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Improvement of a Novel Purification Method of Phycocyanin Pigment from the Microalga *Nostoc Minutum* and Evaluation of Its Anticancer Activity

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ABSTRACT

The efforts to discover anti-cancer drugs from natural sources to treat different types of cancer are of great importance and have received wide attention in recent years. Nowadays, microalgae are a unique source of bioactive compounds used in pharmaceuticals. In this study, the novel Iraqi blue-green alga, *Nostoc minutum* HA-YJ was identified using molecular and morphological diagnostics. DNA was recognized by the PCR technique and registered in the National Center for Biotechnology Information as a genus found in the aquatic environment north of Nineveh (Ain Safni). C-phycocyanin pigment from *Nostoc minutum* was purified using a novel method comprising freezing-thawing, ultrasonication, and ammonium sulfate precipitation, resulting in a purity index of 3.01. After that, dialysis, gel filtration chromatography, and SDS-PAGE electrophoresis were achieved. Two bands were observed corresponding to α (33 kDa) and β (36 kDa) subunits. The anticancer activity of purified PC was investigated using the human breast (MCF-7) and colon (HRT-18) cancer cell lines via MTT assay. The results exhibited a decrease in the percentage of cell viability of both cells in a concentration-dependent manner ranging from 31.25- 1000µg/ mL at 72h. The morphological changes in the cells associated with treatment by 1000µg/ mL were observed under light microscopy.

INTRODUCTION

Microalgae are a diverse kind of aquatic photosynthetic microorganisms that can grow in many environments **(Al-Khafaji *et al*., 2025)**. Cyanobacteria are prokaryotes with photosynthetic functions that use secondary pigments to convert sunlight into energy. They are naturally grown in aquatic environments such as rivers, ponds, streams, and springs **(Jassim & Alghanmi, 2023; Younus *et al*., *al*., 2025)**. Cyanobacteria are famous for accumulating various bioactive compounds and pigments. Particularly, C-Phycocyanin (PC) is a natural blue water-soluble pigment originating in cyanobacteria **(Taqi *et al*., 2024)**. PC has been used as a nutritional component **(Belay *et al*., 1993)**, a food coloring agent, in addition to being utilized in pharmaceuticals **(Asaduzzaman *et***

***al.*, 2024)**. Furthermore, it functions as an antioxidant **(Al-Abbasy *et al.*, 2020)**, with anticancer and anti-inflammatory properties **(Ziyaei *et al.*, 2023)**.

Cancer is now one of the most common harmful health issues in the world, with major social and financial ramifications. One out of every seven deaths worldwide is currently caused by cancer **(Shi, 2018; Ramezani *et al.*, 2023)**. According to a 2020 study by the IARC, the number of cancer diagnoses is increasing and the disease is leading to a considerable number of fatalities among cancer patients. Due to the rising rates of smoking, poor eating habits, and physical inactivity, these instances are predicted to rise in the upcoming years **(Bray *et al.*, 2018)**. Cancer is still a serious health issue despite the healthcare industry's quick advancements. Surgery, radiation, and chemotherapy are used to treat cancer **(Meiyanto *et al.*, 2012)**. Though chemotherapy is a successful cancer treatment, the development of safe, affordable, innovative, and more effective anticancer drugs is still urgently needed **(Sheikh *et al.*, 2018)**. Patients are severely harmed by the anticancer drugs employed in the treatment. Numerous anticancer drugs' toxicity and effectiveness have been examined using various cancer cell lines and cell culture settings **(Choudhari *et al.*, 2020)**.

Biologically active groups found in natural substances are extremely important and are crucial in the development of novel medications **(Calcabrini *et al.*, 2017, Rashan *et al.*, 2023)**. Nowadays, there is a fairly large percentage of medications with anticancer potential that come from natural sources **(Ratovitski., 2017)**. Microalgae species possessing anticancer activities have been the subject of numerous studies **(Martínez *et al.*, 2018)**. In a previous study, the methanolic extract of *Euglena tuba* was shown to have anticancer properties against Dalton lymphoma cells. This effect is due to the chemical profile present in this alga **(Gupta *et al.*, 2024)**. The anticancer activity of the methanolic extract of the microalga *Nannochloropsis oculata* was investigated *in vitro*. In a concentration- and time-dependent manner (400µg/ mL at 72h.), the anticancer activity demonstrated a reduction in the MDA-MB-231 cells' percentage of cell viability **(Wali *et al.*, 2020)**. However, *Nostoc minutum*'s anticancer properties or bioactive components have not been explored. Therefore, this research aimed to develop novel methods for purifying the C-phycocyanin from *Nostoc minutum* and to characterize its chemical properties. Furthermore, the anticancer activity was evaluated by *in vitro* assays.

MATERIALS AND METHODS

1. Collection of algae samples

The samples were collected using a Phytoplankton net to obtain a different type of blue-green algae (*Nostoc minutum*) from various places of the river edge and running water rivers in northern Iraq (Ain Sifni). These samples were placed in sterile glass bottles and were subsequently transferred to the laboratory and subjected to study.

2. Algae cultivation and reproduction

The culture of *Nostoc minutum* was performed in BG-11 medium at pH 8.0 without adding nitrogen sources. Then, it was placed in a conical flask in a shaking incubator at 27±2°C, supporetd with aeration and under the illumination of natural light intensity of 2500 Lux and photoperiod 12/12h.

3. Morphological and molecular diagnosis

Morphological diagnosis aims to ascertain the genus. A light microscope (Olympus mod. IX71; Olympus Corporation, Tokyo) with a coupled digital camera was used to examine the sample. Photomicrographs were taken for morphometric determination in the exponential phase of growth. Taxonomic identification was based on morphological characteristics in the 'classical' taxonomic revisions **(Desikachary, 1959)** and more recent taxonomic revisions **(Komárek, 2010)**. While, molecular diagnosis was implemented to ascertain the genus purity, determining DNA extraction and PCR amplifications after the genomic sequence of the 16sr RNA unit of cyanobacteria **(Moreno *et al*., 1995)**. The DNA was extracted based on the synthesis steps in the kit prepared by the Taiwanese company (Geneaid), according to the method of diagnozing the negative and positive bacteria of Gram dye.

4. Polymerase chain reaction (PCR) technique

The strain of *Nostoc minutum* 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. The sequencing of PCR primer used in this study

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

The total PCR mixture was 20µl in each tube containing genomic DNA, 40 pmol of forward and reverse primers, and 12.5µl of Green Master Mix. The thermal cyclic condition is shown in Table (2) **(Moreno *et al*., 1995)**. The sizes of amplified products were compared with the 1250 bp DNA ladder to determine the exact size of this gene. Purified PCR products were prepared and sequenced in Macrogen (Korean Biotechnology Company, Korea). The sequence was then compared to other sequences using the National Center for Biotechnology Information (NCBI) Genbank library.

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

Step	Function	Temperature °C	Cycles	Time	Stages
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. Purification of C- phycocyanin

Numerous techniques were used to extract C-Phycocyanin from fresh *Nostoc minutum* biomass. According to **İlter *et al.* (2018)**, the cells were collected using a centrifuge at a speed of 4500 rpm for 5 minutes to obtain a precipitate and thus collecting the largest biomass. Biomass was originally lysed by freezing at -20°C for 48 hours, followed by thawing at ambient temperature for 24 hours, promoting cell permeability. The cells were then further ruptured by ultrasonication at 24,000 pulses/15s and were centrifuged at 4,500 rpm for 10min to obtain supernatant. Ammonium sulfate was used to precipitate the dye using different concentrations of 30, 40, 50, 60, and 70% **(Pankaj *et al.*, 2010; Alrushdi *et al*., 2025)**. Each precipitate was individually dissolved in 3ml of Tris-HCl buffer pH=7.8. After extraction, the extract's pH was brought down to 5.5 to confirm that it retained its stability and native blue color, and it was stored at -20°.

6. Dialysis and gel filtration

The precipitate from adding 70% ammonium sulfate was dialyzed according to the method detailed in the study of **Rashan and Al-abbasy (2021)**. The dialyzed solution was loaded into the Sephadex G-50 gel column (1.8 x 120 cm). The separated fractions were monitored at 280nm, collected, and lyophilized. They were dissolved in a Tris-Hcl buffer (0.05 M, pH=7.8) and stored at $^{+}$2Co for 24 hours. Two layers were observed, the upper supernatant layer was taken and dried, which represents the purified C-PC.

7. The biochemical composition

The purity of PC during the extraction process is assessed using an absorbance ratio of A620/A280nm. A620 denotes the peak absorbance of PC, whereas A280 indicates the absorbance of total proteins **(Bennett & Bogorad, 1973)**.

Purity= A620／A280

The PC concentration was evaluated by measuring A620 and A650 following a methodology outlined by researchers **(Silveira *et al*., 2007)**.

PC Concentration (mg／mL) = A620 − (0.474 × A650)／5.34

8. Chemical characterization

The molecular mass of purified PC was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie brilliant blue was utilized

to stain the gel **(Laemmli, 1970)**. C-Phycocyanin subunit mobility was assessed using common protein markers with known molecular weights. The absorbance of the UV–Visible spectrum of the purified PC was monitored at a wavelength range of 200– 800nm using distilled water as a control. Additionally, FT-IR spectroscopy (Agilent Cary 630 FT-IR spectrometer) was analyzed with the range of 600– 4000cm-1 for determining the functional groups of C-Phycocyanin in the *Nostoc minutum* which are responsible for the pigment of alga synthesized **(Patel *et al*., 2005; Al-Burgus *et al*., 2024)**.

9. Cell culture

Two types of cancer cells were used in this study (human breast cancer cell line MCF-7 and colon cancer HRT-18 cells), which were obtained from the Barbara Ann Cancer Center / Detroit, Michigan, USA**.**

The breast cancer cells MCF-7 lines were grown in RPMI medium, while colon cancer HRT-18 cells were cultured in DMEM medium. Both culture mediums were accompanied by 10% heat-inactivated FBS, 1% streptomycin (100 µg/mL), and penicillin (100 U/mL). The cells were sustained and incubated in modified atmospheric conditions, which contained 5% CO2 at 37°C (ambient temperature) **(Safaei *et al*., 2019)**.

10. MTT Assay

The evaluation of cytotoxicity of the purified C-phycocyanin on breast cancer cells (MCF-7) and colon cancer cells (HRT-18) was determined by the MTT assay. Cells were seeded at a density of 3 ×104 cells in a 96-well microplate and were incubated at 37°C for 72h until monolayer confluence was attained. Cytotoxicity was investigated through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is reduced to a purple-blue precipitate of insoluble formazan. The cells were exposed to various concentrations (31.25, 62.5, 125, 250, 500, and 1000µg/ ml). After 72h of infection, MTT dye solution 28µL of 2mg/ ml was added to each well. The incubation lasted for three hours. A total of 100µl of DMSO was supplemented to each well and was incubated for 15min. The optical density (O.D.) was followed at 492nm using a microplate reader. Cytotoxicity% was calculated with the equation:

$$\text{Cytotoxicity\%} = (\text{O.D.}_{\text{Control}} - \text{O.D.}_{\text{Sample}}) / \text{O.D.}_{\text{Control}} \times 100.$$

Where, O.D. Control is the mean optical density of untreated wells and the O.D. is the sample's optical density of treated wells **(Salman, 2022)**.

11. Statistical analysis

Measurements were taken in triplicate, and findings were reported as means ± SD (n=3). Mean separation as well as significance were determined by applying the SPSS software suite. Excel was used to run correlation and regression analyses.

RESULTS AND DISCUSSION

1. Microscopic diagnosis

The cyanobacteria *c* was diagnosed for the first time using a light microscope, as it has a filamentous shape and consists of a series of spherical cells interspersed with larger and spherical cells, as shown in Fig. (1) and exists in the form of colonies. A gelatinous envelope surrounds the strands and multiplies in conditions perfect for the growth of dichotomy. It is present in a stagnant and running aquatic environment.

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

2. Molecular diagnosis DNA purification

After isolating the DNA genetic material and exposing the gel to ultraviolet radiation with a wavelength of 245nm, the light beams appeared of the DNA of the cyanobacteria *Nostoc minutum*, with equal dimensions and a large size (1150) bp DNA. The presence of the clear bands is a clear evidence for achieving the DNA extraction process from the sample, as shown in Fig. (2).

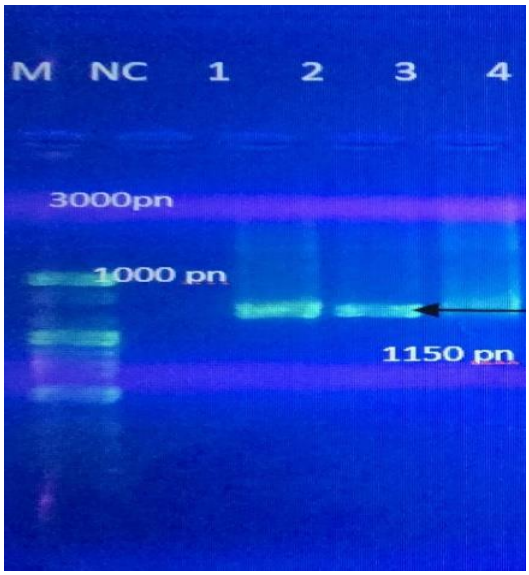

Fig. 2. DNA bands of cyanobacteria in gel electrophoresis

3. Polymerase chain reaction (PCR)

According to the results, the genomic DNA of *Nostoc minutum* was amplified successfully, a company Macrogen (Korea) sequenced 16S rRNA gene of *Nostoc minutum* DNA sequences and analyzed it, and similarity searches were carried out with the Basic Local Alignment Search Tool (BLAST) in NCBI website (http://www.ncbi.nlm.nih.gov). It showed that the sample belongs to *Nostoc minutum* according to the clarification of the sequence of isolation rules studied with the (KY283066.1) rate (100%). It showed that the sample belongs to the taxonomic tree (Fig. 3). The 16sr RNA gene sequence matches the isolate data of genus *Nostoc* sp. in the Genbank **(Ahmed *et al*., 2010)**.

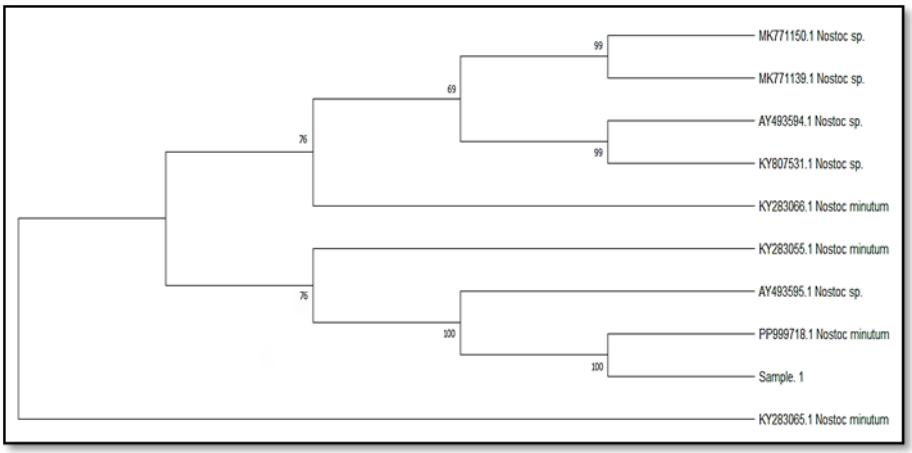

Fig. 3. Taxonomic tree for *Nostoc minutum*

4. Purification of C-Phycocyanin

Crude protein was extracted to purify C-Phycocyanin dye from *Nostoc minutum* by taking different saturation percentages of ammonium sulfate. It was observed that the recovery percentage of C-PC dye increased directly with increasing saturation degree and reached its maximum at 70%. The recovery value of C-PC is the concentration of 0.63mg/ ml, as shown in Table (3).

Table 3. Extraction of C-phycocyanin by ammonium sulfate fractionation (Values are mean of three independent observations)

Ammonium sulfate %	A_{650}	A_{620}	A_{680}	Purity =A620/A280	C-P(mg/ml)
0	0.09	0.28	0.122	0.951	0.17
30	0.11	0.33	0.223	1.479	0.32
40	0.14	0.43	0.272	1.581	0.41
50	0.15	0.45	0.287	1.567	0.43
60	0.16	0.47	0.291	1.615	0.45
70	0.21	0.65	0.211	3.01	0.63

A previous study indicated that the purity and concentration of C-PC were optimum when using 40% ammonium sulfate, with values of 66% and 0.64mg/ ml, respectively **(Thomson *et al.*, 2018)**. After purification by dialysis and gel chromatography, one peak of protein was revealed (Fig. 4). which contains C-PC pigments.

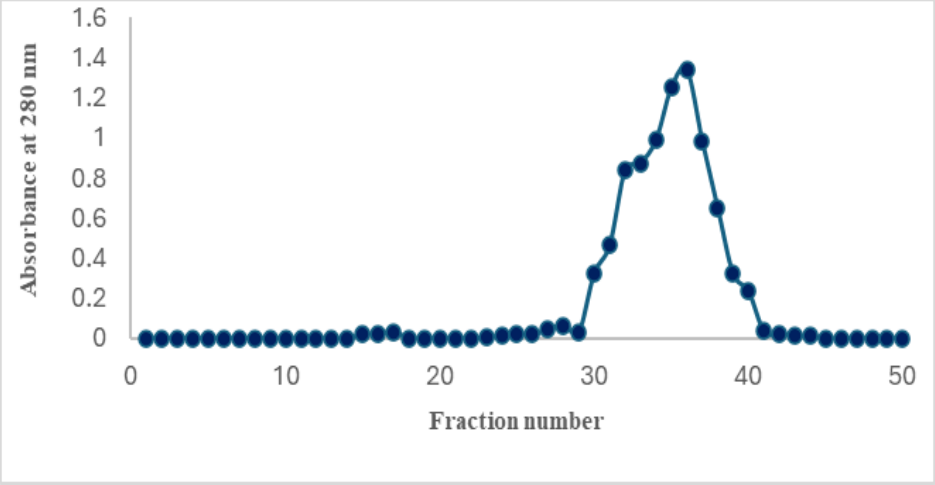

Fig. 4. Gel filteration chromatography using Sephadex G-50

The SDS-PAGE technique was used to determine the molecular mass of purified PC. After staining with Coomassie brilliant blue, two bands were detected, which corresponds to α and β subunits having a molecular mass equals to 33 and 36 kDa, respectively (Fig. 5). The similar result was before obtained in *Limnothrix* sp. NS01 **(Rimbau *et al.*, 2001; Safaei *et al.*, 2019)**. Using the same technique, two subunits (α and β) were also observed for the crude PC from *Spirulina platensis*, and their molecular weights were 17 and 20kDa, respectively **(Bougatef *et al.*, 2024)**.

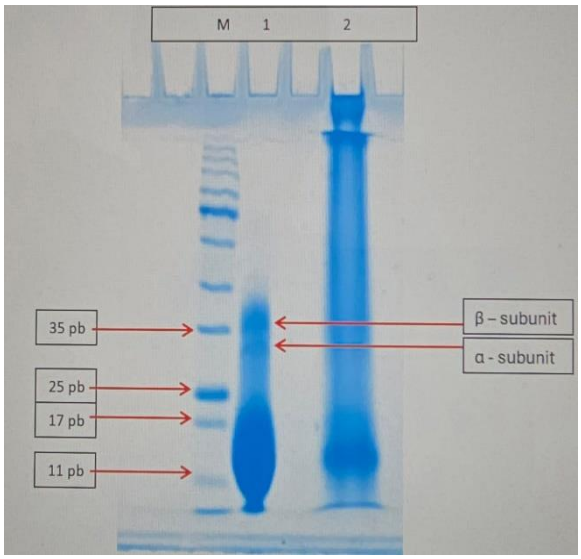

Fig. 5. SDS-PAGE profile of the crude protein from *Nostoc minutum*

The spectral characteristics of purified PC were analyzed at wavelengths ranging from 200 to 800nm. The spectrum indicated two distinct absorption peaks at 304 & 614nm, corresponding to the absorption of proteins (aromatic residues) and phycocyanin, respectively (Fig. 6). Two peaks were observed at 280 and 620nm, a finding which is consistent with similar studies **(Pankaj *et al.*, 2010)**. Comparable results were seen for PC extract from several cyanobacterial strains **(Moreira *et al.*, 2012)**.

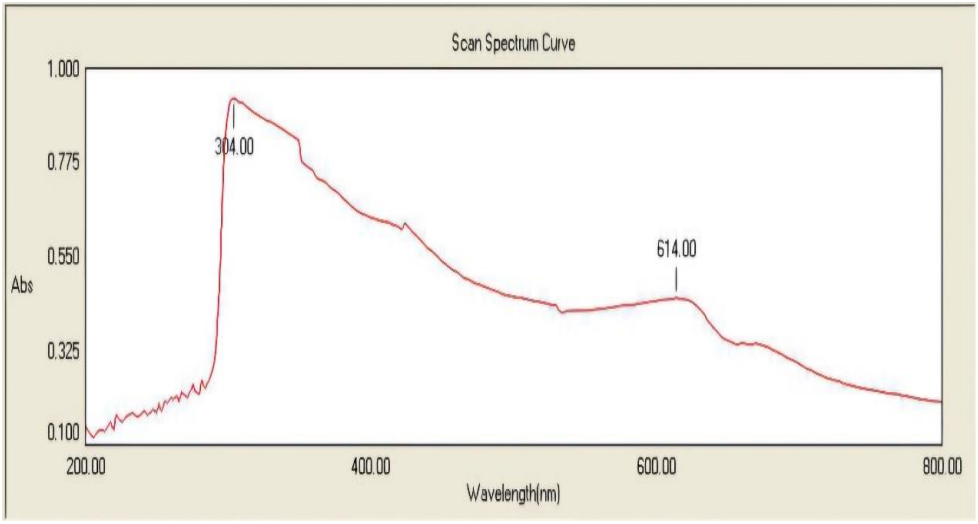

Fig. 6. UV-Vis spectra of proteine

Furthermore, the primary structural properties of PC, purified from Nostoc minutum were analyzed by FT-IR spectroscopy, as shown in Fig. (7). It is well known that, phycocyanin is a molecule characterized by a tetrapyrrole chain, consisting of 4 pyrrole rings, individually containing 4 carbon atoms, 5 hydrogen atoms, and 1 nitrogen atom (C4H5N). Moreover, C-Phycocyanin possesses an amide group. The FT-IR spectrum exhibited distinct characteristics, including an amide I band at 1623cm^{-1} (C=O stretching) and a primary aromatic amine (N−H stretching) at 3271cm^{-1}. An absorption band at 2922cm^{-1} signified the existence of carboxylic acid, whereas an amide II band was observed at 1519cm^{-1}. Similar to previous studies **(Tong *et al.*, 2020; Prabakaran *et al.*, 2020)**, the identified 9 functional groups were recorded to display unique bond stretching at different wavelengths in crude PC obtained from *S. platensis*.

5. Anticancer activity

The purpose of this portion was to assess the probable anticancer activity of purified C-phycocyanin using the MTT assay. Therefore, purified C-phycocyanin was established *in vitro* for the inhibition of cancer cell proliferation using MCF-7 and HRT-18. Experimentally, the concentration-dependent estimation of purified C-phycocyanin activity was evaluated. This was achieved by incubating the mentioned cancer cells with a series of different concentrations of C-phycocyanin ranging from 31.25- 1000µg/ mL for 72h. Our results indicate that C-phycocyanin was able to inhibit cell viability and survival in a concentration-dependent manner (*P< 0.01, ****P< 0.001). It was observed that the concentration of 1000µg/ ml significantly affected the proliferation of MCF-7 and HRT-18 cells, which resulted in an increase in cell toxicity by 69.64 and 71.11%, respectively (Figs. 8, 9).

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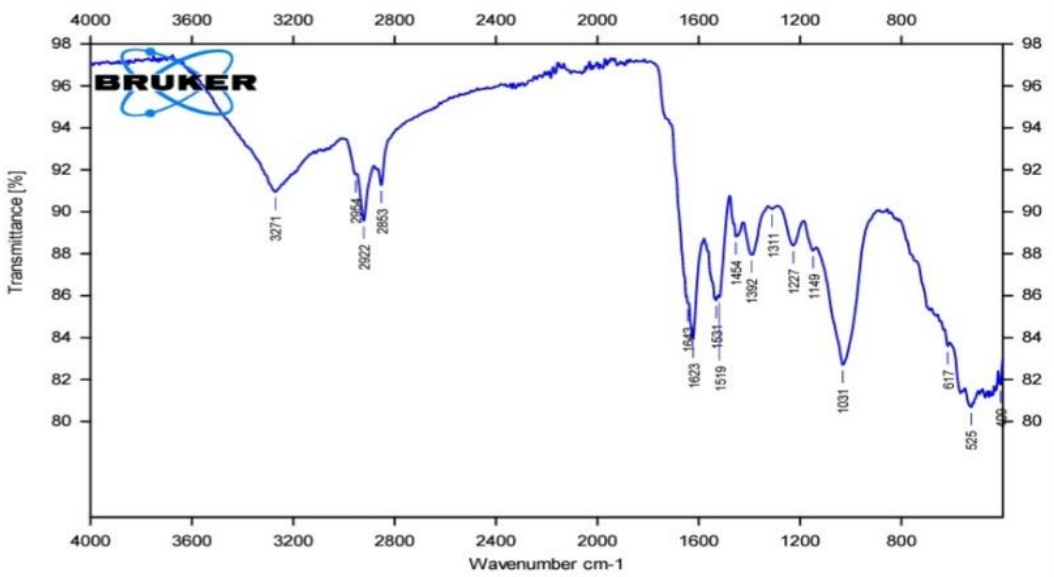

Fig. 7. Characterization of protein FT-IR spectrum

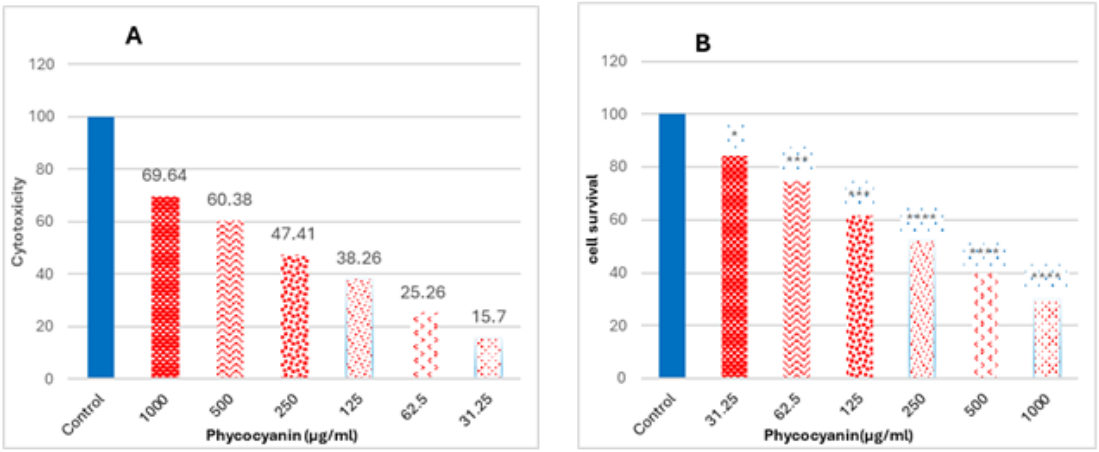

Fig. 8. The anticancer activity of purified c-phycocyanin on the growth of MCF-7 cells at different concentrations for 72h using MTT assay. (A) Cytotoxicity; (B) Cell survival. The results were expressed as cell viability (% of control). The values are demonstrated as mean ± SD (n = 3). Significant at (**P*< 0.01, ****P*< 0.001).

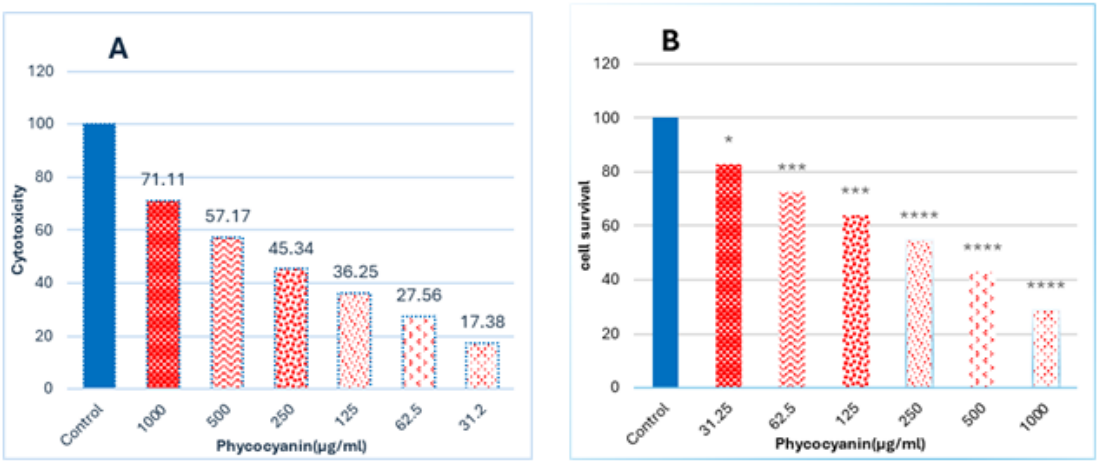

Fig. 9. The anticancer activity of purified c-phycocyanin on the growth of MCF-7 cells at different concentrations for 72h using MTT assay. (A) Cytotoxicity; (B) Cell survival. The results were expressed as cell viability (% of control). The values are demonstrated as mean ± SD (n=3). Significant at (**P*< 0.01, ****P*< 0.001).

Many studies have recently been published on the *in vitro* anticancer activity of certain microalgae species utilizing various cell lines, which is consistent with our findings. The anticancer efficacy of the hexane-derived extract for *Nannochloropsis oculata* microalga was assessed by using the MTT assay on a variety of cell lines, including HEP- 3B, HL- 60, SW- 480, and HCT- 18 **(Sanjeewa *et al.*, 2016)**. The researchers accomplished cytotoxicity and detected a considerable dose-dependent decrease in the viability of cancer cell lines. In another study, crude C-phycocyanin prepared from the cyanobacterium *Spirulina platensis* was recorded with a toxic effect on human breast cancer cells 7-MCF **(Bougatef *et al.*, 2024)**. The methanolic extract of the microalgal *Euglena tuba* exhibited significant and concentration-dependent cytotoxicity against Dalton's Lymphoma Cells **(Gupta *et al.*, 2022)**. In addition, we monitored the morphological changes of cells that might be associated with C-phycocyanin treatment purified from the species *Nostoc mintum*. At 37°C, the study showed that MCF-7 and HRT-18 cells, after being detected under a light microscope, showed a significant decline in cell number at the concentration of 1000µg/ ml compared to untreated cells (Figs. 10, 11).

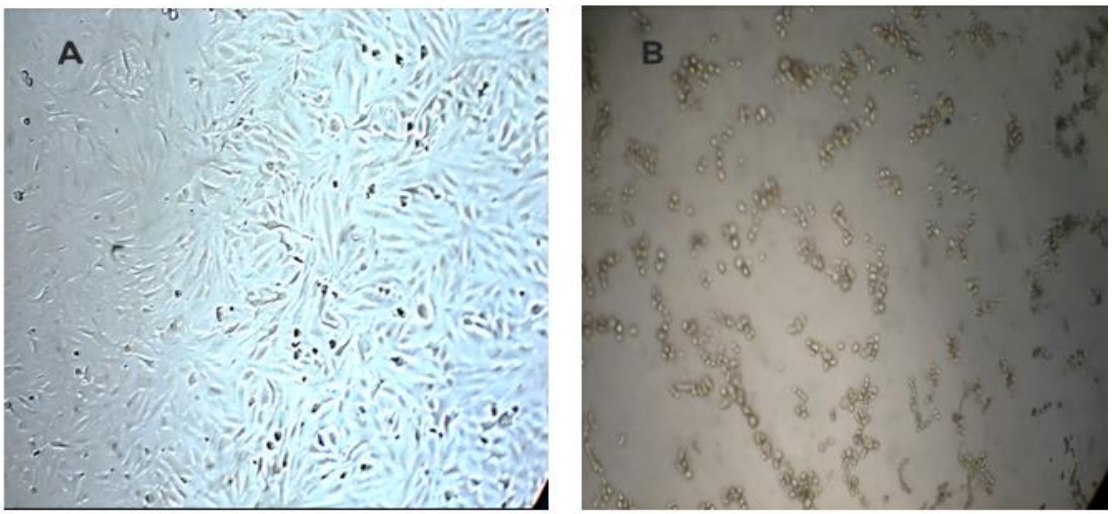

Fig. 10. Impact of purified PC on morphological changes of human breast cancer cells (MCF-7). After incubation at 37°C for 72h, the photomicrograph shows the effect of 1000µg/ ml of purified PC on MCF-7: (A) Untreated MCF-7; (B) Treated MCF-7

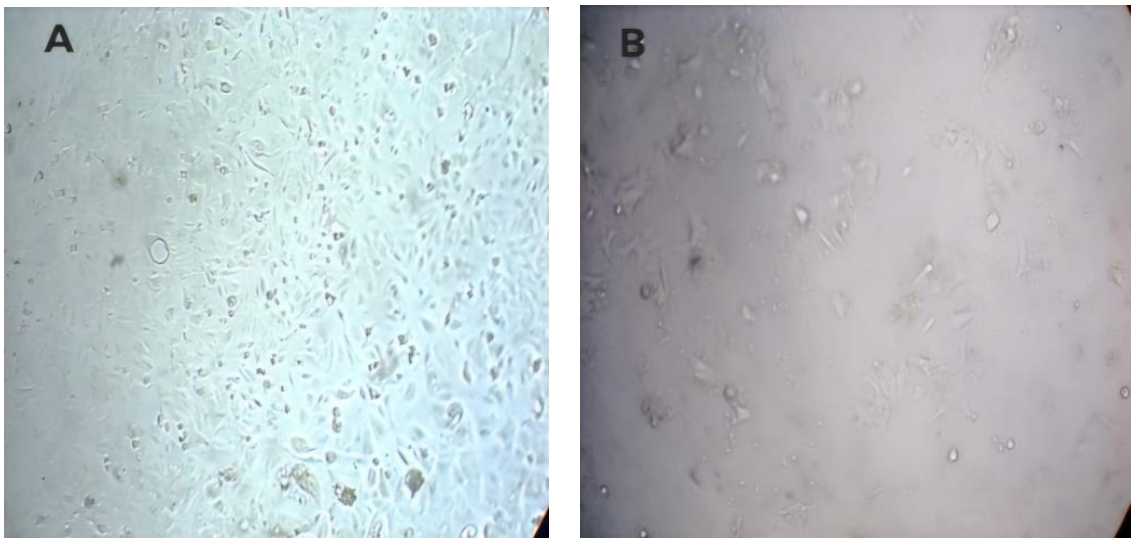

Fig. 11. Impact of purified PC on morphological changes of human colon cancer cells (HRT-18). After incubation at 37°C for 72h, the photomicrograph shows the effect of 1000µg/ ml of purified PC on HRT-18: (A) Untreated HRT-18; (B) Treated HRT-18

CONCLUSION

Several anticancer medicines are now accessible; nevertheless, the reaction to the therapy is weak and associated with significant negative effects. Unfortunately, cancer cells are becoming increasingly resistant to these medications. Considering the primary objective in cancer therapy is to eradicate cancerous cells with minimal harm to normal tissues, limiting the proliferation of these tumor-causing cells, using natural microbe

products might be a good technique for cancer treatment. Cyanobacteria are thought to have been among the earliest photosynthetic creatures to appear on Earth. They are prominent for accumulating numerous bioactive compounds such as C-phycocyanin, which has a massive range of biotechnological requests. In this study, PC was purified from the microalga *Nostoc minutum* via a novel extraction method terminated with gel filtration chromatography. Furthermore, an *in vitro* MTT test was used to assess their impact on proliferation inhibition, with a focus on lines of human cancer cells MCF-7 and HRT-18. The results we obtained revealed that pure PC exhibited substantial proliferation suppression in both kinds of cancer cell lines. It was concluded that the treatment of MCF-7 and HRT-18 with purified C-PC caused inhibition percentages of 69.64 and 71.11%, respectively, at 1000µg/ mL for 72 hours compared to the untreated cells. Therefore, the anti-cancer properties of C-PC make it an encouraging applicant for further advance in cancer therapy.

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