Phycocyanobilin: A Potential Anticancer Therapy —A Tale of a Natural Chromophore

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***Abstract*—Back to nature is a general theme for many research lines nowadays, where several substances that are extracted from natural sources have shown great potential in treating many diseases. Algal compounds, in particular, have drawn significant attention for being of biological importance. As cancer is one of the most diseases which causes people to suffer, all researchers and scientists exert much effort to end that suffering. Unfortunately, almost all the current applied conventional treatment methods exert serious side effects with limited therapeutic abilities. Recently, several algal value-added substances exhibited anticancer potentiality; they can be a promising alternative for cancer treatment. We extracted phycocyanin, a blue biliprotein, from *Spirulina platensis SAM2021*, with content 3.11 mg/ml and purity 0.61. The obtained phycocyanobilin by methanolysis cleavage showed maximum absorption at λ_{max}= 600 nm. It exhibited IC50 108 µg/ml for colorectal cancer cell line HT-29.**

***Index Terms*—algae, cancer, phycocyanobilin, phycocyanin, *Spirulina*, anticancer**

I. Introduction

Cancer is a critical and multifactorial disease burden worldwide. Every year, more than 14 million new cancer cases with a mortality rate of about 8 million are recorded [1]. There are many types of cancer depending on the affected tissue type or a specific organ. Colorectal Cancer (CRC) is the third most prevalent cancer type [2]-[5]. The global burden of Colorectal Cancer (CRC) has been expanding rapidly. Approximately 70% of CRC cases are sporadic cases that were affected by many factors such as smoking, dietary habits, alcohol consumption, and physical activity [6]. At present, primarily the conventional treatment strategies of cancer including invasive or non-invasive treatments such as surgery, radiotherapy, and chemotherapy or a combination of them. Unfortunately, it has limited curative effect with severe side effects. Therefore, finding alternative substances that are suitable, nontoxic and effective as cancer treatment became the prime concern of research and scientists. Previous studies proved that many plants [7] and algae contain valuable substances that can be used as chemotherapeutic agents with anticancer activity [8]. Algae are ubiquitous photosynthetic organisms. They are divided into macroalgae and microalgae. Generally, the microalgae contain a variety of bioactive compounds which find their way in plenty of biotechnological applications [9], and have also been proven to exhibit anticancer activity [8]. *Spirulina platensis* is a filamentous blue green alga (Cyanobacteria), it is basically composed of proteins about 60-70 %, carbohydrates (16 %), vitamins, minerals, essential fatty acids, and pigments, including chlorophylls, carotenoids (Garcia-Lopez, *et al.*, 2020). Particularly, C-Phycocyanin (C-PC) is one of the major and most important pigments produced by *Spirulina platensis*; it is a photosynthetic blue water-soluble pigment [10], [11]. Plenty of researches have proved that phycocyanin can exhibit a remarkable and significant anti-cancer effect against many cancer cell types such as breast, liver, lung, colon, bone marrow and even leukemia in both *In vivo* and *In vitro* assays [12]-[17]. Phycocyanin consists of α (~18 kDa) and β (~21 kDa) subunits. Each subunit is attached to one or more tetrapyrrole chromophores with λ_{max} = 610–620 nm [18]. These chromophores are called phycocyanobilins (PCBs), they are attached to the apoprotein through covalent thioether bonds with a cysteine residue [19]. They became one of the important therapeutic natural substances and applicable in many fields including food supplements due to its antioxidant capacity and medicinal applications due to its anti-inflammatory, antiviral, anticancer, and immunomodulatory effects [19].

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II. Methodology

A. Spirulina platensis Culturing and Growth Parameters

Spirulina (Arthrospira) platensis was purchased from Spirumisr, Egypt. The culture was started with an inoculum (250 ml) using Zarrouk's medium under controlled conditions (temperature 30˚C, pH 9-10, white LED light with 16/8 light/dark cycle) in a 2-liter bench top photobioreactor with RED-LED platform for the light adjustment, the optical density was evaluated at time intervals at 560 nm and 880 nm for growth estimation. After 15 days (log phase) the biomass was harvested using centrifugation (6000 rpm) at room temperature. The collected wet biomass was oven dried at 60-70˚C and kept at under dried conditions for further use.

1) Dry cell weight

At time intervals (3 days), 30 ml of the algal culture was filtered through a pre-weighed filter paper. The filter paper was then oven dried at 60 ˚C till constant weight. The dry weight value was calculated as the difference between the weight of the filter paper before and after drying [20].

2) Estimation of chlorophyll content

An aliquot (5 ml) of the culture was measured spectrophotometrically every 2 days at 645 nm and 663 nm, in order to calculate the chlorophyll content using the following equations ((1), (2), (3)) [21] .

$$Chlorophyll\ a \frac{mg}{l} = 12.7 \times O.D\ 663 - 2.69 \times O.D\ 645 \quad (1)$$

$$Chlorophyll\ b \frac{mg}{l} = 22.9\ \times\ O.D\ 645 - 4.68\ \times\ O.D\ 663 \quad (2)$$

$$Total\ Chlorophyll \frac{mg}{l} = 20.2\ \times\ O.D\ 645\ +\ 18.2\ \times\ O.D\ 663 \quad (3)$$

B. Molecular Identification of Spirulina platensis

The genomic DNA was extracted using FavorPrep Plant Genomic DNA Extraction Minikit. Molecular identification of *Spirulina platensis* was carried out by PCR amplification and sequencing of the *cpcBA-IGS* gene using the CPC1F (5′-GGC KGC YTG YYT GCG YGA CAT GGA-3′) and the reverse primer was CPC1R (5′-AAR CGN CCT TGR GWA TCD GC-3′ [22]. The purified amplicons were sequenced using Big Dye terminator cycle sequencing Kit v.3.1. (Applied Biosystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems, USA) at Macrogen, Inc., Seoul, Korea.

C. Extraction, Purification and Estimation of Phycocyanin

1) Extraction from dried biomass

One gram of oven dried *spirulina platensis* was mixed with 100 ml potassium phosphate buffer (KPB) (0.1 M, pH= 7) for one hour. The solution was subjected to ultrasonication for cell wall disruption. The collected phycocyanin was precipitated by adding 50% of saturated ammonium. The absorbance of the protein was measured by UV-VIS spectrophotometer at 280nm, 620 nm and 652 nm. The resulted phycocyanin was filtered through sephadex G-100 for purification, the final extract was kept at -20 ºC for further use.

2) Phycocyanin content

The content of PC in the crude extract for both was calculated using the following equation (4) [23]:

$$PC\ \left(\frac{mg}{ml}\right) = [OD615 - 0.474(OD652)]\ /\ 5.35 \quad (4)$$

The purity (P) was estimated as ratio between A620/A280, where A620 is the absorbance of phycocyanin and A280 is the absorbance of the other total proteins. The extraction yield of the phycocyanin (mg/g) was calculated by the following equation (5):

$$Y = PC \times V/DB \quad (5)$$

where the PC is the content of the phycocyanin (mg/l), V is the volume of the solvent (ml) and DB is the dried biomass (g).

3) Analytical and spectroscopic analysis of C-PC

The purified C-PC was scanned spectrophotometrically to determine the maximum wavelength. To evaluate the extraction and purification of the phycocyanin pigment, protein electrophoretic patterns was detected and monitoring via Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique (12%) according to [24].

D. Cleavage of the Phycocyanobilin (PCB) from CPC via Methanol Reflux

1) Methanolysis

1 gram of freeze-dried phycocyanin was mixed with 150 ml of pure methanol (98.9%), the solution was refluxed at 110˚C for 16 hours, then the solution was concentrated to 5 ml via rotary evaporator (40˚C, 100 rpm for1 hour), 10 ml chloroform was added and followed by water with vigorous shaking, this step was repeated till the aqueous layer became almost colorless, the green protein debris was discarded, chloroform layer with the pigment was collected and left for air drying [25], [26]. The dried pigment was dissolved in methanol and measured spectrophotometrically.

2) Quantification and standard curve for PCB

The content of the PCB was estimated by (w/v), and a standard curve was performed using different concentrations from the stock solution against the absorbance at the maximum wavelength.

E. Cell Culture, Cytotoxic in Vitro Assay and Statistical Analysis

HT-29: Colorectal Cancer was obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). Cells were maintained in RPMI media supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37 °C. Cell viability was assessed by SRB assay, using phycocyanobilin at various

concentrations (.01, .03, 0.1, 0.3, 1, 3, 10, 100, 300) [27]. The absorbance was measured at 540 nm using a BMG LABTECH®-FLUOstar Omega microplate reader (Ortenberg, Germany). Statistical analysis of IC50 values was calculated from concentration-response curves using an E-max model equation [28].

$$\%\ Cell\ Viability = (100 - R) \times \left(1 - \frac{[D]^m}{K_d^m + [D]^m}\right) + R \quad (6)$$

All experiments were performed in triplicate wells for each concentration. The results are presented as the mean ± Standard Deviation (STD).

III. Results

A. Growth Parameters of Spirulina platensis

The optical density increased with time till reached its maximum absorbance 1.51 and 1.01 after 18 days at 560 nm and 880 nm, respectively and then declined, as shown in Fig. 1. All data were calculated as mean of three independent readings. The Chl b had approximately double the concentration of Chl a, its maximum value (28.18 mg/l). The total chlorophyll content was 59.4 mg/l after 18 days which was its highest value. The Net Dry Cell Weight (NDCW) was increased gradually till reach its maximum value .2213 at 18th day and then declined.

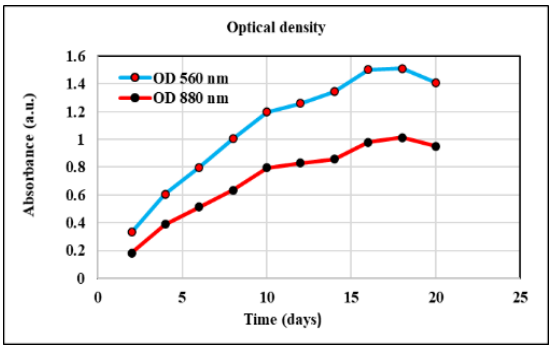

Figure 1. Optical density (nm) of the S. platensis culture at time intervals (Days).

B. Molecular Identification

The amplified product expected size from the Agarose gel electrophoresis was ~ 450 bp. The obtained *CpcBA-IGS* gene partial sequence of our culture was compared with sequences available in the National Center for Biotechnology Information database using BLAST network services (http://www.ncbi.nlm.nih.gov/BLAST). The BLASTn results showed that the identity by about 99 % between our strain and many *Arthrospira platensis* strains. Our Accession number is MW598472.

C. Phycocyanin Extraction and Quantification

1) Extraction of C-PC from dried algal biomass

Phycocyanin was extracted from 1 gram of dried *spirulina platensis*' powder via sonication, the absorbance was measured at 280 nm, 620 nm, and 652 nm at every step of extraction, precipitation and purification. After the calculations, it is clear that the crude extract had the lowest concentration (0.13 mg/ml) and purity (0.26). In contrast, the purity of extract after ASP and Sephadex G100 increased to reach approximately 0.9 which is higher than the food grade (≥ .7), Sephadex G100 sample had the highest content (3.11 mg/ml) and yield (59.12 mg/g) as shown in the following Table I.

TABLE I. The Content, Purity, and Yield of the Extracted Phycocyanin during Extraction and Purification Steps

	C-PC (mg/ml)	Purity	Yield (mg/g)
Crude extract	0.131	0.264	11.16
ASP	0.336	0.918	6.4
Sephadex G100	3.11	0.61	59.12

2) UV-Visible spectroscopic measurement and SDS-PAGE

Both the referenced phycocyanin and the extracted sample exhibit approximately the same peak with maximum wavelength at 620 nm. The SDS-PAGE analysis of the purified C-PC sample showed two bands corresponding to α- and β- subunits with molecular weight 17 kDa and 21 kDa, respectively. Lane A represents the bands corresponding to the molecular weight of protein marker, and lane B represents the loaded protein of extracted C-PC from S. platensis, as shown in Fig. 2.

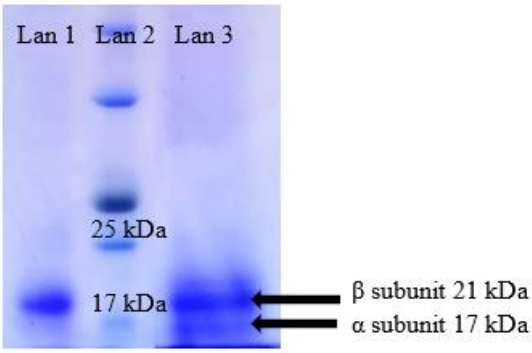

Figure 2. C-PC protein separated on SDS-PAGE. Lane 1 represents the standard C-PC, Lane 2 protein marker, Lane 3 the extracted C-PC from *S. platensis*.

D. UV-Visible Absorption Spectrum, Quantification and Standard Curve of PCB

The cleaved PCB via methanolysis method exhibited maximum absorbance at wavelength ~ 600 nm. The w/v concentration of the obtained PCB was about 3.3 mg/ml.

E. Cytotoxic in Vitro Assay

The results showed that phycocyanobilin has cytotoxic effect on HT-29 colorectal cancer cells by IC50 (108 µg/ml), we also found that the concentration (300 µg/ml) caused high toxicity (viability % 4.5).

IV. Discussion

There was a great interest in using natural compounds for treatment. Microalgae are a valuable source of secondary metabolites that exert multiple biological activities. We chose *Spirulina (Arthrospira) platensis* due to its nutritional value and therapeutic potentialities [29].

It has gained worldwide attention for use as human food supplements and pharmaceuticals. In the present study, our results revealed that the *S. platensis* is a good source for phycocyanin; its content was higher during the exponential phase than the decline phase. Many previous studies proved that C-PC has an anticancer effect on different cancer types [16], [30]. It can arrest cancer cell cycle; this was described in breast cancer MDA-MB-231 [31] and colon cancer HT29 [32]. We supposed that C-PC's anticancer potentiality is mainly due to its chromophore PCB because it is the active part of the protein structure. Konícková *et al*. proved that PCB has a cytotoxic effect on pancreatic cancer PA-TU-8902 cell viability [33]. Bilirubin was estimated to have anticancer potentialities. As the PCB is similar in structure to bilirubin, it might exert its effects in the same manner. Bilirubin exerts its anticancer effect mainly on mitochondria [34] and intracellular signalization [35]. Our results proved that PCB has a cytotoxic effect on colorectal cancer HT-29. To our knowledge, this is the first study that approved the anticancer effect of PCB on the colorectal cancer HT-29 via *in vitro* assays.

V. Conclusion

Empirically, phycocyanin is a constitutive pigment of *S. platensis*. We extracted it with good purity via the ultrasonication method. The cleavage of PCB can be performed well via methanolysis. It was outstanding that PCB exhibited potential cytotoxicity ability against CRC; We can conclude that the anticancer potentiality of the phycocyanin is mainly attributed to its chromophore (PCB). Sum of all, PCB can be considered a good candidate for cancer treatment in the long run.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

Suzan A. Rashed conceived the main idea, performed the practical part, writing, editing, and submission.

Ahmed Osman, Sherif F. Hammad and Moustafa El Dakak supervised the whole work, and revised the manuscript. Islam A. Khalil conceived and interpreted the spectroscopic analysis.

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Prof. Ahmed Osman received his Ph.D. degree in Biochemistry (Molecular Parasitology) in 1994 through a joint supervision program between Ain Shams University (ASU), Egypt and School of Medicine and Biomedical Sciences, State University of New York (SUNY) at Buffalo, USA. Dr. Osman joined Professor Philip LoVerde's laboratory in USA in 1996 as a post-doctoral fellow. Dr. Osman returned to his home institution in ASU after spending 12 years in Prof. LoVerde's lab, where he gained strong experience in different fields of life sciences including molecular biology, immunology, animal cell culture and vaccine research. After his return, Dr. Osman served as a Dean of postgraduate study and research and chairman of the Department of Biochemistry in the Faculty of Science, Ain Shams University. During his career, Prof. Osman supervised more than 50 MSc and Ph.D. students in SUNY-Buffalo, ASU, Cairo University and Egypt-Japan University of Science and Technology (E-JUST) and authored and co-authored more than 60 research articles in peer-reviewed journals including The Journal of Biological Chemistry, PLoS one and PLoS pathogens and vaccine as well as 6 book chapters in famous book series such as Methods in Molecular Biology (Humana press). Dr. Osman has recently joined Biotechnology program in E-JUST, where he is leading research in different fields of biomedical sciences such as molecular oncology, human viral infections (such as HCV and COVID-19) and neuromuscular disorders.

Sherif F. Hammad is one of the expert Egyptian medicinal chemists especially in the field of API industrial production. Dr. Hammad was graduated in Faculty of pharmacy, Alexandria University in 1997 with an excellent with honor grade after receiving the best ideal student award (first ranked over the university). He started his academic career in Helwan University where he earned a master's degree in Pharmaceutical chemistry followed by a Medicinal Chemistry PhD degree from Auburn University in Alabama State in the US and a postdoctoral fellowship in University of Maryland Baltimore County. His work in the US focused on the multistep total synthesis of DNA minor groove alkylators, carbocyclic nucleosides and other modified nucleotides targeting cancer, tuberculosis and Hepatitis C virus. Upon returning to Egypt, he started his professional career both academically as an assistant professor of Medicinal Chemistry in Helwan University and other Universities as adjunct professor as well as industrially as a Research and development (R&D) consultant in Pharco group for pharmaceutical industries. He shared in the startup and establishment of the most modern API production facility in the Middle East (Pharco B for Chemicals) at the role of R&D and technical director with a significant contribution in the treatment of more than 2 million Egyptian patients from HCV in the last five years. He joined EDA (Egyptian Drug Authority) from its beginning as API and Quality consultant and He is the major R&D consultant for EVA Pharma in API projects.

Dr. Moustafa M. Eldakak has received his bachelor's degree in Genetics and Biotechnology, College of Agricultural Sciences, Alexandria University with first ranking and honors in 2005. After the completion of his Master's degree in 2008, in the field of molecular genetics and Cytogenetics, he got an assistantship to study his Ph.D. at South Dakota State University, USA. He joined the molecular biology program in the Bio/Micro department, where he studied the different levels of gene expression regulations and interactions through the cutting-edge technologies of genomics, transcriptomics, proteomics and metabolomics. After the completion of his Ph.D. in 2015, and returning back to Alexandria University, he applied these technologies along with others like DNA barcoding. Moreover, he applied the concept of biotechnology with many microorganisms like microalgae and others for their manipulation for industrial and environmental benefits.

Dr. Islam A. Khalil received his B.Sc. in pharmaceutical sciences from Misr University for Science and Technology in 2003, and M.Sc. (2009) and Ph.D. (2013) in pharmaceutics from the Faculty of Pharmacy, Cairo University, Egypt. Currently, Islam is an associate professor of pharmaceutics at the College of Pharmacy and Drug Manufacturing, Misr University for Science and Technology, Egypt since 2019. He worked previously as a postdoctoral research fellow in an American university in Cairo (Egypt), Zewail City for Science and Technology (Egypt), Brigham and Women's Hospital - Harvard Medical School (USA), and Northeastern University (USA). His research activities are focusing on designing, developing, and evaluating of nanomedicines for various biomedical applications especially therapeutics and tissue regeneration. Also, he has a considerable industrial research and development experiences from 2014 with Orchidia Pharmaceutical Industry, Zi Diligence, and EVA Pharma.