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Innovative Isolation of *Nostoc minutum* Protein for Antibacterial Applications

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ABSTRACT

The study successfully identified a new Iraqi blue-green alga, *Nostoc minutum* HA-YJ, through both morphological and molecular diagnostics, and recorded it in the gene bank for the first time as a genus found in northern Nineveh (Ain Sifni). This cyanobacterium thrived in BG11 medium and is notable for its bioactive compounds, particularly its protein content. In this study, the protein was extracted, purified, and continuously monitored. Chromatography revealed a single peak, confirming the presence of pure protein. Both spectrophotometry and Fourier Transform Infrared (FTIR) spectroscopy were employed to identify the protein. UV absorption peaks were observed at 304 and 614nm in the protein extracts. FTIR analysis showed distinct features, such as an aromatic primary amine (N-H stretching) at 3202cm^{-1} and an amide I band at 1638cm^{-1} (C=O stretching vibration). Additionally, an absorption peak at 2891 cm^{-1} indicated the presence of carboxylic acids, and the amide II band appeared at 1437cm^{-1}. The optimal concentrations of purified protein (10.0 and 7.5mg/ ml) were effective against all bacteria tested, with Gram-negative bacteria being particularly sensitive. The protein was tested against a variety of pathogenic bacteria, including both Gram-positive and Gram-negative species. At concentrations of 5.0mg/ ml, the protein exhibited moderate antibacterial activity, while 2.5mg/ ml showed no effect. Notably, *E. coli* displayed high sensitivity to the protein, whereas *Staphylococcus aureus* showed resistance.

INTRODUCTION

Nostoc minutum is an isolate of Cyanophyta that is widespread in freshwater environments. Nostoc can be found in soil, on moist rocks, at the bottoms of lakes and springs, and occasionally in marine habitats. It may also be found in terrestrial temperate, desert, tropical, or polar environments **(Ku *et al*., 2012)**. The *Nostoc* is a genus of Gram-negative photosynthetic cyanobacterium **(Rathwa & Patel, 2020)**. Many species of *Nostoc* possess an outer layer and extensive inner matrix of polysaccharides, giving them their "jelly-like" or gelatinous appearance, and also help protect them from their environment and can assist in the absorption of moisture. This ability allows Nostoc

minutum to survive under stressful conditions, such as fluctuating temperatures, drought, salt stress, desiccation, UV radiation, and infection by pathogens **(Tamaru *et al.*, 2005)**. This alga is classified as follows: Division: Cyanophyta, Class: Cyanophyceae, Order: Nostocales, Family: Nostocaceae, Genus: *Nostoc*. Microalgae are living microorganisms characterized by their ability to produce a wide range of high-value bioactive compounds using sunlight, CO2, and water **(Al-Hayali *et al.*, 2020)**. Among these, blue-green microalgae/cyanobacteria represent the most diverse category of phototrophic organisms and are recognized as a primary source of chemical compounds, including nutrients, lipids, and vitamins **(Hussein & Ausama, 2021; Yaqub *et al.*, 2023)**. Cyanobacteria are among the most abundant biological sources of bioactive compounds derived from secondary metabolism. They are significant sources of proteins, phenolics, alkaloids, and carotenoids **(Hayali *et al.*, 2020)**. Currently, there is significant focus on utilizing cyanobacterial proteins as potent antibacterial agents for managing and controlling human and plant diseases. Numerous studies have identified that proteins produced by cyanobacteria possess a broad range of biotechnological applications, including antibacterial, anticancer, biosensing properties, and catalytic functions **(Atwan & Hayder, 2020; Abd & Mohammed, 2021)**. Cyanobacterial proteins are distinguished by their low economic cost, non-toxicity, and interfacial safety, enabling their use in secondary medicine **(Wu *et al.*, 2017)**. There are two primary approaches for protein extraction: chemical and physical, with the physical method being the most favored strategy **(Abbasi *et al.*, 2016)**. Cyanobacteria-based protein extraction is regarded as one of the most effective methods due to its environmental sustainability, safety, cost-effectiveness, ease of handling, rapid protein production, and superior chemical stability compared to other biological manufacturing techniques involving bacteria, fungi, yeasts, and viruses **(Vanlalveni *et al.*, 2018)**. Therefore, cyanobacteria-based protein extraction offers new possibilities as an alternative to antibiotics, addressing the issue of multiple drug resistance (MDR) shown by several plant- and human-infecting pathogens **(Morell & Balkin, 2010)**. Cyanobacteria represent a viable global resource, with yearly production surpassing 35.1 million tons **(FAO, 2022)**. They are a rich source of various bioactive compounds, including plant hormones, antioxidants, antimicrobials, and antivirals, making them valuable in the antibiotic industry **(Nabti *et al.*, 2016; Galal *et al.*, 2024)**. This study focused on methods of protein extraction from *Nostoc minutum* and its effects on pathogenic bacteria.

MATERIALS AND METHODS

1. Algae collection, isolation and cultivation

The microalga/cyanobacterium was collected from various locations along the river edge and the running water of the Spring (Ain Sifni) using a phytoplankton net. Water temperatures ranged from 20 to 30°C, with a pH of 7.9. The samples were transferred to

the lab in clean bags or bottles to identify the algae types present and to select the pure isolates for culture. After isolating the pure algae, it was cultured in BG11 medium. All isolates were grown under light with a photon flux density of 70 Lux at 28°C. The cyanobacterial cells were transferred to fresh medium multiple times and were then grown on BG11 agar plates containing 1.2% agar for one to three weeks. The filamentous cyanobacterial cells were isolated and transferred to a liquid medium. The isolates were then cultured in a 2L bottle, aerated with a simple flow of air, resembling a photobioreactor, and placed under a 12/12-hour light/dark photoperiod **(Al-Shahery, 2021)**.

2. Morphological identification

The cell morphologies were examined using a light microscope (Olympus model IX71; Tokyo) coupled with a digital camera. Species classification was performed using taxonomic references, which included both 'classical' taxonomic revisions **(Geitler, 1932; Desikachary, 1959)** and more recent taxonomic revisions **(Willame, 2006; Komárek, 2010)**.

3. Molecular identification

A total of 10mL cultures were harvested by centrifugation at 3500rpm for 10min. The concentrated cells were placed into 1.5mL microcentrifuge tubes and preserved at -20°C until DNA extraction. Molecular diagnosis aims to confirm the genus identity by determining the DNA and performing PCR amplification of the 16S rRNA gene sequence of cyanobacteria **(Moreno *et al.*, 1995)**. DNA was extracted using a specialized kit for cyanobacteria (Geneaid, Taiwan), following the method outlined for diagnosis.

4. (PCR) Polymerase chain reaction technique

The cyanobacterial strain was identified by 16S RNA or rDNA amplification and sequencing using the cyanobacterial-specific primers for *Nostoc* sp. **(Qasim *et al*., 2012)**, as shown in Table (1).

Table 1. Primers Cyanobacteria

Gene	PCR Primers
CYA1492-Reverse	5´- CTACGGGCTACCTTGTTACGA-3´
CYA27-Forward	5´- AGAGTTTGATCCTGGCTCAG3´

The total PCR mixture was 20µl in each tube, containing genomic DNA, 40 pmol of forward and reverse primers, and 12.5µl of Master Mix. The thermal cycling conditions, as shown in Table (2), were followed according to **Nubel *et al.* (1997)**. The sizes of the amplified products were compared with a 1250 bp DNA ladder to determine the exact size of the gene. Purified PCR products were prepared and sequenced by Macrogen (Korean Biotechnology Company, Korea). The sequence was then compared

with other sequences in the National Center for Biotechnology Information (NCBI) GenBank library.

Table 2. Thermal cycle program **(Nubel *et al*., 1997)**

Step	Function	Temperature °C	Cycles	Time	Stage
1	Initial denaturation	95	1	5 min	1
2	DNA denaturation	95	40	1min	2
3	Primer annealing	65	40	1min	2
4	Template elongation	72	40	1min	2
5	Final elongation	72	1	10min	3
6	Incubation	15	1	Hold	3

5. Estimating the amount of external cyanobacterial total protein in medium

The amount of protein was measured using the Biuret method on the tenth, fifteenth, and twentieth days of the culture's growth. To prepare the Biuret reagent, 1.5 grams of hydrated copper sulfate ($CuSO_4$) and 6 grams of sodium potassium tartrate ($Na_3C_4H_4O_6$) were dissolved in 500ml of sterile distilled water with continuous stirring. Then, 300ml of 10% sodium hydroxide (NaOH) was added while stirring. Finally, the volume was adjusted to 1 liter with sterile distilled water.

6. Extraction of protein

After growing the cyanobacterium in glass vials for 15 days, the culture reached the stationary phase. The *Nostoc minutum* cells were collected using a centrifuge at 3000rpm for 5 minutes to obtain a precipitate and to maximize biomass recovery. The precipitate was then placed in a freezing chamber at 4°C for 24 hours. Afterward, the cells were thawed for 1 hour, and this process was repeated for 3 consecutive days. Subsequently, the precipitated cells were disrupted using an ultrasonic device with a power of 24,000 pulses/s for 15 seconds, with intermittent pauses to cool the device and to maintain a temperature of 4°C. The process was repeated several times to ensure complete cell lysis. The lysed cells were then placed in the centrifuge at 3000rpm for 10 minutes to collect the cellular debris. To precipitate the protein, ammonium sulfate was added to a concentration of 70%. The precipitate was dissolved in 2mL of Tris-buffer solution (pH = 7.5). Ammonium sulfate was removed by dialysis **(Alabbas *et al.*, 2022)**. Protein concentration was measured using a spectrophotometer at a reading of 280nm.

7. Chemical characterization

The molecular weight of pure phosphatidylcholine was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie Brilliant Blue was employed to stain the gel **(Laemmli, 1970)**. The mobility of protein subunits was evaluated using standard protein markers with established molecular weights. The absorbance of the UV–visible spectrum of the purified protein was recorded across a wavelength range of 200–800nm, utilizing distilled water as a control. FT-IR

spectroscopy (Agilent Cary 630 FT-IR spectrometer) was conducted in the range of 600–4000cm^{-1} to identify the functional groups of protein in *Nostoc minutum* that are responsible for the pigment generated by the alga **(Patel *et al.*, 2005)**.

8. Antibacterial activity of purified of protein against pathogenic bacteria

The antibacterial activity of the purified protein was evaluated by preparing different concentrations (2.5, 5.0, 7.5, and 10.0mg/ ml) and by testing them against five types of pathogenic bacteria: *E. coli, K. pneumoniae, P. aeruginosa, S. aureus,* and *S. epidermidis*. The bacterial strains were cultured on Mueller-Hinton agar medium. Four wells were created on the medium to place the different concentrations of the purified protein. The cultures were then incubated at 37°C for 24 hours. Antibacterial activity was assessed by measuring the inhibition zone around each sample **(Ghazala *et al.*, 2022)**.

9. Statistical analysis

All measures were conducted in triplicate, and the results were expressed as means ± SD (n = 3). Mean separation and significance were assessed using the SPSS software package. Correlation and regression analyses were performed using the Excel program.

RESULTS AND DISCUSSION

1. Morphological diagnosis

The cyanobacterium *Nostoc minutum* is composed of filaments of moniliform cells, surrounded by a gelatinous envelope and multiplies under natural conditions by binary fission. When present, akinetes are larger than vegetative cells and have subglobose to ovate appearances. Long rows of akinetes are observed between trichomes, either devoid of heterocytes or interspersed with them **(Afify *et al.*, 2023)**. A somewhat brownish spore is present on the round, smooth-surfaced, granular ripe akinetes **(Fidor, 2019)**. These cells are Gram-negative bacteria with highly structured cell walls **(Perkins *et al.*, 1981)**, as shown in Fig. (1).

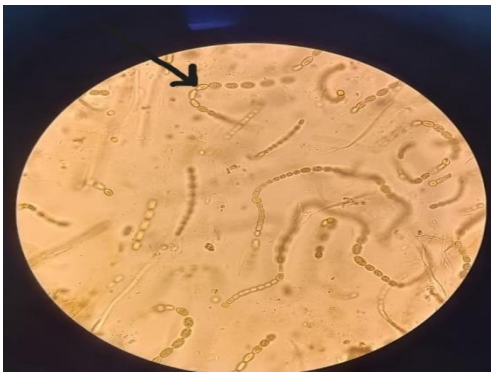

Fig. 1. *Nostoc minutum* 40x magnification power

2. Molecular diagnosis

The DNA diagnostic analysis revealed that the DNA of the cyanobacterium *Nostoc minutum* had a base pair size of 1200bp, with additional fragments ranging between 1000 and 1500bp, as shown in Fig. (2).

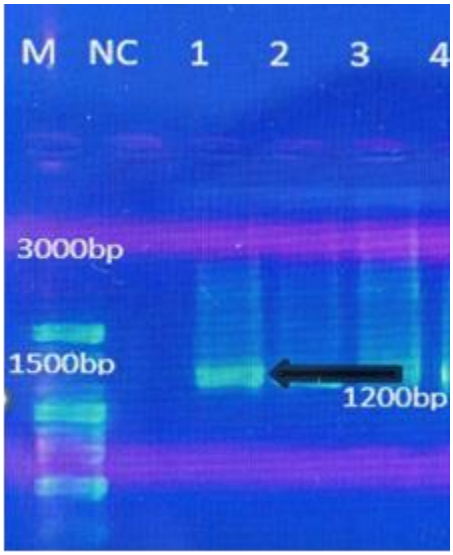

Fig. 2. DNA Bands of cyanobacteria in gel electrophoresis

3. Polymerase chain reaction (PCR)

The 16S rDNA genomic sequence was matched with isolate data registered in GenBank **(Ahmed *et al.*, 2010)**, an essential technique for identifying cyanobacterial species and genomic sequences. This method is closely related to studies by **Manuelov (2014)** and **Thompson *et al.* (2018)**. Molecular diagnosis plays a crucial role in accurately identifying cyanobacterial species **(Shalini *et al.*, 2007)**. The sample (1), which was studied and molecularly diagnosed, was recorded in the National Center for Biotechnology Information (NCBI). This molecular analysis was conducted at the Technical Research Center at Northern Technical University in Mosul. The results showed that the sample belongs to *Nostoc minutum* according to the taxonomic tree shown in Fig. (3). The sequence of the isolated strain (sample 1, PP999718) matched the control strain by 98%, and this was the first isolation of this species recorded in Iraq.

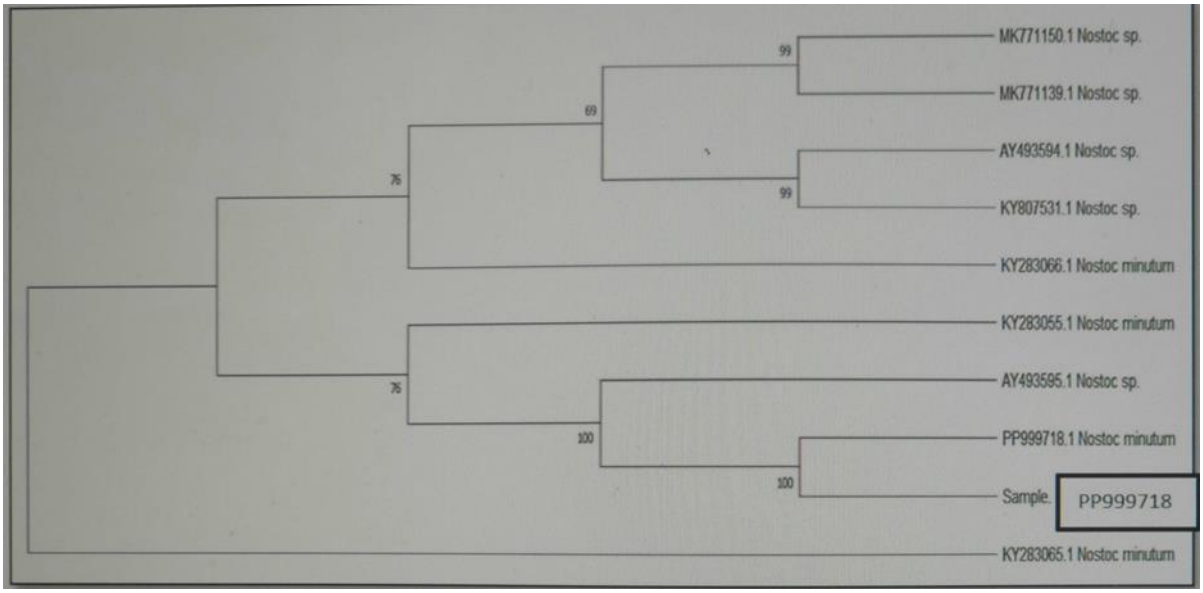

Fig. 3. Taxonomic tree *Nostoc minutum*

4. Protein purification

The crude protein of *Nostoc minutum* was successfully isolated and purified. The protein concentration increased when fractionated with 70% ammonium sulfate **(Pankaj *et al.*, 2010)**. Following purification, dialysis and gel chromatography were employed, revealing a single protein peak, as shown in Fig. (4). The molecular weight of the protein was determined after precipitation with 70% ammonium sulfate.

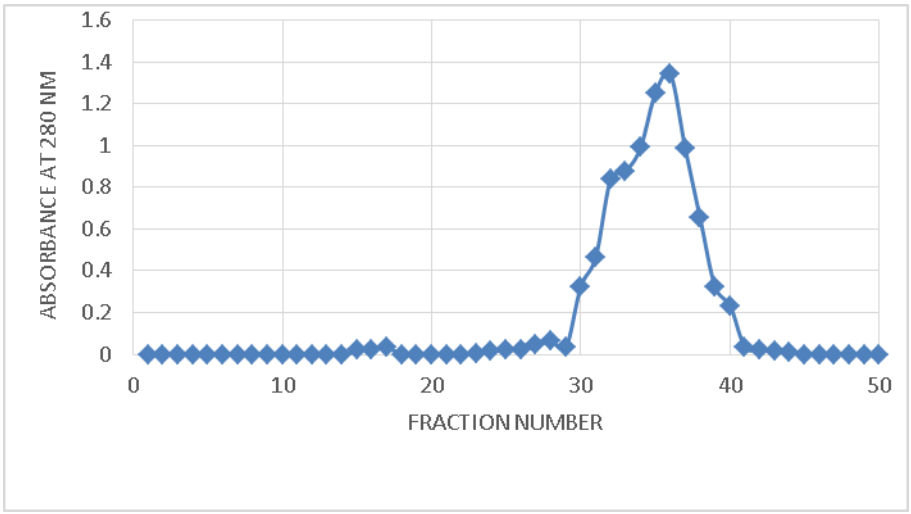

Fig. 4. Gel filteration chromatography using Sephadex G-50

The SDS-PAGE method was used to determine the molecular weight of the isolated protein. The SDS-PAGE results, stained with Coomassie Brilliant Blue, revealed two bands corresponding to the α and β subunits of the protein, with molecular weights of approximately 18 and 13kDa, respectively (Fig. 5). A similar result was previously observed for the protein from *Limnothrix* sp. NS01 **(Rimbau *et al.*, 2001)**. The α and β subunits represent the fundamental components of the protein. Each subunit consists of an open-chain tetrapyrrole molecule, with a thioether linker connecting the apoprotein to it **(Bougatef *et al.*, 2024)**.

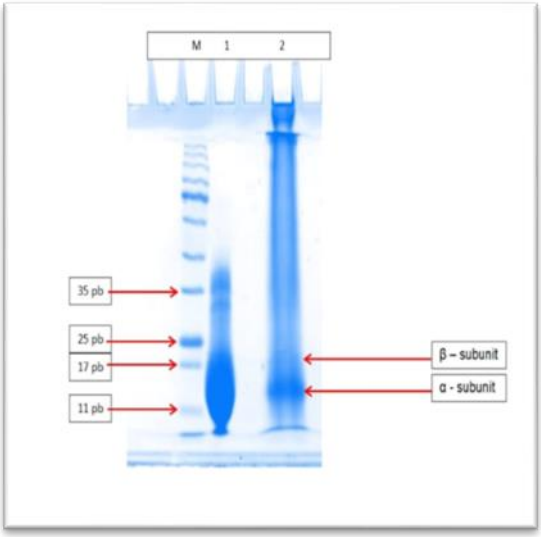

Fig. 5. SDS-PAGE profile of the crude protein from *Nostoc minutum*

UV-vis spectroscopy was used to examine the spectrum characteristics of protein in the 200–700nm wavelength range. The protein crude had two distinctive absorption peaks at 304 and 614nm, as seen in Fig. (6). Comparable results were seen with protein extract from further strains of cyanobacteria. Despite reaching analytical grade and being suitable for a wide range of applications, the protein product still requires further optimization for specific uses (**Moreira *et al.*, 2012)**.

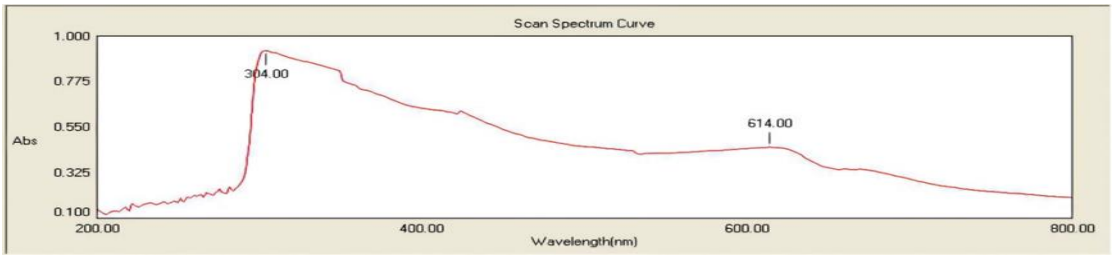

Fig. 6. UV-Vis spectra of protein

Furthermore, FT-IR spectroscopy was used to examine the protein's primary structural characteristics after it was isolated from *Nostoc minutum*, as illustrated in Fig. (7). As it is well known, proteins are molecules with an open tetrapyrrole chain, which is made up of four pyrrole rings with one nitrogen atom and four carbon atoms each (C4H5N). Additionally, protein has an amide group. The FT-IR spectra showed unique features, such as the aromatic primary amine (N-H stretching) at 3203cm^{-1} and the amide I band at 1638cm^{-1} (C=O stretching vibration). The presence of carboxylic acids was indicated by an absorption peak at 2891cm^{-1}, while the amide II band was seen at 1437cm^{-1}. This study is consistent with earlier research **(Tong *et al.*, 2020)**. **Prabakaran *et al.* (2020)** identified nine functional groups with unique bond stretching at different wave numbers in the crude protein obtained from *S. platensis*. The current sample used in the *Nostoc* investigation showed absorption bands that closely matched those reported in the literature for protein, derived from nine functional groups, each exhibiting distinctive bond stretching at various wave numbers in the crude protein from *Nostoc minutum*.

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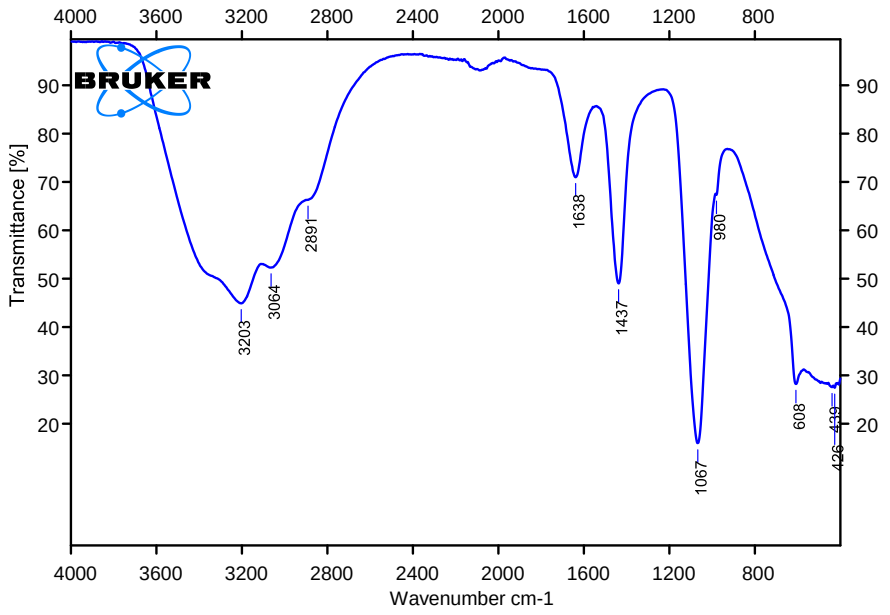


Fig. 7. Characterization of protein FT-IR spectrum

5. Estimating the amount of external algae total protein in medium

The protein content was measured using the Biuret method on the tenth, fifteenth, and twentieth days of the culture. The highest protein value, 90µg/ cm³, was recorded on the fifteenth day, while the lowest amount of protein was observed on the twentieth day (Fig. 8). Fig. (8) shows the protein content in the culture filtrate **(Rosales *et al.*, 2017)**.

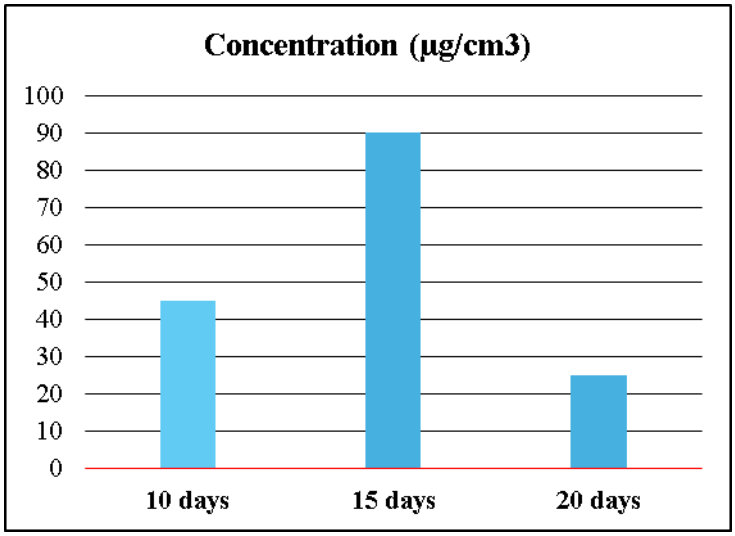


Fig. 8. The amount of protein (micrograms/cm3) leachate extract

To calculate the averages for the development periods of Nostoc minutum, protein content (from both cell extract and culture filtrate) was estimated on the tenth, fifteenth, and twentieth days, as shown in Table (3). The average protein concentration for the cell extract during these periods was 45, 90.25, and 90.25µg/ cm^3, respectively. Regarding the protein leaching from the culture, the percentages were 50.1, 100.4, and 31.2%, respectively.

Table 3. The average protein for the external protein in medium

Average development periods	Protein extract depositor	Protein cytophilic precipitate
Day 10	45.0 µg/cm^3	Ab 50.1 µg/cm^3
Day 15	90.20 µg/cm^3	Ab 100.4 µg/cm^3
Day 20	20.01 µg/cm^3	Ab 31.20 µg/cm^3

6. The antibacterial activity for purified protein

The antibacterial activity of the purified protein was assessed against Gram-positive (*S. epidermidis* and *S. aureus*) and Gram-negative (*E. coli*, *K. pneumoniae*, and *P. aeruginosa*) bacteria by measuring the inhibition zone areas. The results indicated that the purified protein was effective against most of the tested strains (Table 4). The bacterial strains exhibited varying responses, with the diameter of the inhibition zone increasing in proportion to the amount of protein. Among the Gram-positive bacteria, the maximum inhibition zones were observed for *S. aureus* (25 mm, 17 mm) and *S. epidermidis* (24mm, 13mm) at 10mg/ mL and 7.5mg/ mL, respectively. No minimum concentration inhibition zones were observed for other Gram-positive strains. Gram-negative bacteria were generally more resistant to the protein's inhibitory effects than Gram-positive bacteria. The least sensitive strain was *P. aeruginosa*, with inhibition zones ranging from 24 to

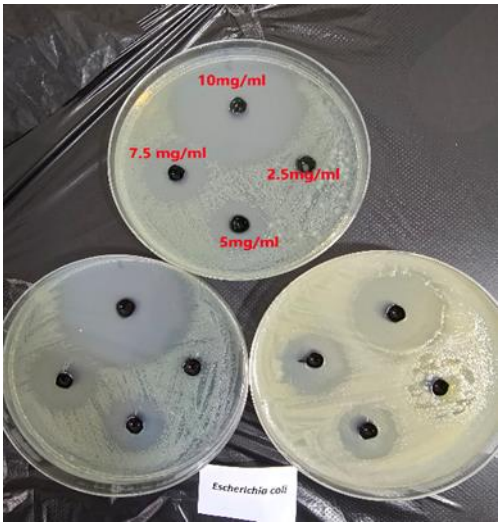
16mm at concentrations of 10 and 7.5mg/ mL. In contrast, the most sensitive strains were *K. pneumoniae* (26mm, 14mm) and *E. coli* (30mm, 20mm) at 10 and 7.5mg/ mL, respectively. The observed variations in bacterial sensitivity to the protein may be due to differences in their bacterial cell wall composition.

Numerous studies have suggested that proteins exhibit an enhanced efficacy against microbial infections. **Safari *et al.* (2019)** found that protein derived from *S. platensis* was more effective against Gram-positive than Gram-negative bacteria. Additionally, **Fan *et al.* (2013)** reported that proteins had specific antibacterial activity against *S. aureus, S. epidermidis*, and *K. pneumoniae*. In an *in vitro* study, **Osman *et al.* (2015)** evaluated the antibacterial properties of proteins against *S. aureus, E. coli*, and *K. pneumoniae* using the agar well-diffusion method. Their findings suggest that this innovative protein demonstrates antibacterial capabilities and may serve as a partial substitute for certain antibiotics. Furthermore, **Sarada *et al.* (2011)** showed that proteins effectively countered *E. coli, K. pneumoniae, P. aeruginosa*, and *S. aureus* (Fig. 9).

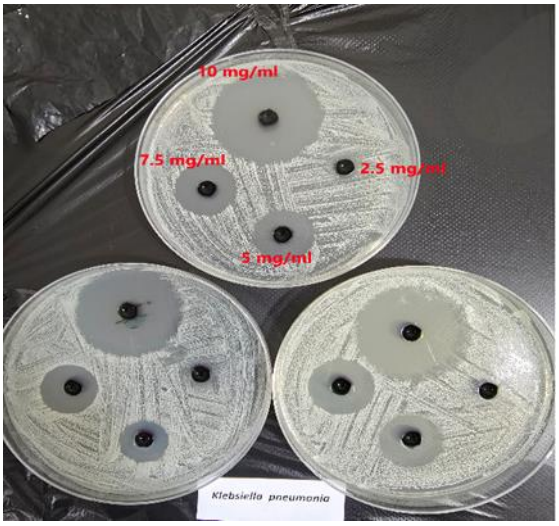
Table 3. Inhibition zones (mm) of antibacterial activity of protein

Bacterial strains / Protein concentration (mg/ml)	2.5	5.0	7.5	10.0
E.coli	3*	15*	21*	30*
Staph.aureus	0	9	17	25
Staph.epidermis	3	10	13	24
P.aeruginosa	0	6	17	25
K.pneumoniae	0	6	14	26

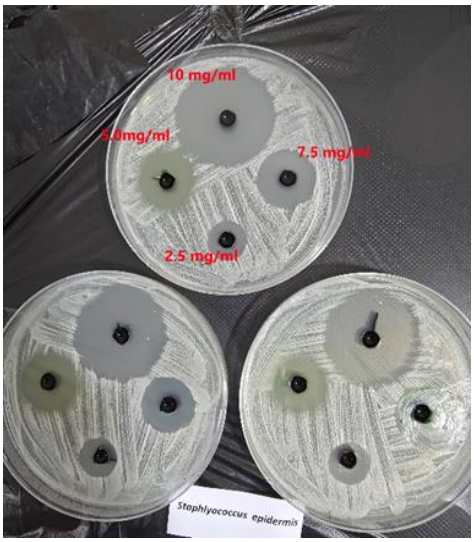
* The number is inhibition zone in mm.



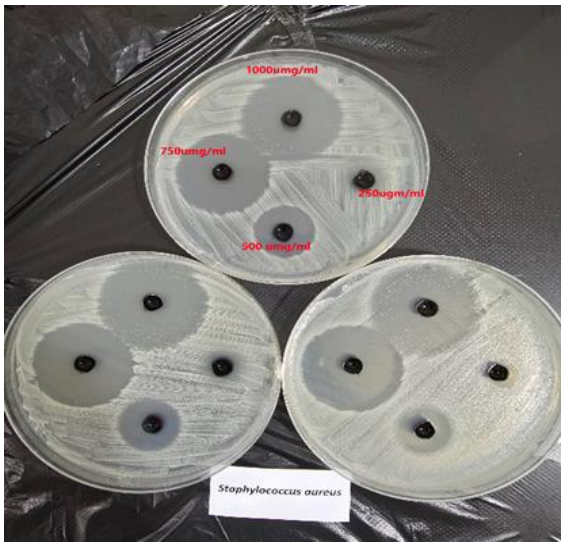
E. coli



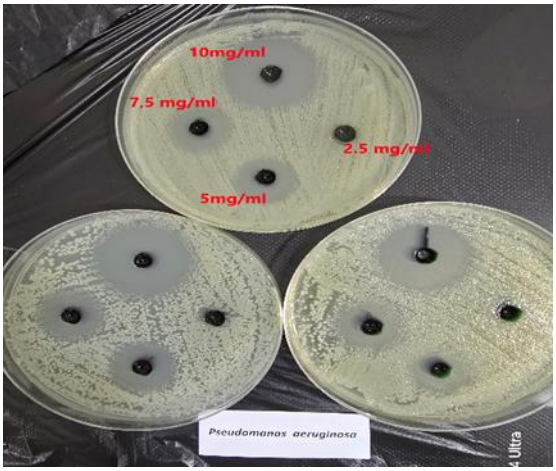
K.pneumoniae



Staph.epidermis



Staph.aureus



Pseudomans aerugionsa

Fig. 9. Antibacterial assay of protein as shown in the present study

CONCLUSION

In this study, the results confirmed the effectiveness of protein extraction from the blue-green alga *Nostoc minutum* in inhibiting the growth of pathogenic bacteria and reducing their harmful effects. Based on our preliminary findings, the crude protein from Nostoc minutum demonstrated strong antimicrobial activity against a broad range of pathogenic Gram-positive and Gram-negative bacteria. However, further in-depth investigations are needed. Future research should focus on identifying the molecular mechanisms underlying this activity, with the goal of developing practical applications for these proteins in the production of effective antibiotics and therapeutic drugs.

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