Research Paper

Enhanced Production and Characterization of Cyanophycin Granule Polypeptide from *Nostoc* sp.: A Sustainable Bioactive Polymer

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Cyanobacteria, or blue-green algae, produce cyanophycin granule polypeptide, a nitrogen storage polymer composed of aspartic acid and arginine, as a survival mechanism. This study aimed to enhance cyanophycin granule polypeptide production in *Nostoc* sp. under nitrogen-limited BG-11 medium, achieving a maximum yield of 7.5 % of the cell dry weight (wt/wt). Cyanophycin granule polypeptide characterization by Fourier transform infrared spectroscopy revealed characteristic amide vibrations at 1654 cm^{-1} and 1542 cm^{-1}, confirming peptide bonding. Reverse-phase high-performance liquid chromatography analysis revealed a sharp, well-defined peak for cyanophycin granule polypeptide at 5.664 min, confirming its presence and characteristic elution profile. Additionally, ^{1}H nuclear magnetic resonance spectroscopy confirmed the presence of arginine and aspartate residues. Antioxidant assays demonstrated significant free radical scavenging activity, with 46 % and 44.5 % inhibition observed in the 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid and hydrogen peroxide assays, respectively. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on L929 murine fibroblast cells showed a concentration-dependent decrease in cell viability, with cyanophycin granule polypeptide reducing viability to 81 % at 100 µM, indicating mild cytotoxicity at higher concentrations. These findings highlight *Nostoc* sp. as a sustainable source of bioactive cyanophycin granule polypeptide with strong antioxidant potential and limited cytotoxic effects, supporting its application in biomedical and biopolymer research.

Key words: Cyanophycin granule polypeptide, nitrogen limitation, antioxidant activity, L929 fibroblast cells, biopolymer

Cyanobacteria have significantly contributed to the production of atmospheric oxygen for about 2.4 billion years[1]. Their ability to thrive over geological time is due to several vital adaptations, including the use of Hydrogen sulphide (H2S) as an alternative electron donor in photosynthesis, survival in low-oxygen and sulphide-rich environments, and strong resistance to ultraviolet radiation. These features have ensured their long-term ecological success[2]. Cyanobacteria are responsible for nearly 20 %-30 % of the world's total primary photosynthetic production[3]. Described as blue-green algae, they are considered to be amongst the initial oxygenic photosynthesizes and are essential to photosynthesis in a range of environments. These microorganisms are widely distributed in both aquatic and terrestrial environments and can exist as single cells, colonies, or filamentous forms[4,5].

Although microscopic, these organisms become visible when they aggregate into colonies, often forming crust-like layers or water blooms[6]. Because of their efficient photosynthetic system and minimal requirement for light, water, carbon dioxide, and trace nutrients, they may grow rapidly[7]. However, any

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limitation in these elements can significantly affect both biomass yield and biochemical composition.

To enhance the production of valuable compounds such as pigments, lipids, or carbohydrates, microalgae are often cultured under stress conditions[8]. One such substance is the intracellular Cyanophycin Granule Polypeptide (CGP) that acts as a form of energy, carbon, and nitrogen storage[9]. Remarkably, these microorganisms can enzymatically fix atmospheric nitrogen into organic molecules under ambient conditions[9]. This ability gives a nitrogen-rich input to ecosystems, thereby boosting their overall productivity[10].

Cyanophycin granules are intracellular inclusions commonly found in many cyanobacteria and consist of a compound known as CGP, or cyanophycin[11]. This polymer is synthesized non-ribosomally and is chemically identified as multi-L-arginyl-poly-L-aspartate. It is found in several heterotrophic bacteria in addition to cyanobacteria, where it acts as a transient storage molecule for energy, carbon, and nitrogen[12]. Structurally, cyanophycin has a backbone composed of aspartic acid with arginine residues forming the side chains. These two amino acids typically occur in nearly equal proportions[13].

During the night, when photosynthesis is inactive, cyanophycin plays a key role in protecting the nitrogenise enzyme from oxygen-induced damage during nitrogen fixation. With the return of daylight, cyanophycin is degraded to release the fixed nitrogen, leading to the nitrogen fixation's termination[14]. Serving as a nitrogen reserve, cyanophycin adapts to environmental conditions to support algal metabolism under nitrogen-limited conditions. The stored nitrogen can be redirected toward the synthesis of essential enzymes and other macromolecules[15]. In nitrogen-deficient substrates, it is notable that many microalga species can divide for several days as their metabolic pathways adapt and rebalance[16].

Despite its biological importance and industrial potential, detailed structural characterization of CGP has been challenging due to its insolubility and heterogeneity. In this context, Nuclear Magnetic Resonance (NMR) spectroscopy has appeared as a valued device for investigating the molecular structure and dynamics of CGP. Both solution-state and solid-state NMR techniques have been employed to elucidate the secondary structure, hydrogen bonding patterns, and side-chain interactions of CGP polymers[17]. These insights are essential for understanding the physicochemical behaviour of CGP *in vivo* and for engineering derivatives with improved solubility functional properties.

With β-sheet-like conformations in the poly aspartate backbone and limited mobility in the arginine side chains, recent solid-state NMR studies have shown that CGP adopts a largely organized structure[18]. These discoveries have given insight into how cyanobacteria aggregate and form granules. Moreover, advances in high-field NMR apparatus and isotopic labelling have further enhanced spectrum resolution, enabling the assignment of specific residues and improved understanding of interactions between molecules.

In this study, CGP was extracted and characterized from *Nostoc* sp. with a focus on enhancing its accumulation under nutrient-stress conditions. Cultivation in nitrogen-depleted BG-11 medium promoted CGP biosynthesis, indicating that nitrogen limitation acts as a regulatory signal for polymer production in cyanobacteria. The extracted CGP was structurally analysed using Fourier Transform Infrared (FTIR) spectroscopy, confirming characteristic functional groups associated with its amino acid composition. Chemical purity and retention behaviour were subsequently evaluated using reverse-phase High-Performance Liquid Chromatography (HPLC), confirming the sample's suitability for biochemical applications. Further molecular characterization was conducted through proton ^{1}H NMR spectroscopy, which verified the presence of constituent residues and provided insights into the structural organization of the polymer. In addition to structural validation, CGP was evaluated for its antioxidant activity using 2, 2′-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) and Hydrogen peroxide (H_2O_2) radical scavenging assays, demonstrating effective free radical neutralization in both assays. Cytotoxicity assessment using L929 murine fibroblast cells revealed optimistic biocompatibility at lower concentrations, with a concentration-dependent response observed at higher levels. These results highlight the potential of CGP derived from *Nostoc* sp. as a bioactive compound suitable for applications in biomedical and sustainable polymer research

MATERIALS AND METHOD

Strain source:

The cyanobacterial strain *Nostoc* sp. BDU 10241

was obtained from the National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli, India. We sincerely acknowledge NFMC for providing this strain.

Culture conditions:

The *Nostoc* sp. strain was grown in BG-11 medium following the procedure described by Rippka *et al*[19]. For nitrogen-limited cultivation, Sodium nitrate ($NaNO_3$) was excluded from the medium. Cultures were maintained under a 12 h light and 12 h dark cycle with an illumination of 1500 lux. Prior to inoculation, all glassware was properly cleaned, and the medium was sterilized at 121° under 15 psi pressure for 30 min. The pH was set to 7.5±1 to facilitate optimal growth.

Chlorophyll-a content measurement:

Chlorophyll-a content was used as a measure of cyanobacterial growth. A 10 ml culture was centrifuged at 8000 rpm for 10 min, and the pellet was extracted with 5 ml of 80 % acetone at 4° overnight. After centrifugation, absorbance was measured at 663 nm using a Ultra Violet (UV) spectrophotometer, following the method of Arnon[20].

Determination of total protein content:

Total protein concentration was determined using the Bradford assay[21]. A 0.1 ml sample was diluted to 1 ml, mixed with 1 ml of Coomassie Brilliant Blue G-250 reagent, and incubated for 10-15 min. Absorbance was measured at 595 nm using a UV-Visible spectrophotometer, with Bovine Serum Albumin (BSA) used as the standard.

Isolation and purification of CGP:

Cyanobacterial biomass was collected by centrifugation at 8000 rpm for 15 min at 4° and resuspended in BG-11 medium. Cell disruption was carried out by sonication[22], followed by centrifugation (8000 rpm, 15 min) to clarify the lysate and collect the pellet. For CGP purification, the pellet was sequentially washed with 2 % (v/v) Triton X-100 to remove lipids and with distilled water (three times) to eliminate residual contaminants. The pellet was then treated with 0.1 N HCl to solubilize non-CGP proteins[23]. After centrifugation (8000 rpm, 15 min), the supernatant containing CGP was neutralized to pH 7-8 using 1 N Sodium hydroxide (NaOH), dialyzed against distilled water[18], and lyophilized for storage at -20°. Arginine content was quantified using the Sakaguchi assay by measuring absorbance at 520 nm[24].

FTIR analysis of CGP:

FTIR spectroscopy was performed to identify the functional groups present in CGP extracted from *Nostoc* sp. The analysis was performed using a PERKIN ELMER spectrum One spectrophotometer fitted with a universal Attenuated Total Reflection (ATR) accessory. The UV spectra were recorded in the range of 4000-400 cm^{-1}, averaging 8 scans at a resolution of 4 cm^{-1}. Background spectra were collected before each measurement, and the sample spectra were processed using IR Expert software[25]. The presence of amide I and amide II bands, along with other characteristic peaks, was analysed to confirm the structural integrity and chemical composition of CGP[26].

HPLC analysis of CGP:

HPLC analysis of purified CGP was performed using an RP-HPLC system (Shimadzu) equipped with lab solutions software. The instrument was coupled with a reverse-phase C18 column (250×4.6 mm, 5 µm; e.g., Agilent Zorbax SB-C18). Sample preparation involved dissolving CGP in 0.1 N HCl, followed by filtration through a 0.22 µm membrane syringe filter. A 20 µl aliquot was injected via auto sampler. Separation was achieved using a gradient mobile phase consisting of: (A) 0.1 % (v/v) Trifluoroacetic Acid (TFA) in water and (B) 0.1 % (v/v) TFA in acetonitrile, at a flow rate of 1 ml/min. The gradient program was: 5 % B (0-2 min), 5 %-80 % B (2-25 min), and 80 % B (25-30 min). CGP detection was performed at 210 nm[23,27].

^{1}H NMR analysis of CGP:

^{1}H NMR analysis of CGP was performed using a Bruker AVNeo 500 MHz spectrometer (Bruker BioSpin GmbH, Germany). The CGP sample in 0.1 N HCl, and spectra were acquired at 302.2 K. The following acquisition parameters were used: 64 scans, 10 000 Hz spectral width, and 1.638 s acquisition time. Free Induction Decay (FID) data were collected by 32 768 points (TD), zero-filled to 65 536 (SI), and processed using an exponential window function with 0.3 Hz line broadening. All spectra were analysed using Top Spin software (v4.2.0, Bruker). Chemical shift assignments were determined by comparison with published values for CGP and its constituent amino acids[28,29].

***In vitro* Study on CGP:**

Antioxidant activity: The antioxidant potential of CGP was evaluated using ABTS and H_2O_2 radical scavenging assays. The ABTS assay was performed following the method of Arnao *et al*.[30]. with slight modifications. The ABTS radical species was formed by reacting 7 mm ABTS with 2.4 mm potassium persulfate, followed by incubation in the dark for 14 h. The resulting solution was diluted with Phosphate-Buffered Saline (PBS) to achieve an absorbance of 0.706±0.01 at 734 nm. CGP samples were then mixed with 1 ml of this ABTS solution, and the absorbance was measured at 734 nm after 7 min. The radical scavenging activity was calculated using the formula:

((Abs control-Abs sample)/Abs control)×100

Where, Abs control and Abs sample refer to the absorbance of the control and the sample, respectively. Ascorbic acid was used as a reference standard.

For the H_2O_2 scavenging assay, the method described by Ruch *et al*.[31], was followed. Different concentrations of CGP were prepared in 50 mm phosphate buffer (pH 7.4) to a final volume of 0.4 ml and mixed with 0.6 ml of 2 mm H_2O_2 solution. After vertexing, the reaction mixture was incubated for 10 min, and absorbance was recorded at 230 nm. Phosphate buffer served as the blank, and ascorbic acid was used as the standard. The percentage of H_2O_2 scavenging activity was calculated using the same formula as above.

Cell culture and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cytotoxicity assay: Cytotoxicity of CGP extracted from *Nostoc* sp. was assessed using the MTT assay on L929 murine fibroblast cells, as described by Mosmann[32]. The CGP sample was dissolved in 0.1N HCl, and serial two-fold dilutions were prepared over a concentration range of 0 to 100 µM. A parallel treatment group using 0.1N HCl alone was also included to assess the solvent's effect independently. L929 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium supplemented with 10 % inactivated Fetal Bovine Serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37° in a humidified 5 % Corbon dioxide (CO_2) atmosphere. Cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated for 24 h. Following this, cells were treated with either CGP in 0.1N HCl or 0.1N HCl alone for another 24 h. After incubation, 100 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for 4 h. Formazan crystals formed by viable cells were dissolved in 100 µl Dimethyl Sulfoxide (DMSO), and absorbance was taken at 590 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells. The IC_{50} values were determined using nonlinear regression analysis with Origin software[33].

RESULTS AND DISCUSSION

In the current research, *Nostoc* sp. was grown on BG-11 medium in Nitrogen-sufficient ($NaNO_3$) and nitrogen-free (without $NaNO_3$) conditions to compare growth performance and CGP yield. Microscopic observation of cells exhibited typical morphological features including uniseriate trichomes in mucilaginous sheaths (fig. 1), which are typical *Nostoc* sp., morphological features.

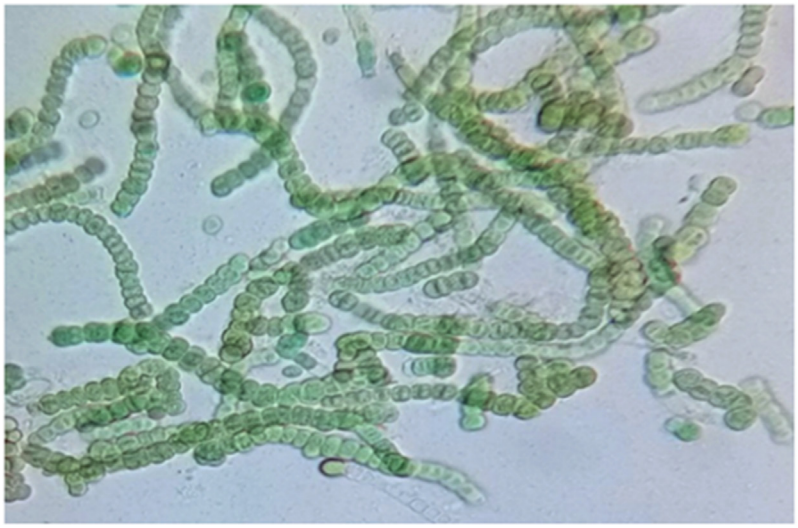

Fig. 1: Microscopic image of *Nostoc* sp. showing filamentous structure with rounded cells and heterocyst

Growth was also observed with chlorophyll-a content, used as an indicator of growth. As shown in fig. 2a, chlorophyll-a was maximum on d 24, reaching 2.3 µg/ml in cultures supplemented with nitrogen and 1.7 µg/ml in nitrogen-deprived cultures, reflecting decreased growth upon nitrogen limitation. The pattern was the same in the case of total protein content, which was maximum at 27.5 µg/ml in control cultures and 19.3 µg/ml under nitrogen deprivation on d 20 (fig. 2b).

Significantly, CGP production was highly stimulated under nitrogen-deprived conditions. As revealed in fig. 2c, CGP reached a maximum of 7.5 % of Cell Dry Mass (CDM) on d 20 during nitrogen-starved cultures *vs.* 4.8 % under standard conditions. These findings assure that *Nostoc* sp. under nitrogen-limitation conditions adapts to reorganize metabolic flux for producing CGP, a nitrogen-storage biopolymer. This is consistent with earlier reports of heightened reserve polymer formation under nutrient stress[34,35].

FTIR spectroscopy was performed to determine the functional groups in *Nostoc* sp. biomass and the CGP extracted from it. The FTIR spectra of (a) *Nostoc* sp. biomass and (b) the isolated CGP are indicated in fig. 3, revealing prominent absorption peaks. The CGP spectrum showed typical peaks related to O-H and N-H stretching vibrations (3436-3417 cm^{-1}), C–H stretching (2923.72 and 2426.54 cm^{-1}), and strong amide I (1654 cm^{-1}) and amide II (1544 cm^{-1}) bands, which are characteristic of peptide and protein folds[36,37]. Extra peaks for C-N stretching and alkyne groups also confirm the existence of amino acid residues, especially arginine and aspartic acid. These results validate the presence of amino acid constituents, i.e., arginine and aspartic acid, reflecting the proteinaceous nature of the isolated CGP[27,38].

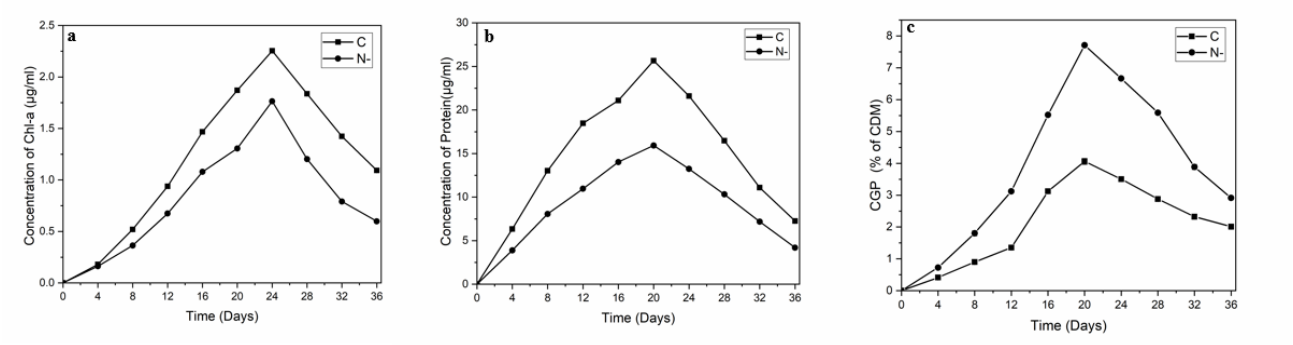

Fig. 2: Growth and CGP production in *Nostoc* sp. under control (C) and nitrogen deprived (N-) conditions, (a): Chlorophyll-a concentration (µg/ml); (b): Protein content (µg/ml) and (c): CGP content (% of CDM) over 36 d
Note: (■): C and (●): N-

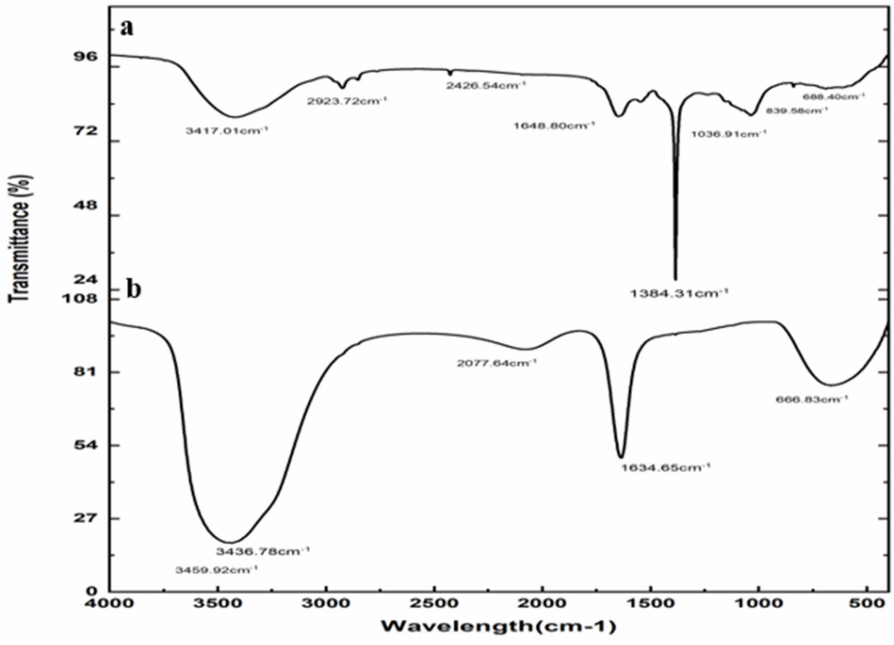

Fig. 3: FTIR spectra of (a) *Nostoc* sp. Biomass and (b) purified CGP. Distinct peaks indicate the biochemical differences between the crude biomass and the extracted polymer

RP-HPLC of the CGP showed a major symmetrical peak at 5.664 min, which represented 99.78 % of the peak area (fig. 4). Two small peaks were present at 14.360 min (0.084 %) and 24.491 min (0.132 %), which can be attributed to the remaining impurities or buffer components. The thin, symmetrical shape of the principal peak and the flat baseline suggest high sample purity, low degradation, and efficient extraction with no column overloading Such retention properties are in line with those earlier HPLC traces previously described for cyanophycin[39,27]. The level of purity (>99.7 %) found in this work is well beyond the levels generally obtained from crude extracts[27], testifying to the efficient isolation of CGP with minimal contamination. This outcome is an appearance of the dependability and effectiveness of the purification technique employed in this examination.

The ^{1}H NMR spectrum of the purified CGP confirmed its structure as a poly (L-aspartic acid-co-L-arginine) copolymer (fig. 5). Aliphatic proton signals were observed between 1.00 and 1.95 ppm, corresponding to the β- and γ-methylene groups of both amino acids. The α-methylene protons of aspartic acid appeared at 2.56-2.94 ppm, showing characteristic downfield shifts due to deshielding by the adjacent carboxylate group. The δ-methylene protons of arginine resonated at 2.70-2.87 ppm. Broad peaks in the 3.26-4.78 ppm range were attributed to the backbone α-protons and side-Chain Methine (CH) groups, with peak broadening indicative of the polymeric nature and polydispersity. These spectral features are consistent with previously reported ^{1}H NMR data for cyanophycin isolated from *Synechocystis* sp. PCC 6803 and Anabaena sp.[22,23], confirming the presence of the β-Asp-Arg repeating unit. Thus, ^{1}H NMR provided detailed insights into the monomeric composition and structural organization of CGP synthesized under the specified culture conditions.

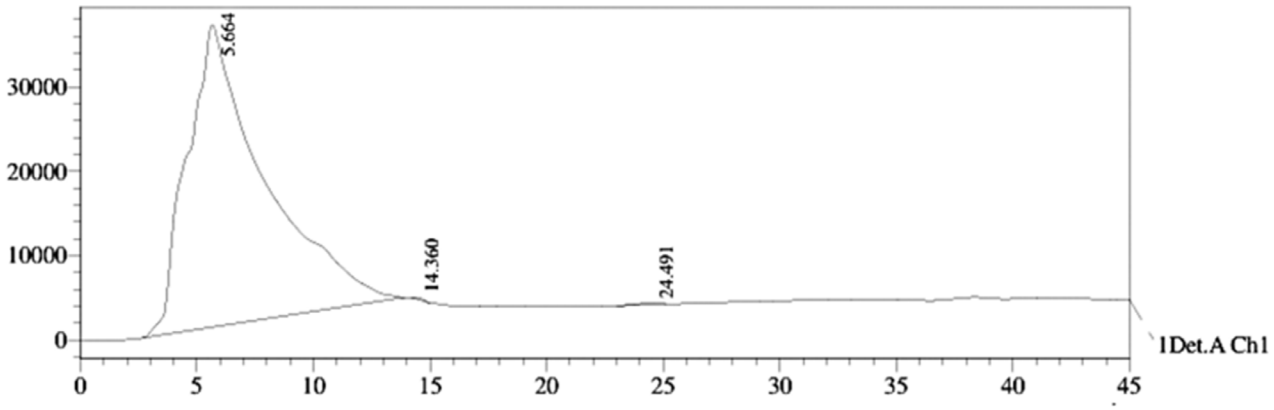

Fig. 4: HPLC chromatogram of CGP showing distinct peaks representing its main amino acid components

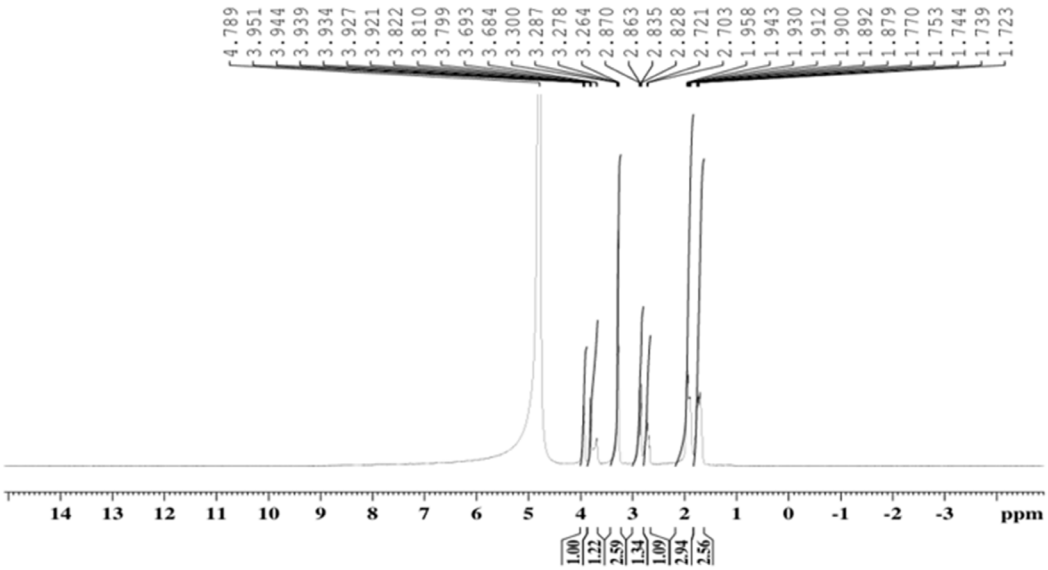

Fig. 5: Proton NMR spectrum of CGP showing characteristic chemical shifts corresponding to aspartic acid and arginine residues

The antioxidant activity of CGP was assessed through the ABTS radical scavenging assay. CGP had a concentration-dependent scavenging effect between 20-120 µg/ml, and an inhibition of 46 % was recorded at 120 µg/ml (fig. 6a). Though the activity was lower than ascorbic acid at corresponding concentrations, the finding shows moderate antioxidant activity. The ABTS assay, reliant on quenching of ABTS radicals formed upon reaction of ABTS with potassium persulfate, is used extensively to evaluate the antioxidant activity of peptides and proteins[40,41]. The activity reported here for CGP is in agreement with existing reports on the radical-scavenging activity of bioactive peptides from microalgae.

CGP also demonstrated concentration-dependent scavenging activity towards H_2O_2, with a maximum inhibition of 44.5 % at 120 µg/ml (fig. 6b). This also attests to its antioxidant ability. The capacity of CGP for the scavenging of H_2O_2 could be due to its amino acid content, especially residues like arginine and aspartic acid, which are documented to participate in free radical scavenging[42]. These results are consistent with previous reports of the antioxidant activities of bioactive peptides isolated from cyanobacterial sources[43].

Cytotoxicity of CGP was tested with the MTT assay in L929 fibroblasts. Solvent control with 0.1N HCl did not influence cell viability, which was always above 94 % and within the range of 0-100 µg/ml concentration. This is in agreement with earlier reports showing that mildly acidic conditions have minimal effect on fibroblast viability[44]. IC_{50} for 0.1N HCl was greater than the highest concentration tested, thus confirming the lack of solvent cytotoxicity.

However, the viability of CGP-treated cells decreased in a concentration-dependent manner, going from 100 % at 0 µg/ml to 81 % at 100 µg/ml. The decrease suggests mild cytotoxicity at higher concentrations, even though the CGP IC_{50} value was not reached at the tested concentration (>100 µg/ml). The outcomes are consistent with those obtained with the modified MTT assay, which was preceded by the reduction of tetrazolium dye[33]. Microscopic analysis also validated these results: While cells treated with CGP showed slight morphological changes characteristic of early cellular stress, cells treated with 0.1N HCl had normal morphology similar to the untreated control.

Overall, the findings show that L929 fibroblast cells tolerate CGP well and that moderate concentrations of the protein do not significantly harm them. The lack of significant morphological damage and the slight reduction in viability indicate that CGP has good biocompatibility. These results bolster the potential use of CGP in biomedical domains where low cytotoxicity and functional bioactivity are crucial, such as drug delivery systems, wound healing scaffolds, and antioxidant biomaterials (fig. 7 and fig. 8).

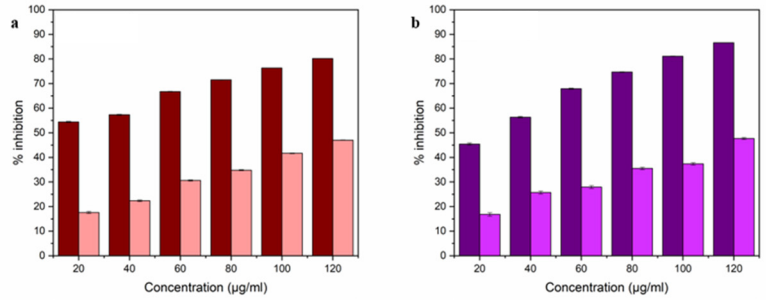

Fig. 6: Antioxidant activity of CGP from *Nostoc* sp. Evaluated by (a) ABTS and (b) H_2O_2 scavenging assays
Note: (a): (■): Ascorbic acid and (■): CGP and (b): (■): Ascorbic acid and (■): CGP

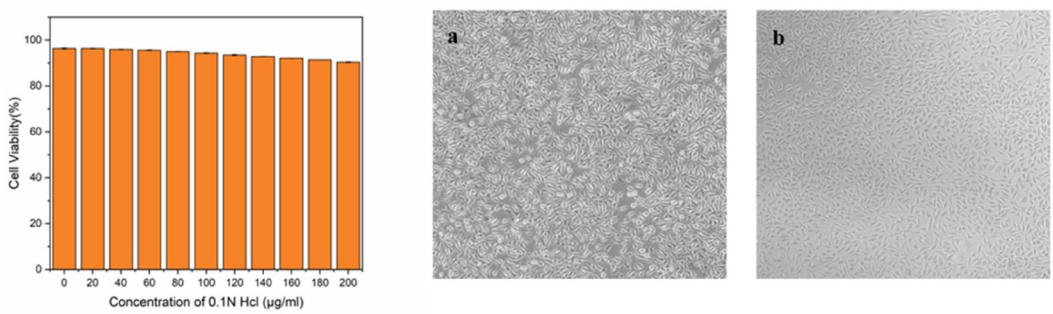

Fig. 7: Inhibitory effect of 0.1N HCl on L929 cells. (Left) cell viability remained above 90 %. (Right) Morphology of control (a) and (b) showed no major changes

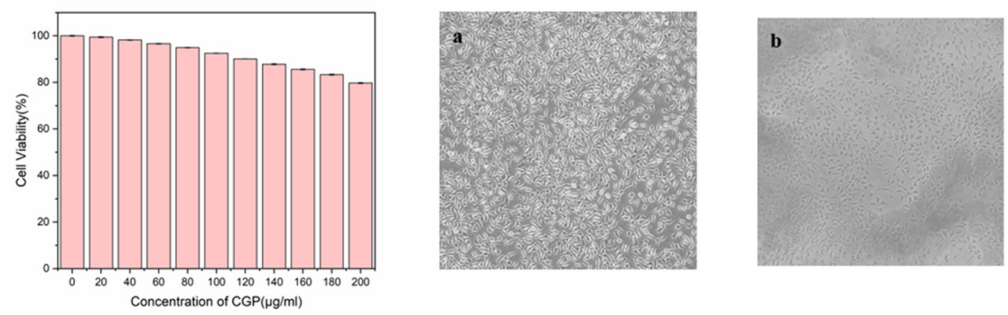

Fig. 8: Inhibitory effect of CGP on L929 cells. (Left) cell viability decreased with increasing concentration of CGP. (Right) Morphology of control (a) and treated cells (b) showed concentration dependent changes

This study highlights the extraction and purification of CGP from *Nostoc* sp. cultured under nitrogen-deprived conditions. Spectroscopic and chromatographic analyses confirmed the structural integrity and purity of the isolated polymer. Functional evaluations showed that CGP possesses moderate antioxidant activity and low cytotoxicity toward L929 fibroblast cells, supporting its potential as a bioactive and biocompatible compound. These findings suggest that purified CGP from cyanobacteria may have valuable applications in biomedical and antioxidant-related research. Further research is essential to explore its performance in advanced biological systems and material formulations.

Conflict of interests

The authors declared no conflict of interests.

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