion method against 4 bacterial pathogens. For this purpose, Two Gram positive (*S. aureus* and *Bacillus* spp.) and two Gram negative bacteria (*E. coli* and *P. aureginosa*) were used as test pathogens obtained from the Pakistan institute of medical sciences (PIMS), Islamabad. For antimicrobial testing nutrient broth media was prepared by dissolving 13 g of nutrient broth powder in 500 ml of distilled water and autoclaved at 121°C for 15 min. Each test pathogen was inoculated separately into sterile nutrient broth and incubated at 37°C for 24 hours. Nutrient agar medium was prepared by dissolving 23 grams in distilled water in 1000 ml and autoclaved for 15 min. at 121°C then pour the medium into sterile Petri plates and then allowed to solidify. After solidification sterile cotton buds were used to spread all known test pathogenic bacteria on 4 agar plates, then by using sterile blue tips 4 wells were made with the size of 9-10 mm. Three wells were loaded with 70µl of culture supernatant of isolated cyanobacterial isolates and 30 µl of nutrient agar and fourth one was loaded with DMSO as a negative control after that plates were placed for incubation at 37°C for 24 hours. After incubation, the antimicrobial activity was assessed by measuring the diameter of zone of inhibition (in mm) using a ruler (Balouiri *et al*., 2016).

Preservation: Glycerol stocks (30% glycerol in PBS) were prepared and stored at -20° C.

Table 1. PCR protocol.

PCR reagents	Stock Conc.	Working concentration	Vol/Rec
DNA template			1µL
Reverse primer	10 µM	0.2 µM	0.4 µL
Forward primer	10 µM	0.2 µM	0.4 µL
Buffer	10 X	1X	2 µL
MgCl2	25 mM	2.5 Mm	2 µL
DNTPS	10 mM	0.2 mM	0.4 µı
Taq polymerase	5U/µL	1.5 U	0.3 µL
Deionized H_2O			13.5 µı
Final Volume			20 µı

Table 2. Zone of inhibition shown by isolated cyanobacterial isolates against *Pseudomonas aeruginosa, Bacillus* spp., *S. aureus* and *E.coli*. Nine isolates showing different zones opposition to test pathogens.

	Pseudomonas	*Bacillus* spp.	*S. aureus*	*E.coli*
S(B)	0mm	6mm	8mm	12mm
S(E)	4.5mm	0mm	7.5mm	10mm
S(D)	6mm	9.5mm	8.5mm	9mm
S(A)	2mm	0mm	9mm	7mm
SA(O)	0mm	5.5mm	0mm	9mm
S(C)	8mm	15 mm	6mm	5mm
S(F)	5.5mm	14mm	3mm	0mm
S(G)	0mm	7.5mm	12mm	4mm
S(H)	0mm	11mm	0mm	2mm

DNA extraction & PCR: DNA was extracted using a chemical method (Neilan *et al*., 1995) (Table 1). Fresh cyanobacterial cultures were washed with phosphate-buffered saline (PBS) and STE buffer (Sodium Tris-EDTA) twice and followed by treatment with TE buffer. Phenol and chloroform extraction was used to isolate DNA. Multiple centrifugation and transfer steps were performed to purify the DNA and finally DNA was collected in Eppendorf tube and stored at -20°C. PCR amplification was performed using universal cyanobacterial 16S rRNA primers targeting the V3-V4 region, as previously described by (Nübel *et al*., 1997). The forward primer sequence was AGAGTTTGATCCTGG CTCAG and the reverse primer sequence was CACCTT CCGGTACGGCTAC.

Gel electrophoresis: A 0.8% agarose gel with ethidium bromide was used to visualize amplified DNA bands under UV light using 500 bp marker.

Results

A total of nine cyanobacterial isolates were obtained from freshwater sample collected from different location of Haripur. The isolates were coded as S(B), S(E), S(D), S(A), SA (O), S (C),S(F), S(G), and S(H). The antimicrobial activity of these isolates against *Pseudomonas aeruginosa, Bacillus spp*., *Staphylococcus aureus*, and *Escherichia coli* is presented in Table 2. The results showed variable inhibitory effects among the isolates. The largest inhibition zone against *Bacillus spp*. was produced by isolates S(C) (15mm), followed by S(F) 14mm) and S(H) (11mm). Against *S. aureus* the highest inhibition was observed for isolates S (G) (12mm). In the case of *E.coli,* isolate S (B) showed the greatest activity with a 12mm inhibition zone.

Antimicrobial activities of isolated cyanobacterial isolates: The antibacterial activities of all 9 isolated cyanobacterial isolates were tested against bacterial pathogens that cause severe human infections including *S. aureus, E. coli, Bacillus* spp*.,* and *P. aeruginosa*. Total of 9 cyanobacterial isolates exhibited antibacterial activities against test pathogens **(**Fig. 2).

Colony morphology by gram staining: All 9 isolated cyanobacterial isolates were identified on the basis of morphology of the Gram negative rods of *Microcystis* spp*., Oscillatoria* spp*.,* and *Nostoc* spp. (Fig. 3).

Simple staining results: Simple staining under microscopy showed filamentous appearance of *Nostoc spp.,* the rod like structure of *Oscillatoria* spp*.*, and the round colonies of *Microsystis* spp*.* (Fig. 4).

Morphological characteristics and simple staining results: Based on morphological characteristics and simple staining the isolated cyanobacterial isolates were tentatively identified at the generic level. 4(50%) of them were smooth circular, slimy, dark green *Oscillatoria* spp*., 3(30%)* smooth circular, slimy, green as a *Nostoc* spp*,* and 2(20%) irregular circular, slimy, green *as* a *Microcystis* spp*.,* were identified (Table 3).

Biochemical tests result: Three main biochemical tests were performed to characterize cyanobacterial isolates based on their biochemical reactions. These included Catalase, Nitroginase, and Oxidase tests. All cyanobacterial isolates were exhibited positive catalase oxidase and nitrogenase activity (Table 4).

Molecular characterization: The isolated cyanobacterial isolates were further analyzed for molecular identification.

Bands of DNA: DNA was extracted using chemical method, and distinct DNA bands were observed through Gel electrophoresis (Fig. 5).

Table 3. Morphological and staining analysis results of isolated cyanobacterial isolates from fresh water samples.

Isolates code	Margin	Texture	Color	Gram staining
S(A)	Smooth circular	Slimy	Dark green	Negative rods
S(E)	Smooth circular	Slimy	Green	Negative rods
S(D)	Smooth circular	Slimy	Dark green	Negative rods
S(B)	Smooth circular	Slimy	Green	Negative rods
SA(O)	Irregular circular	Slimy	Green	Negative rods
S(C)	Smooth circular	Slimy	Green	Negative rods
S(F)	Irregular circular	Slimy	Green	Negative rods
S(G)	Smooth circular	Slimy	Dark green	Negative rods
S(H)	Smooth circular	Slimy	Dark green	Negative rods

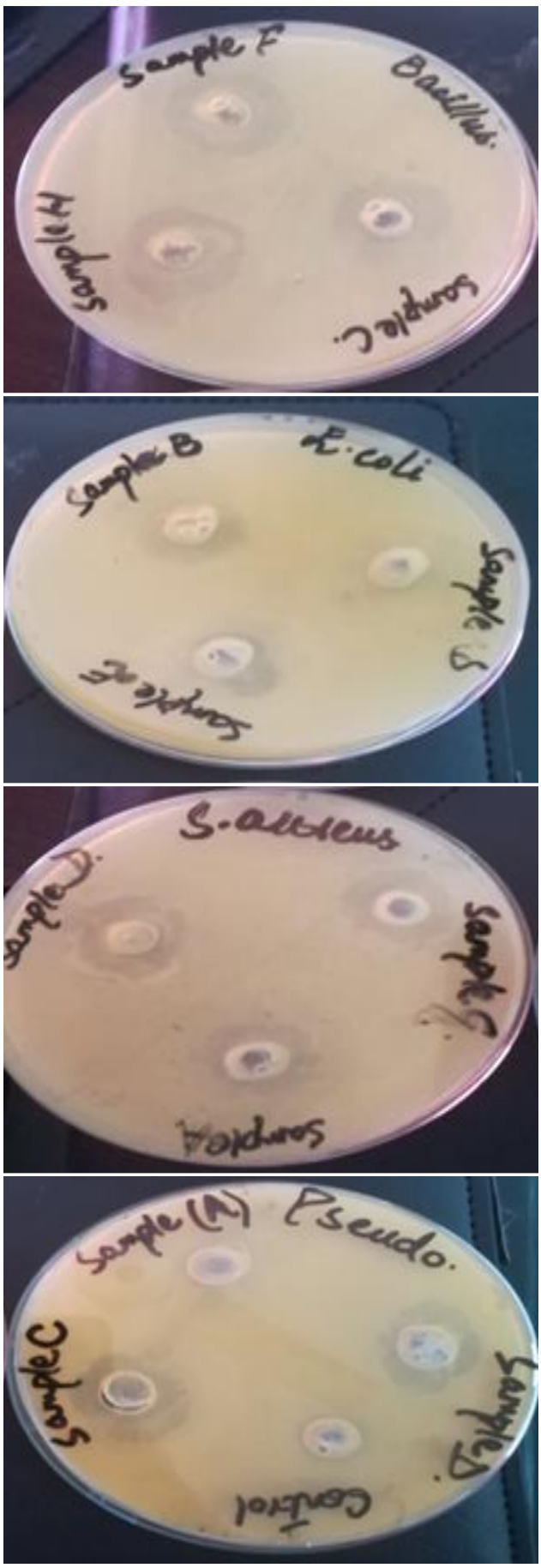

Fig. 2. Antibacterial activities of isolated cyanobacterial isolates against test pathogens *S. aureus, Pseudomonas, E.coli* and *Bacillus* spp., exhibited by clear Zone of inhibition.

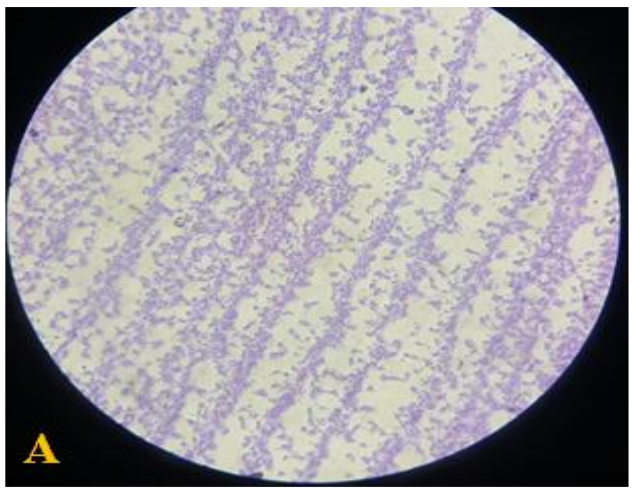

Fig. 3. Gram negative rods of isolates show colony morphology of isolated cyanobacterial isolates.

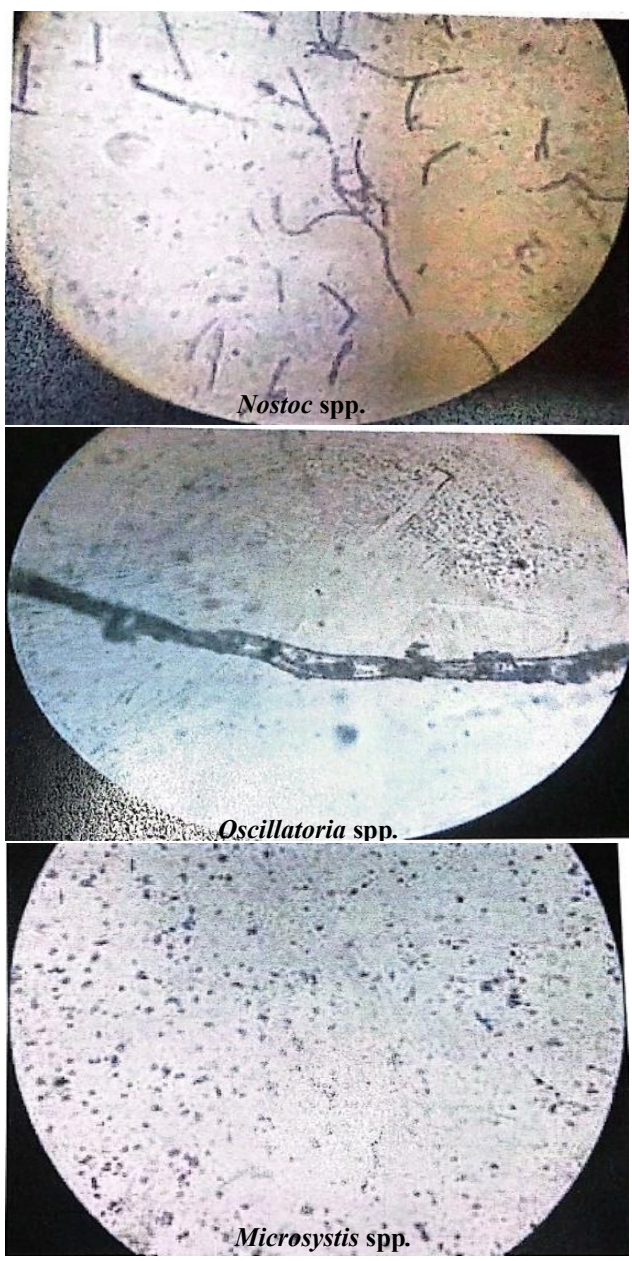

Fig. 4: colony morphology of cyanobacterial isolates by simple staining under microscope.

Table 4. Biochemical tests were performed to characterize cyanobacterial isolates on the basis of their biochemical reactions.

Strains	Oxidase	Catalase	Nitrogenase
S(B)	Positive	Positive	Positive
S(E)	Positive	Positive	Positive
S(D)	Positive	Positive	Positive
S(A)	Positive	Positive	Positive
S A (O)	Positive	Positive	Positive
S(C)	Positive	Positive	Positive
S(F)	Positive	Positive	Positive
S(G)	Positive	Positive	Positive
S(H)	Positive	Positive	Positive

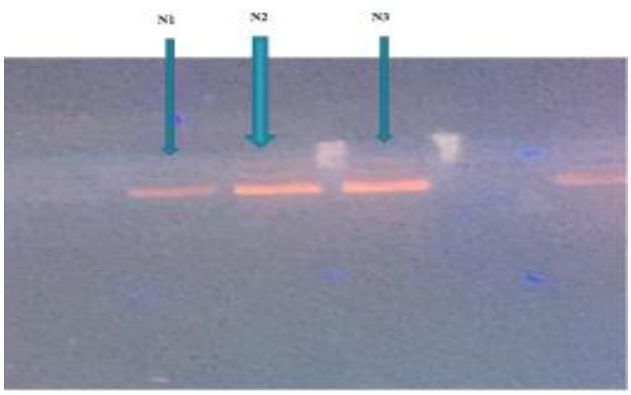

Fig. 5. DNA bands observed on Gel electrophoresis.

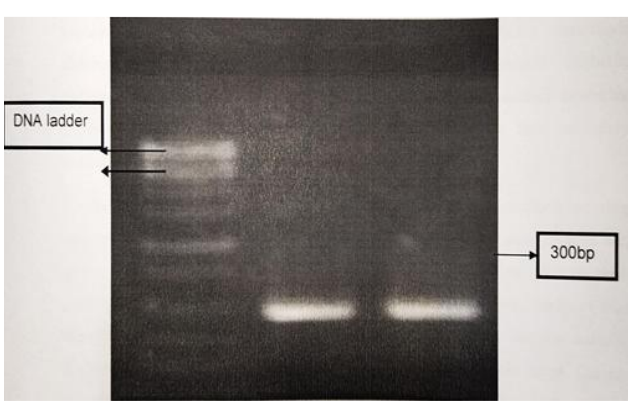

Fig. 6. Amplified PCR product.

GEL analysis of Amplified DNA: An amplified product of approximately 300 base pairs observed when compared with the 500 bp DNA ladder. Intact bands were detected in samples S (B) and S (G) loaded in wells 2 and 3, respectively.

Specific PCR primers (V3-V4) were used to characterize Cyanobacteria. PCR analysis showed intact band of 300 bp (Fig. 6). Specific ITS region (V3-V4) was present in Cyanobacteria and Molecular analysis using partial 16S rRNA gene amplification (~300bp) confirmed the cyanobacterial origin of selected isolates. However, this fragment length was insufficient for species or strain level discrimination. The V3-V4 region provided preliminary genus level confirmation only. Further identification using longer rRNA sequences or additional molecular markers is recommended for precise taxonomic resolution. Although the initial identification was done on the basis of morphological and microscopic analysis, the molecular identification also confirmed the presence of different isolates of Cyanobacteria.

Discussion

The need for novel antimicrobial agents has increased due to the emergence of pathogens that are resistant to multiple drugs. Variety of secondary metabolites with noteworthy biological activities, such as antimicrobial, anticancer, and anti-inflammatory effects are produced by cyanobacteria, making them a promising source (Gupta *et al*., 2013; Jeong *et al*., 2020). The antimicrobial potential of bioactive substances such as terpenes, phenols, fatty acids, and halogenated aliphatic is well documented in both fresh water and marine cyanobacteria (Kannan *et al*., 2010). The current study aimed at preliminary isolation and screening antimicrobial nine cyanobacterial isolates for antimicrobial activity rather than detailed taxonomic resolution from streams of Haripur city. Most of the isolates resembled, *osillatoria* spp., *nostoc* spp., and *microcystis* spp., against *Staphylococcus aureus, Escherichia coli, Bacillus* spp., and *Pseudomonas aeruginosa*. A study conducted in Saudi Arabia for the isolation of cyanobacteria from Riyadh desert soil exhibited isolated 7 cyanobacterial species including, *Nostoc linckia, Spirulina platensis, Phormidium autumnale, Tolypothrix distorta, Microcystis aeruginosa, Dunaliella salina* and *Chlorella vulgaris* (Al-Wathnani *et al*., 2012). Another study also showed strong inhibitory activity particularly against *S. aureus, B. subtilis* and *S.sonnei* (Yalçın, 2020). Similarly, extracts from *phormidium, Geitlerinema, Arthrospira, leptolyngbya* and other freshwater cyanobecteria demonstrated antimicrobial activity against pathogens such as *MRSA, E. coli, Salmonella typhimurinum* and *Klebsiella pneumoiae*, with a dihydro-2H-pyran-2-one derivative identified as key compound (Srivastava *et al*., 2017). in another comparative screening,12 out of 61 cyanobacterial strains-including marine, terrestrial, and freshwater isolates-showed strong activity against *Mycobacterium tuberculosis* and *Bacillus anthracis*, particularly in heterocyst-forming species (Chlipala *et al*., 2009). In comparison the present study assessed the antimicrobial potential of acetyl acetate extract of 3 isolated cynobacterail strain including *Nostoc* spp., *Microcytosis* spp., and *Oscillatoria* spp., using the same ager well diffusion method. The extract exhibited notable antimicrobial effects, with the highest zone of inhibition recorded at 15 mm against *Bacillus spp.* and 12 mm against *S. Aureus*, these findings are aligned with previous observations (Al-Wathnani *et al*., 2012). These results confirm that cyanobacteria possess bioactive compounds capable of effectively inhibiting Gram-positive bacteria, and highlighting their potential as a source of natural antimicrobial agents. Result of the study depict that these cyanobacterial isolates could be utilized in pharmaceutical industry for the production of novel antibiotics against tested human pathogens(Shah *et al*., 2017). Another study in Saudi Arabia illustrated that chloroform extract could be used for the extraction of antimicrobial agent as it exhibited strong inhibitory action as compared to acetone and ethanol(Vijayakumar *et al*., 2011). According to this study, Most of the strains of these bacteria are active against *S. aureus, E. coli, P. aeruginosa,* and *Bacillus spp.* Specific ITS region (V3-V4) were present in Cyanobacteria and molecular analysis using partial 16S rRNA gene amplification (~300bp) confirmed the cyanobacterial origin of selected isolates. However, this fragment length was insufficient for species or strain level discrimination. The V3-V4 region was sufficient for preliminary genus level

confirmation only. Further identification longer rRNA sequences or additional molecular markers is recommended for precise taxonomic resolution. Although the initial identification was done on the basis of morphological and microscopic analysis, the molecular identification also confirmed the presence of different isolates of Cyanobacteria. It is advised to perform additional molecular characterisation (16S rRNA sequencing) and compound identification (GC-MS analysis).

Conclusion

On the basis of morphological and growth characteristics, 50% of the isolates resembled with *Oscillatoria spp.,* 30% resembled *Nostoc* spp., and 20% were similar to *Microcystis* species of the phylum cyanobacteria. The cyanobacterial isolates isolated from different fresh water streams of Haripur demonstrated the capability to produce bioactive compounds against *Pseudomonas aeruginosa, Bacillus* spp*., Staphylococuss aureus* and *Escherchia coli*. The study suggests that these isolated cyanobacterial isolates could serve as potential candidates for pharmaceutical industry in the development of novel therapeutics.

Competing Interests: The authors declare that there is no competing interest to declare.

Authors Contribution: Sundas Nisar is the lead author of this article, having conducted the entire research process. Muhammad Fayaz Khan, as corresponding author, wrote, reviewed, and guided the project to ensure its quality. Muhammad Ilyas made significant contributions by conducting statistical analyses and offering expertise in data interpretation. Fahad-ur-Rehman provided assistance with sample collection, facilitating the groundwork for the study. Additionally, Sohaib contributed significantly to various laboratory tasks, including the PCR analysis, ensuring the experimental procedures were carried out effectively.

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