

BJP

Bangladesh Journal of Pharmacology

Research Article

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Cyanobacterial phycoerythrin purified from marine *Lyngbya* sp. induces apoptosis in lung carcinoma cells

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Article Info

Received: 27 June 2015
Accepted: 17 August 2015
Available Online: 26 September 2015
DOI: 10.3329/bjp.v10i4.23896

Cite this article:

Madamwar D, Kaushal A, Patel DK, Desai SN, Upadhyay K, Devkar RV. Cyanobacterial phycoerythrin purified from marine *Lyngbya* sp. induces apoptosis in lung carcinoma cells. Bangladesh J Pharmacol. 2015; 10: 770-78.

Abstract

Phycoerythrins from various cyanobacterial sources have been extensively studied globally for their therapeutic potentials. This study shows the apoptotic potential of cyanobacterial phycoerythrin isolated, purified and characterized from marine *Lyngbya* sp. A09DM on human lung carcinoma cells (A549 cell). Results indicated that purified cyanobacterial phycoerythrin accounted for a dose-dependent decrement in cell viability, mitochondrial membrane potential and an increment in lactate dehydrogenase release. Higher dose of cyanobacterial phycoerythrin caused loss of cell viability and nuclear condensation/fragmentation. Flow cytometry accounted for more number of cells in G₀/G₁ phase and an increase in total number of early apoptotic cells (39.5%). Overall, this study is the first to report on apoptotic property of cyanobacterial phycoerythrin from marine *Lyngbya* sp. A09DM against A549 human lung carcinoma cells and requires a detailed scrutiny to establish its anti-cancer potential.

Introduction

Lung cancer accounts for about 17% of the newly reported cases of cancer and 23% of the total cancer deaths that occur annually primarily due to nicotine intake and various occupational hazards (Report on carcinogenesis, 2011). Extensive use of chemotherapy for alleviating symptoms and for treating lung cancer also involves undesired physiological effects. In recent times, metal complexes (Singh et al., 2012; Vyas et al., 2013; Vyas et al., 2014; Ramadevi et al., 2015; Thakore et al., 2015), herbal extracts (Sankhari et al., 2012; Patel et al., 2012), structured nano sized particles (Valodkar et al., 2011; Valodkar et al., 2012) or novel proteins from bacterial or fungal origins (Huang et al., 2002) have been extensively researched as a therapeutic option.

Phycobiliproteins are light-harvesting accessory pigments with immunomodulatory, anti-inflammatory, anti-oxidant and nutritional properties (Belay, 2002; Rodríguez-Sánchez et al., 2012). Cyanobacterial phycoerythrin (absorption- 520 to 580 nm and emission peak at 575 ± 10 nm) is used as a dietary supplement (Romay et al., 2003) that also inhibits growth of tumor cells (Huang et al., 2002; Pan et al., 2013; Madamwar et al., 2015). However, cytotoxic property of proteins from a marine cyanobacteria *Lyngbya* sp. against carcinoma cells has so far not been reported.

This study investigates the impact of cyanobacterial phycoerythrin on A549 lung carcinoma cells wherein, alterations in cell morphology, cell cycle and possible induction of apoptosis has been scrutinized in detail.



Materials and Methods

Chemicals

Sephadex G-150 powdered matrix (bead-diameter 20-300 μm and fractionation range 5-300 kDa) was purchased from GE Healthcare UK Limited (UK), 2, 4, 6-tripyridyl-s-triazine and ferric chloride (hexahydrate) from Sigma, USA; protein molecular mass standard from Bangalore Genei (India); sodium dodecyl sulfate, electrophoresis grade acrylamide and bisacrylamide from Merck (Germany). Phosphate buffer saline, Dulbecco's Modified Eagle Medium, trypsin phosphate versene glucose solution, trypsin and methylthiazolyl-diphenyltetrazolium bromide (MTT) were purchased from HiMedia Laboratories Pvt. Ltd. (India). Fetal bovine serum was purchased from Biosera (UK) and dimethyl sulfoxide was purchased from the Sisco Research Laboratories Pvt. Ltd. (India).

Isolation and growth condition

The enrichment, isolation and purification of the cyanobacterial cell culture were carried out according to Shah et al. (2001). Samples were isolated from the rocky shores of Bet Dwarka, Okha and estuarine mouth of river Daman Ganga and further cultivated in ASN III medium (Waterbury and Stanier, 1981) with 12:12 light:dark (L:D) cycles at $27 \pm 2^\circ\text{C}$ and illumination of 36 W white fluorescent lamps at a flux density of 130 $\mu\text{mol photons.m}^{-2}.\text{sec}^{-1}$. Identification by 16S-rRNA gene sequencing showed that the isolated strain was *Lyngbya* sp. A09DM (accession number: HM446280).

Extraction

Marine *Lyngbya* sp. A09DM culture was harvested within 30 days of inoculation (Soni et al., 2006). Cyanobacterial cell mass was washed with 1 M Tris Cl buffer (pH 8.1) containing 3 mM sodium azide. One volume of washed cell mass was resuspended in five volumes of the same buffer and subjected to repeat freeze-thaw cycles of -30°C and 4°C shocks for the release of phycobiliprotein. The cell debris was removed by centrifugation at 17,000 xg (Kubota 6500, Bunkyo-Ku, Tokyo, Japan) for 40 min and the supernatant was termed as crude extract.

Purification

Steps of purification were carried out as described by Parmar et al., (2011).

Ammonium sulfate precipitation

The crude extract was subjected to two step ammonium sulfate precipitation (20% and 70%) followed by centrifugation at 15,000 xg for 40 min at 4°C . The supernatant were discarded and the pellet was resuspended in 10 mM Tris Cl buffer (pH 8.1). The extracts, thus, obtained were termed as 70% ammonium sulfate cut that was further subjected to dialysis for 24 hours against 100

times volume of MilliQ water containing 3 mM sodium azide.

Gel permeation chromatography

Dialysed 70% ammonium sulfate cut was applied on a sephadex G-150 column (45 cm x 1.5 cm, bed height 35 cm) pre-equilibrated and eluted with 10 mM Tris Cl buffer (pH 8.1). The flow rate was maintained at 60 mL/hour using peristaltic pump (Model P1; Pharmacia, Sweden). Bright pink color elutes were collected as 1 mL fractions.

Biochemical estimations and characterization of protein

The protein contents were determined by the method of Lowry et al., (1951) with bovine serum albumin as the standard. Phycoerythrin from all the three steps- crude, dialysed 70% ammonium sulfate cut and gel permeation chromatography elutes were characterized by UV-Visible spectrophotometer (Analytik Jena AG Specord® 210, Germany). The amount of cyanobacterial phycoerythrin, cyanobacterial phycocyanin, and cyanobacterial allophycocyanin was calculated according to equations of Bennett and Bogorad (1973). The ferric reducing ability of plasma assay was performed according to Benzie and Strain (1996). A fresh cocktail solution was prepared by mixing solution A (300 μM acetate buffer- pH 3.6), solution B (10 mM TPTZ 2, 4, 6-tripyridyl-s-triazine prepared in 40 mM hydrochloric acid) and solution C (20 mM ferric chloride hexahydrate) at a ratio of 10:1:1. Later, 30 μL sample and 90 μL distilled water was added to 900 μL of cocktail solution and the mixture was incubated at 37°C for 4 min. Absorbance was read at 593 nm and was recorded against a reagent blank. The purified phycoerythrin was also characterized in terms of the molecular masses of the subunits. Non-denaturing and denaturing polyacrylamide gel electrophoresis was carried out using 15% polyacrylamide slab gels (Singh et al., 2009) and the same was visualized by silver staining (Garfin, 1990). For calibration, marker proteins ranging from 6 kDa to 43 kDa (aprotinin- 6 kDa, lysozyme- 14.3 kDa, soyabean trypsin inhibitor- 20.1 kDa, carbonic anhydrase- 29 kDa and ovalbumin- 43 kDa) were used. Bilin chromophore was detected using zinc-acetate staining as per Brekelman and Lagarias (1986), and the bilin fluorescence was observed under UV light using AlphaEase FC Imaging System (Alpha Innotech Corp., USA).

Cell line and culture

Human lung carcinoma (A549) cells were obtained from National Centre for Cell Science, Pune, India and were seeded (1×10^5 cells/T25 flask) and cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution at 37°C with 5% in water jacketed CO_2 incubator (Thermo

scientific, forma series II 3111, USA). Cells were sub-cultured every third day by trypsinization with trypsin phosphate versene glucose solution. Reagents used herein were filtered through 0.22 μ filter (Millipore Biomedical Aids Pvt. Ltd, Pune) prior to their use for the experiment. A549 cells were maintained for a period of 24 hours in absence or presence of phycoerythrin at a cell density of 5.0×10^3 cells/well in 96 well plate for MTT and LDH assay and 1×10^5 cells/well in 6 well plate for other assays mentioned herein.

Cell viability assay

A549 cells (7×10^3 cells/well) were maintained in 96-well culture plates (Tarson India Pvt. Ltd.) for 24 hours with or without phycoerythrin (10, 25, 50, 100, 200 μ g/mL). Later, 10 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/mL) was added to the wells and plates were incubated at 37°C for 4 hours. Subsequently, the culture media were discarded and wells were washed with phosphate buffered saline. The resultant formazan formed was dissolved in 150 μ L of DMSO and absorbance was read at 540 nm in BIOTEK ELX800 Universal Microplate Reader (USA).

LDH release assay

Cellular integrity of A549 cells was measured with LDH release assay, wherein cells were maintained in 96 well plates with or without phycoerythrin for 24 hours as mentioned above. Later, supernatant from each well was collected and LDH was assayed with commercially available kit (Reckon diagnostics Ltd., India) on Merck microlab L 300 semi-autoanalyzer (India). Based upon the readings obtained, percentage cytotoxicity was calculated.

Intracellular oxidative stress

Intracellular oxidative stress due to reactive oxygen species generation in A549 cells was studied using 2,7-dichlorodihydrofluorescein diacetate (CM-H₂-DCFDA) staining. Cells were treated with cyanobacterial phycoerythrin for 18 hours as per the method mentioned above. Later, the cells were incubated with 7.5 μ M DCFDA at 37°C for 30 min and cells were photographed in Leica DMRB fluorescent microscope (Germany) using Canon Power Shot camera.

Mitochondrial membrane potential

The changes in mitochondrial membrane potential were measured using the fluorescent cationic dye rhodamine 123 (RHO 123). A549 cells were incubated for 24 hours with or without cyanobacterial phycoerythrin as mentioned above. Later, cells were incubated with 1 μ M RHO 123 for 10 min at 37°C and the fluorescence was determined at an excitation and emission wavelengths of 485 and 530 nm, respectively using spectrofluorometer (Jasco FP-6300, Japan) and expressed as fluorescence

intensity units.

Assessment of cyanobacterial phycoerythrin-induced cell cycle analysis and apoptosis

Assessment of nuclear morphology was done using DAPI staining. A549 cells (5×10^4 cells/well) were plated into 6-well plate. After 80% confluence was achieved, the cells were treated with or without different concentrations of cyanobacterial phycoerythrin at 37°C for 24 hours. Single-cell suspensions of treated cells were washed with phosphate buffered saline and fixed with 70% ethanol for 20 min at room temperature. Cells were re-washed with phosphate buffered saline and stained with DAPI (0.6 μ g/mL in phosphate buffered saline) for 5 min. Nuclear morphology (condensed/fragmented nuclei) was examined under Leica DMRB fluorescence microscope (Germany).

Apoptotic cells were studied using AO/EB staining. A549 cells (1×10^5 cells/well) were maintained in 6 well plates for 24 hours with or without cyanobacterial phycoerythrin as described earlier. At the end of experiment period, cells were collected using TPVG solution. One microliter of dye mixture (1 mg/mL AO and 1 mg/mL EB in phosphate buffered saline) was mixed with 9 μ L of cell suspension (0.5×10^6 cells/mL) on a clean microscope slide and examined and photographed under Leica DMRB fluorescence microscope (Germany). A minimum of 300 cells were counted in every sample to calculate percentage cell death.

Cells (1×10^6 cells/well) were cultured with or without phycoerythrin as mentioned earlier for 24 hours. After incubation, the cells were washed once in ice-cold phosphate buffered saline and subjected to cell cycle analysis. Briefly, 1×10^5 cells were fixed in 4.5 mL of 70% (v/v) cold ethanol for 30 min, centrifuged at 400 \times g for 5 min. Supernatant was removed and cells were washed with 5 mL of phosphate buffered saline. Cells were then re-suspended in 0.5 mL of phosphate buffered saline and 0.5 mL of DNA extraction buffer (Mix 192 mL of 0.2 M Na₂HPO₄ with 8 mL of 0.1% Triton X-100 v/v) was added. The pH was adjusted to 7.8. Cells were incubated at room temperature for 5 min and then centrifuged at 400 \times g for 5 min. Supernatant was discarded and cells were re-suspended in 1 mL of DNA staining solution (200 mg of PI in 10 mL of phosphate buffered saline + 2 mg of DNase free RNase). Cells are then incubated for at least 30 min at room temperature in the dark and the cell cycle distribution was then analyzed on a flow cytometer (BD FACS Aria III, USA) using FlowJo (Oregon, USA).

Annexin V/propidium iodide double staining assay was used to quantify apoptosis, according to the manufacturer's protocol (Alexa Fluor 488 annexin V/dead cell apoptosis kit, Invitrogen). After incubation, cells were harvested using TPVG solution and washed with ice-cold phosphate buffered saline and suspended

in 100 μ L of 1x binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4). To this mixture, 5 μ L of annexin V- Alexa Fluor 488 conjugate and 1 μ L of propidium iodide solution were added to each cell suspension and incubated for 15 min at room temperature in the dark. Samples were analyzed on flow cytometer (BD FACSAria III, USA) using FlowJo (Oregon, USA). Double staining of cells with Alexa Fluor 488 annexin-V and propidium iodide enables the discrimination of live cells (Alexa Fluor 488-PI⁻), early apoptotic (Alexa Fluor 488⁺PI⁻), late apoptotic (Alexa Fluor 488⁺PI⁺) or necrotic cells (Alexa Fluor 488-PI⁺).

Statistical analysis

Data was analyzed for statistical significance using one way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test and results were expressed as mean \pm SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego, California, USA.

Results

Precipitation of cyanobacterial phycoerythrin could not be achieved in first step because the supernatant of 20%

ammonium sulfate cut in 70% ammonium sulfate was not efficient for its extraction. Hence, in the second step, the contents were dialyzed overnight and precipitate of cyanobacterial phycoerythrin with a purity ratio of 1.66 was obtained. In the final step, fractionation of the resultant cyanobacterial phycoerythrin was done with sephadex G-150 to achieve a purity ratio of 3.67 and a final yield of 65.2%. The purity of cyanobacterial phycoerythrin was monitored at each step by UV-Visible spectroscopy and gel electrophoresis. The purity ratio was monitored at each step by overlay spectra using UV-Visible spectroscopy. The absorbance maxima (λ_{max}) for phycoerythrin was recorded at 562 nm. The purity ratios of phycoerythrin to total protein (A_{562}/A_{280}), phycoerythrin to phycocyanin (A_{562}/A_{620}), and phycoerythrin to allophycocyanin (A_{562}/A_{650}) were 3.57, 4.09 and 11.41, respectively (Table I).

Non-denaturing gel electrophoresis of purified cyanobacterial phycoerythrin showed two bands corresponding to phycoerythrin-I and phycoerythrin-II, suggesting homogeneity of purified cyanobacterial phycoerythrin (Figure 1a). Denaturing gel electrophoresis of cyanobacterial phycoerythrin showed the presence of four bands having molecular masses of 23, 20, 18.5 and 18 kDa (Figure 1b). These bands were

Table I

Determination of spectrophotometric purity and concentration of phycobiliproteins

Purification stages	A_{562} (nm)	A_{620} (nm)	A_{650} (nm)	A_{562}/A_{280}	A_{562}/A_{620}	A_{562}/A_{650}	C-PE (mg/mL)	C-PC (mg/mL)	C-APC (mg/mL)	Total protein (mg)
Crude extract	0.31	0.06	0.01	0.81	5.33	36.83	0.03	0.01	-0.01	13.21
Dialyzed 70% ammonium sulfate cut	0.45	0.14	0.07	1.66	3.25	6.33	0.03	0.02	0.01	10.74
Gel permeation chromatography	0.70	0.17	0.06	3.57	4.09	11.41	0.06	0.03	0.01	8.61

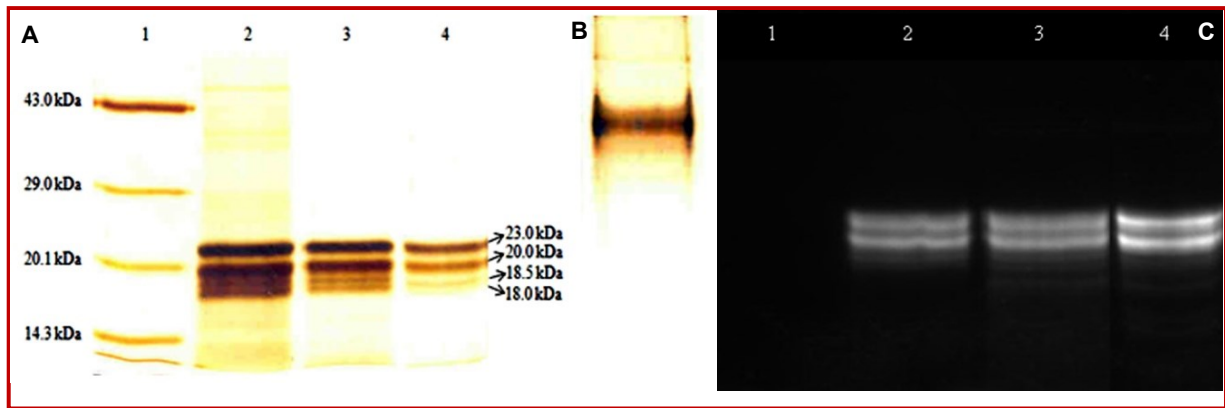


Figure 1: Detection of proteins by silver staining (A) and biliproteins by zinc-assisted fluorescence enhancement method (C). Lanes in (A) and (C): 1. Protein molecular mass marker; 2. Crude extract; 3. Ammonium sulfate cut (70%); 4. Pure PE eluate from gel permeation chromatography. Molecular mass marker had no biliproteins and hence, no fluorescence was detected. 5 mg protein was loaded in each lane. (B) Silver stained 12% native gel electrophoresis of purified cyanobacterial phycoerythrin from *Lyngbya* sp. A09DM

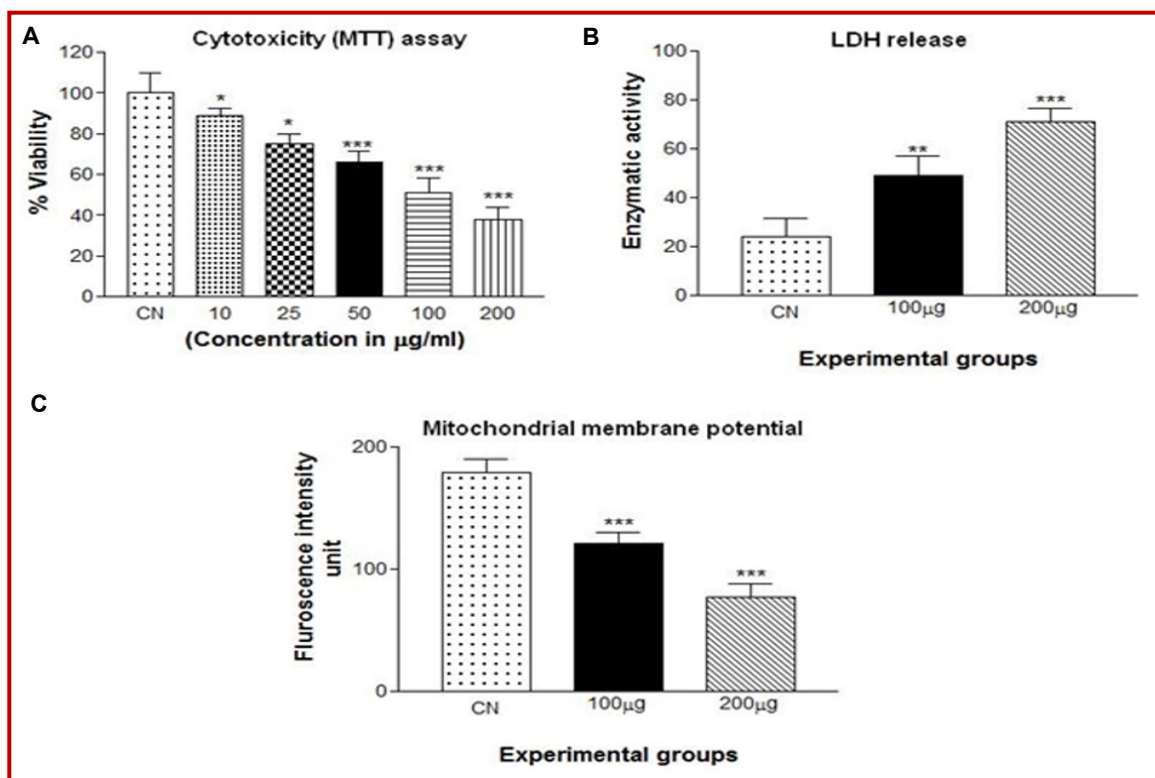


Figure 2: (A) Cytotoxicity assay of A549 cells following 24 hours cyanobacterial phycoerythrin treatment. (B) LDH release from untreated cells and 24 hours cyanobacterial phycoerythrin treated cells. (C) Mitochondrial membrane potential of untreated and 24 hours cyanobacterial phycoerythrin treated cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

obtained in the last step of purification and corresponded to α and β subunits of each of phycoerythrin-I and phycoerythrin-II. The presence of bilin chromophore group attached to the phycoerythrin subunit was confirmed by zinc-acetate staining that showed characteristic orange fluorescence under UV light. No fluorescence was observed in molecular mass standard due to the absence of bilin-linked peptides. These results also confirmed attachment of bilin chromophore groups to the subunits of phycoerythrin (Figure 1c).

The antioxidant capacity of purified cyanobacterial phycoerythrin obtained herein was 48.45 μM AEAC as per the results obtained in FRAP assay. Cytotoxic potential of cyanobacterial phycoerythrin against lung carcinoma cell line is shown in (Figure 2a). Cells treated with cyanobacterial phycoerythrin showed a dose dependent inhibitory effect. Also, there was a significant dose dependent increment in LDH release following cyanobacterial phycoerythrin treatment as compared to untreated control cells (Figure 2b). Overall, a maximum cell growth inhibition (>80%) and LDH release was recorded at 200 $\mu\text{g}/\text{mL}$ dose of cyanobacterial phycoerythrin.

The fluorimetric analysis of cyanobacterial phycoerythrin treated samples recorded a dose-dependent decrement in MMP as compared to untreated group. The lowest MMP was recorded in 200 $\mu\text{g}/\text{mL}$ dose of

phycoerythrin (Figure 2c).

The apoptosis induced by phycobiliprotein in A549 cells was confirmed by AO/EtBr staining. The cells of untreated control group showed green fluorescence indicating viability whereas, these cells were progressively less in number in a dose-dependent manner in phycoerythrin-treated group. More number of yellow colored cells was present in 100 $\mu\text{g}/\text{mL}$ phycoerythrin-treated group. The same indicates the early apoptotic stage. However, 200 $\mu\text{g}/\text{mL}$ phycoerythrin-treated group showed more number of reddish or orange stained cells that symbolize late apoptotic phase (Figure 3a). DAPI staining phycoerythrin-treated cells showed higher number of cells with condensed nuclei in phycoerythrin-treated groups as compared to control group (Figure 3b). The cell cycle analysis (Figure 4a) showed 93.1 % in G_0/G_1 and 4.5% of cells in sub G_0/G_1 phase in control group whereas, 15% in G_0/G_1 and 83.3% of cells in sub G_0/G_1 phase in phycoerythrin-treated group. Flow cytometric assay with annexin V/propidium iodide double staining of control and phycoerythrin-treated cells showed that apoptosis rate was increased after phycoerythrin-treatment as they recorded 39.5% annexin V positive (early apoptotic) and 0.51% annexin V and propidium iodide positive cells (late apoptotic) compared to 0.9% and 0.6% in control group (Figure 4b).

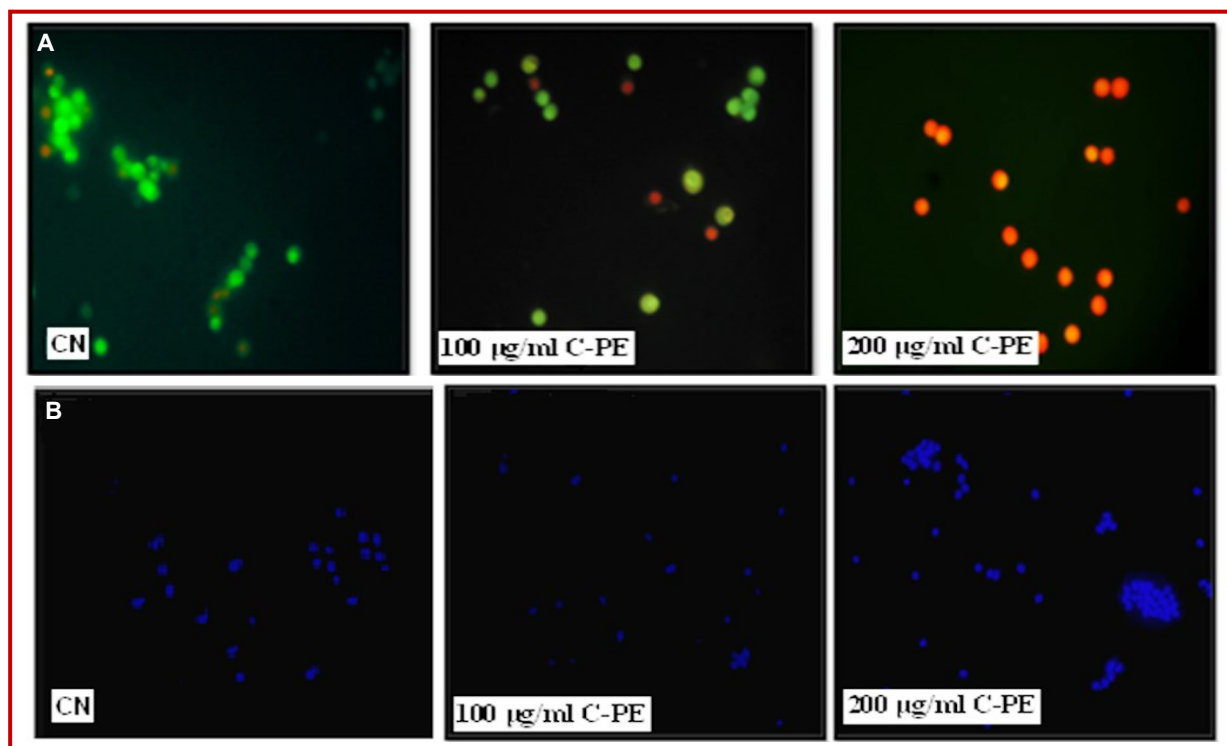


Figure 3: (A) AO and EB stained A549 cells; CN-untreated cells, 100 and 200 µg cyanobacterial phycoerythrin treated with low and high doses respectively. Yellow or orange fluorescence indicates nonviable cells. (B) DAPI stained CN-untreated cells, 100 or 200 µg cyanobacterial phycoerythrin-treated with low and high doses respectively

Discussion

This study elaborates the method of purification of phycoerythrin from a novel isolate *Lyngbya* sp. A09DM and its subsequent cytotoxicity against A549 lung carcinoma cells. Culturing of *Lyngbya* sp. under white light can be considered as an efficient source for obtaining cyanobacterial phycoerythrin with a high purity ratio of 3.67. Also, the two-step ammonium sulfate precipitation method is equally efficient for purification of cyanobacterial phycoerythrin and these results are in agreement with Rito-Palomares et al., (2001). Cytotoxicity (MTT) assay of cells treated with cyanobacterial phycoerythrin indicated a dose-dependent impact on cell viability wherein, higher dose (200 µg/mL) was most potent and accounted for maximum cell growth inhibition (>80%). Transmembrane potential of mitochondria is an important parameter to assess mitochondrial function and overall health of cell (Patel et al., 2013; Gajaria et al., 2015). In the present study, a dose-dependent decrement in mitochondrial membrane potential was recorded following cyanobacterial phycoerythrin treatment suggesting possible mitochondrial dysfunction. These results provide supportive evidence on cyanobacterial phycoerythrin-induced cytotoxicity recorded in the MTT assay. Any damage to the plasma membrane of an animal cell causes leaching out of LDH from cytoplasm into the media and the same is measured as an

indication of the extent of cell damage (Devkar et al., 2012). Hence, biochemical estimation of LDH is a reliable and popularly used parameter to assess cell damage. In our study, high levels of LDH are in support of the results obtained in cytotoxicity (MTT) assay.

Experiments with natural anticancer agents have reported that heightened intracellular oxidative stress also accounts for cell death by causing damage to the plasma membrane (Gajaria et al., 2015). But in our study, the control and treated cells showed comparable levels of weak fluorescence suggesting that the cyanobacterial phycoerythrin failed to initiate oxidative stress in A549 cells. At this stage, it was assumed that, cyanobacterial phycoerythrin induced cell death was possibly due to the trigger of apoptotic pathway. Inhibition of a tumor due to the arrest of cell division or cytotoxicity and subsequent induction of apoptotic cell death are key features for treating any type of cancer. The down-stream pathway of apoptotic cascade is reportedly triggered in presence of cytochrome c that leaches out from the transitional pores of mitochondrial membrane (Jiang and Wang, 2004). This event results due to an altered mitochondrial membrane potential as observed in our study. Also, presence of more number of EB positive cells (red/orange) observed herein, further corroborate the findings that cyanobacterial phycoerythrin is instrumental in inducing cytotoxicity.

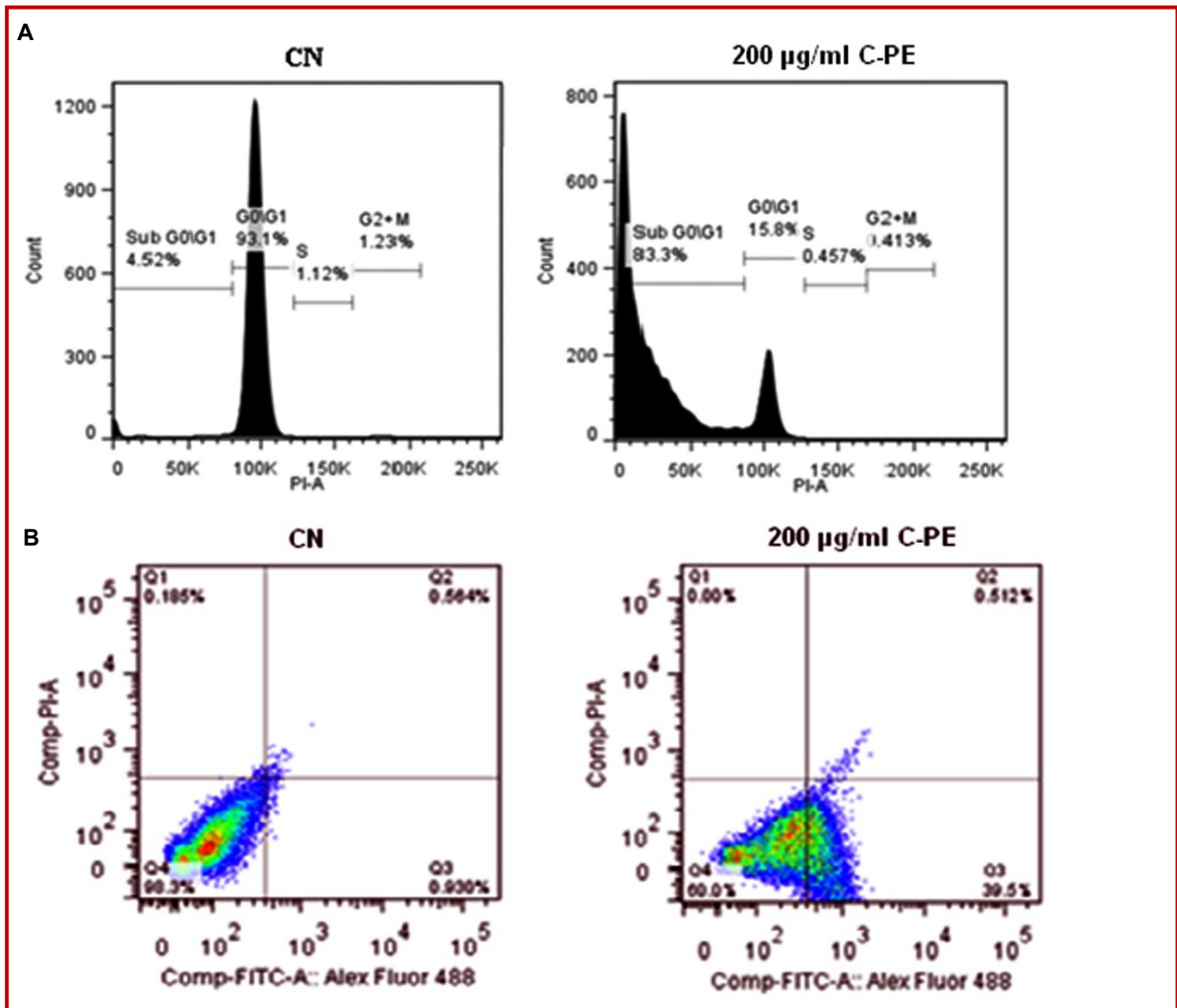


Figure 4: (A) FACS analyses of A549 cells indicate more number of cells in sub G0/G1 phase (83.3%) following cyanobacterial phycoerythrin treatment as compared to untreated cells (4.52%). (B) Annexin V-FITC/PI apoptosis detection assay shows higher number of apoptotic cells in cyanobacterial phycoerythrin treated group. CN- Untreated; 200 µg/mL cyanobacterial phycoerythrin high dose

As compared to the lower dose (100 µg/mL), the higher dose (200 µg/mL) showed qualitative evidence of higher extent of cell damage in form nuclear fragmentation with possible indications of an early apoptosis (Figure 4a). Chromatin condensation, nuclear fragmentation, nuclear shrinkage and formation of apoptotic bodies are characteristic features of apoptosis (Elmore, 2007). Hence, higher dose was chosen for further cell cycle analysis. Flow cytometric analysis revealed that, cyanobacterial phycoerythrin-treated cells showed cell arrest in sub G0/G1 phase (83.3%) as compared to the control. Further confirmation of cyanobacterial phycoerythrin-induced apoptosis was obtained with annexin V-propidium iodide staining wherein, a large number of early apoptotic (39.5%) and only few late apoptotic (0.51%) cells were observed. These observations provide compelling evidence on cyanobacterial phycoerythrin induced apoptosis of A549 cells

Conclusion

Cyanobacterial phycoerythrin induced altered mitochondrial membrane potential triggers apoptosis in human lung carcinoma cells. This study is the first to report on apoptotic potential of cyanobacterial phycoerythrin from marine *Lyngbya* sp. A09DM and the findings warrant a detailed scrutiny on its efficacy as an anti-cancer agent.

Conflict of Interest

The authors declare that they have no competing interests

Acknowledgement

RVD acknowledges DST-SERB for financial support in the

form of Major research project No. SR/SO/AS-45/2011 and MSU-DBT-ILSPARE for infrastructural and technical support.

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