Asian Journal of Green Chemistry 2 (2018) 364-379

Contents lists available at Avicenna Publishing Corporation (APC)

Asian Journal of Green Chemistry

Journal homepage: www.ajgreenchem.com

Orginal Research Article

Extraction and purification of phycocyanin from spirulina platensis and evaluating its antioxidant and anti- inflammatory activity

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ARTICLE INFORMATION

Received: 29 March 2018
Received in revised: 5 May 2018
Accepted: 9 May 2018
Available online: 12 June 2018

DOI: 10.22034/AJGC.2018.63597

KEYWORDS

Phycocyanin
Spirulina Platensis
NADPH Oxidase

ABSTRACT

Phycocyanin is a blue pigment and water-soluble biliprotein from the spirulina platensis. It has great medical and medicinal properties and has been used as a nutritional supplements. Phycocyanin is also a natural and powerful antioxidant and anti-inflammatory. In this study, the phycocyanin of Spirulina platensis was extracted by using two enzymatic and ultrasonic methods and purified by ammonium sulfate precipitation and dialysis. The UV spectrophotometer absorption of the extracted sample showed a broad peak range at 280, 615, and 652 nm. By using The FT-IR results confirmed the structure and molecular bonds from extracted and purificated phycocyanin. The concentrations obtained at the enzymatically was 0.405 mg/mL and for sonication 0.422 mg/ mL. By using the SDS PAGE method, bands of phycocyanin were identified and the molecular weight was determined between 19-14 KD. The antioxidant and anti-inflammatory activity of the phycocyanin was approved with the reaction by DPPH and HOCl.

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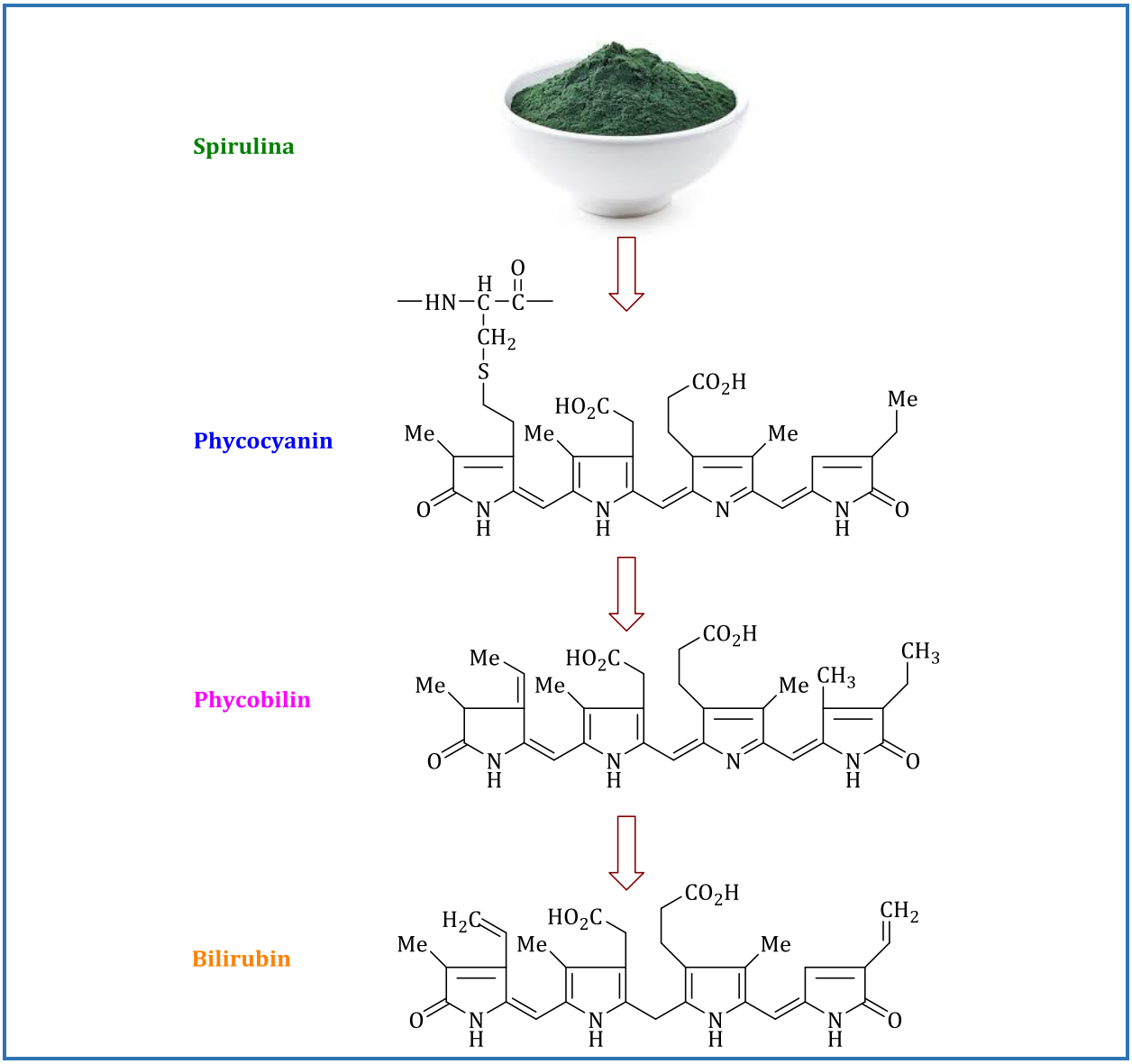
Graphical Abstract



Introduction

Cyanobacteria are the prokaryotic and photosynthetic microorganisms which have been adapted to a wide range of environmental conditions [1]. Spirulina is a string and spiral cyanobacteria with soft walls comprised of sugar and protein, and that is why it is easier to digest than the other algae. Also it has a 60% protein rate and contains all of the essential amino acid necessary for human health [2]. In addition, it is a rich source of vitamin B12 and gamma-linolenic acid (GLA) [3]. There are two classes of photosynthetic pigments in cyanobacteria. The first floor contains a water-soluble proteins that called phycobiliproteins. Another class is carotenoids and chlorophyll, are insoluble in water and

have small molecular weight [4]. Phycobiliproteins based on spectral properties are divided into three categories: phycoerythrin (Red), phycocyanin (Blue) and allo-phycocyanin (Cyan). As shown in Figure 1, in phycobilisome, the phycoerythrin at the tip of the bars, the phycocyanin adjacent to the nucleus, and at the other end of the bars is alophycocyanin [2, 5]. Phycocyanin is the main harvest sunlight pigment in cyanobacteria [6]. Phycocyanin is an oligomeric protein and consists of α and β subunits that can be traymr $(\alpha\beta)_3$ or hexamer $(\alpha\beta)_6$ interact. Phycocyanin is usually trimer and three chain tetrapyrrole that covalently binds to cysteine thioeter [7, 8]. It also has excellent spectral properties, such as high absorption and strong fluorescent emission factor [9].

Phycocyanin mainly has been used as a dietary nutritional supplements and it was as powerful anti-inflammatory, anticancer, antiviral and neuroprotective and hepatoprotective [7, 10]. Micromolar concentrations of phycocyanin are able to reduce the concentration of the peroxyl radicals in half, which indicates its antioxidant activity for this compound [11]. Phycocyanin is homologous of biliverdin and in mammalian cells is converted by biliverdin reductase to phycocyanorubin which has structural similarity to bilirubin. Bilirubin inhibit NADPH oxidase activity strongly in human cell culture [12]. In Figure 2, the structural similarity of Phycocyanin, phycobilin, bilirurin and bilirubin has been identified. Phycocynin has been shown to mimic the inhibitory effect of biliverdin/ bilirubin on NADPH oxidase activity [13]. NADPH oxidase in inflammatory conditions is the main source of superoxide activated and cause oxidative stress [14]. The function of this enzyme, molecular oxygen reduction in ROS (Such as superoxide) and reduced form nicotinamide adenine dinucleotide phosphate (NADPH) also acts as a cofactor for this reaction. These enzymes by transfer electrons from oxygen produce superoxide [15].

Oxidative stress is a major source of inflammation, causing the loss of dopaminergic neurons which results in neurological disorders such as Parkinson's disease [12]. Parkinson's disease is a chronic progressive disease of the central nervous system in the elderly and is rarely seen in young people. It is characterized by advanced and gradual muscular tightness, shaking and losing motor skills and after Alzheimer's is the most common malignant neurological disorder [16]. Oxidative stress and inflammatory processes that lead to the accumulation of α-synuclein in the dopaminergic cells and causing the death of these cells [17]. α-synuclein presynaptic protein that has been implicated in the etiology of Parkinson's. This protein involved in many neurological processes. Extracellular release of α-synuclein is Correlation with increases inflammatory cytokine and ROS [18]. NADPH oxidase can produce hypochlorous acid so that: NADPH oxidase produces superoxide anions (O^-) from NADPH and oxygen, hydrogen peroxide (H_2O_2) is also produced from superoxide anion by superoxide dismutase. Then myeloperoxidase produces hypochlorous acid (HOCl) from

H_2O_2 and anion chloride (Cl^-) (Figure 3) [19]. The anti-inflammatory action of phycocyanin done by removing HOCl**.** HOCl readily reacts with thiol and thioether groups [20].

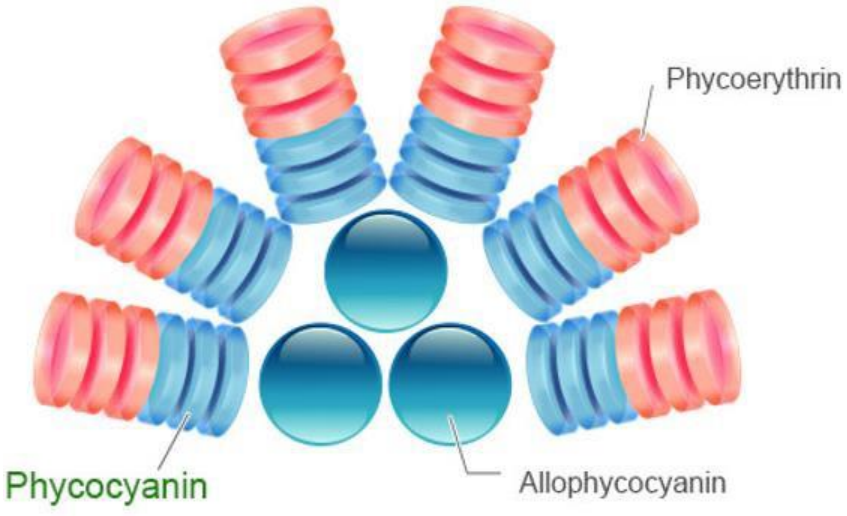
Phycocyanin by inhibiting NADPH oxidase prevents damage to dopaminergic neurons and progression of Parkinson's disease. Therefore, it seems that NADPH oxidase can be the ideal target for potential drug intervention, given that it plays an important role in α-synoclinic and neuronal activation. So phycocyanin with antioxidant and anti-inflammatory properties may be effective in the treatment of Parkinson's disease [21]. To date, there is no cure for this disease, but its symptoms with chemical drugs can be relieved or controlled [16]. Currently, L-doopa is used to treat the disease, which leads to chronic oxidative stress and mitochondrial damage caused by dopamine metabolism [22]. So phycocyanin has antioxidant, anti-inflammatory, and neuroprotection effects and potential benefits of its use in the prevention and treatment of pathological disorders associated with oxidative stress and inflammation are effective. Application of biotechnology in food science, treatment, immunology, identification, cosmetics and pharmaceuticals. The primary potential of these molecules are as natural dyes color in the industry [23].

Experimental

Materials and methods

Dried and powdered spirulina platensis, was bought from super food company and for extraction of phycocyanin were used enzymatic and ultrasound methods, to compare the two methods.

Figure 1. The stracture of phycobilisomes



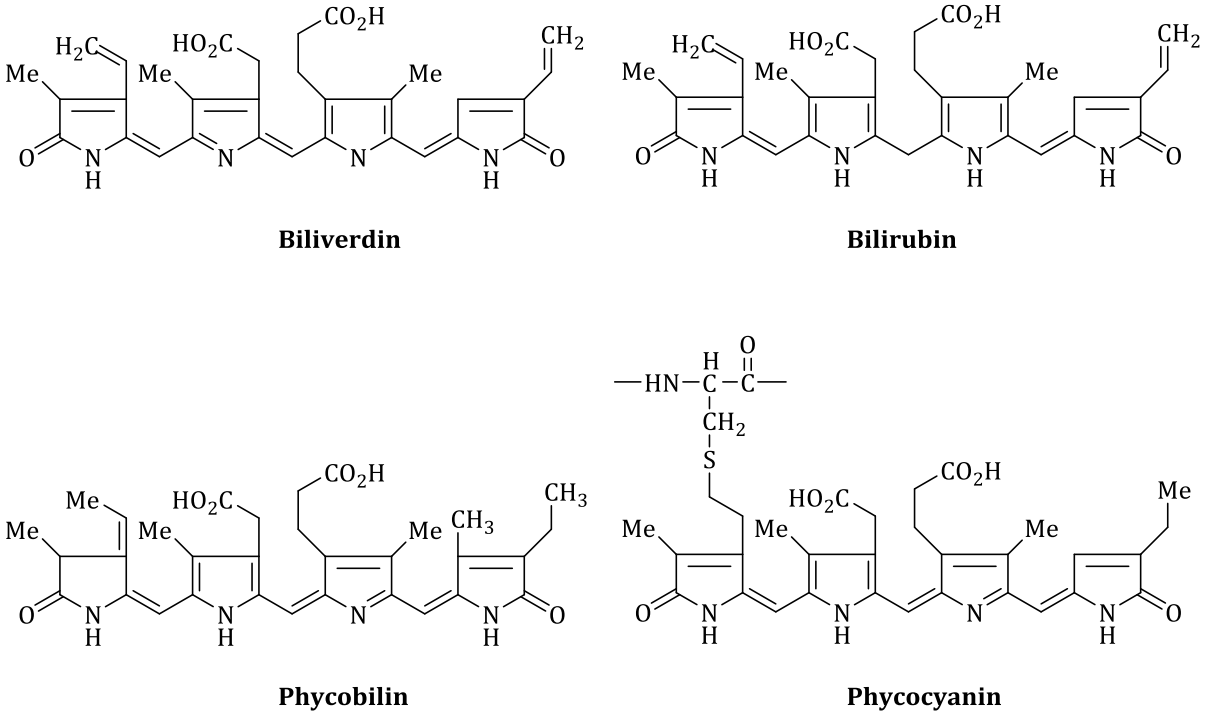
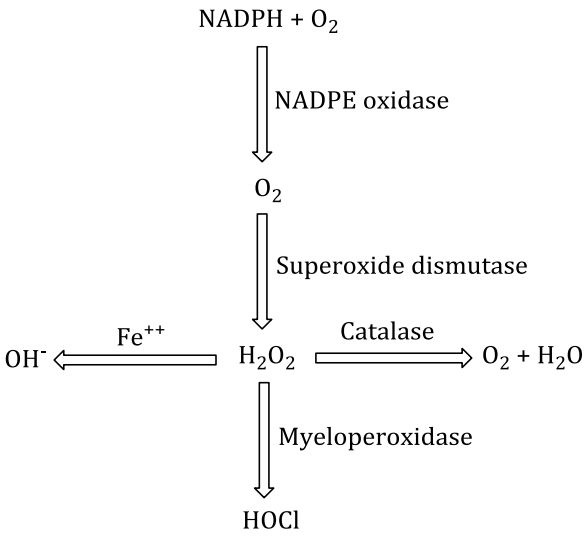


Figure 2. Comparison structural of phycocyanin with its homologues

Figure 3. HOCl production pathway by NADPH oxidase



Extraction of phycocyanin by enzymatically method

2 g of the dried spirulina was dissolved in 200 mL sodium phosphate buffer 0.1 M, pH = 7, containing 10 mmol EDTA and 100 μg/mL lysozyme enzyme. Then the mixture was mixed and stirred for 24 h at 30 °C to digestive cell wall of the algae by enzymatic method. After 24 h, the solution was

centrifuged in 4 °C for 1 h at 8000 rpm. The supernatant was then that the cyan color holding to continue of extraction process and the sediment containing the cell wall of algae discarded.

Extraction of phycocyanin by ultrasonic method

The dried spirulina was solved in distilled water at a ratio 1:25 (w/v) for 24 h at 4 °C. Then the solution was irradiated at 40 kHz for 1 h, and centrifuged at 10000 rpm in 4 °C for 15 min. The sediment was discarded and the supernatant was collected. The crud extract was kept in 4 °C and pH = 7.

Purification of phycocyanin by ammonium sulfate precipitation

Solid ammonium sulfate was gradually added to the crude extract to achieve 50% saturation while stirring and shaking. It was stirring for 1 hour and was kept overnight in 4 °C and dark condition. Then this solution was centrifuged at 10000 rpm for 30 min in 4 °C. The colorless supernatant was discarded and blue sediment containing phycocyanin was dissolved in small volume of sodium phosphate buffer (0.025 M for enzymatically method and 0.005 M for ultrasonic method), pH = 7 and was kept, in a brown bottle in 4 °C.

Partial purification of phycocyanin by dialysis

The obtained crude from the ammonium sulfate precipitation stage, was dialyzed for 24 h in 4 °C, against the phosphate buffer with the same concentration as it was solved.

Analysis by uv-spectroscopy

To detect the presence of UV absorbing, a UV-Visible spectrophotometer (Shimadzu-Japan- model: UV- 2550) was emplyed. The spectral range of the phycocianine are about 520 to 680 nm. Where, A615 is absorbance at 615 nm, A652 is absorbance at 652 nm, 5.34 is constant factor.

Analysis by fourier transfer infrared spectral (FT-IR)

The FT-IR spectroscopy was used to investigate the chemical structure and molecular bonds of compounds. The samples were blended with KBr and then the IR spectral analysis was performed in a fourier transmission infrared spectrophotometer (JASCO-4200).

Analysis by thin layer chromatography (TLC)

The phycocyanin was exposed to thin layer chromatography (TLC) in order to separate the existing bioactive compounds. The TLC plates of silica-gel (Merck) was used for this experiment.

Using a microcapillary tube, a small drop of pigment extract and standard of phycocyanin were placed on the TLC plate, and kept in the TLC tank which was saturated with methanol 100%. When the methanol reached 2 cm below the top, the plates were taken out of the tank and detected for the spots. R_f values of the spots were calculated.

High performance liquid chromatography or HPLC

Phycocyanin was identified by HPLC method. The column C18 (250 x 4.6 mm) was used. The mobile phase was a mixture of methanol and ammonium acetate 3%, in the ratio of 7:3, v/v. The temperature was set at 25 °C and wavelength at 615 nm. The phycocyanin obtained from both methods was injected into HPLC (SY-8100).

Polyacrylamide gel electrophoresis or SDS PAGE

SDS PAGE was carried out to reconfirm the purity of phycocyanin. Electrophoresis of dialyzed sample in polyacrylamide gel was carried out in a vertical chamber using 15% polyacrylamide gel with % 0.1 SDS and gels were stained by coomassie blue G250. The molecular markers and standard of Phycocyanin were used to identification the bands.

Assessment of anti-inflammatory and antioxidant activity

To determine the anti-inflammatory property of the phycocyanin, the reaction mixtures constituted of HOCl (NaOCl, 25/5%) and phycocyanin in the ratio of 1:6.5 were incubated for 30 min at 25 °C. Ascorbic acid or vitamin C was used as a control sample (250 mL phycocyanin and 500 μL ascorbic acid). The absorbance was measured by UV spectrophotometer (Shimadzu), the absorption of phycocyanin decreased at 615 nm and increased at 290 nm.

The antioxidant activity of the phycocyanin was measured by 1-diphenyl-2-picrylhydrazyl (DPPH assay). 0.3 mm of DPPH in 100% ethanol was solved and 3 mL was added of the phycocyanin dissolved in ethanol at different concentration. The solution was shaken and inside at room temperature for 30 min and the absorbance was measured by UV Spectrometer (Shimadzu) at 517 nm. The scavenging activity was determined and ascorbic acid (Vitamin C) was used as the control sample.

$$\%RSA = \text{Absorbance of control} - \text{Absorbance of sample} / \text{Absorbance of control} \times 100$$

Statistical calculations

Concentration obtained in the different stages of both enzymatic and ultrasound methods, was performed by using t-test by SPSS software. A value of $p<0.05$ was considered to be statically significant.

Results and discussion

The most important requirement for obtaining phycocyanin from Spirulina, choosing a suitable extraction method. Extraction of intracellular material, was done by destroying the cell wall. In the present study, using the enzymatic and sonication methods for the extraction of PC. In the enzymatic method, lysozyme enzyme was used to break the cell walls of algae, and EDTA and sodium phosphate buffer helps to release phycocyanin from the cell to the outside. In sonication method, ultrasonic waves was used to break down the cell walls of spirulina. Then the blue pigment was precipitated by 50% saturation with ammonium sulfate. The precipitate was purified by dialyzing against phosphate buffer. The absorbance at the end of each stage of extraction and purification are measured, then the purity and concentration of the sample was calculated (Table 1 and 2). UV absorption was determined by a spectrophotometer at 280, 615, and 652 nm. The purified phycocyanin has the highest absorption at 615 nm. Figures 4 and 5 has shown the UV-spectrophotometer from the enzymatic and sonication extraction, A: crude extraction step, B: ammonium sulfate purification step, C: dialysis, D: phacocianine standard. As it is seen, purified phycocyanin in the dialysis stage And its concentration has increased.

IR spectral analysis was performed and the functional groups of phycocyanin was determined which is in areas 1101.15, 1459.85, 1638.23, 3465.46, groups of C–O stretching in alcohol groups C=C in aromatic groups, C=C in Alkene groups, N–H stretching vibrations presence of amine (Protein) groups, O–H in Alcohol or N–H in Amide groups, has specified. (Table 3 and Figure 6 and 7).

In this study, thin layer chromatography (TLC) was used to characterization of phycocyanin. Silica gel was used as absorbents to separate the more polar substrates. The plate developed in methanol 100% showed spots of phycocyanin chromophore. The R_f values for all three samples (samples obtained from enzymatically, sonication method and standard of phycocyanin) was equal to was 0.88.

The concentrations of phycocyanin at high performance liquid chromatography (HPLC) with column C18 and methanol ammonium acetate 3% as a mobile phase, for the samples obtained from enzymatically was 0.405 mg mL and sonication was 0.422 mg/mL. Figure 8, has shown the HPLC graph, for phycocyanin obtained from enzymatic method and Figure 9, has shown the HPLC graph for phycocyanin obtained from sonication method.

Table 1. Concentration of phycocyanin obtained from spirulina platensis from enzymatic and ultrasound

Purification step	Enzymatic method (mg/mL)	Ultrasound methods (mg/mL)
Crude extract	0.230	0.238
$(NH4)_2SO_4$ Percipitation	0.272	0.303
Dialysis	0.323	0.345

Table 2. Purification data of phycocyanin obtained from spirulina platensis from enzymatic and ultrasound methods

Purification step	Method (Absorbance raito)			
	Enzymatic A615/A280	ultrasonic A615/A280	Enzymatic A652/A280	Ultrasonic A652/A280
Crude extract	0.703	0.713	0.678	0.699
$NH_4)_2SO_4$Percipitation	0.886	1.047	0.756	0.872
Dialysis	1.866	2.471	1.368	2.013

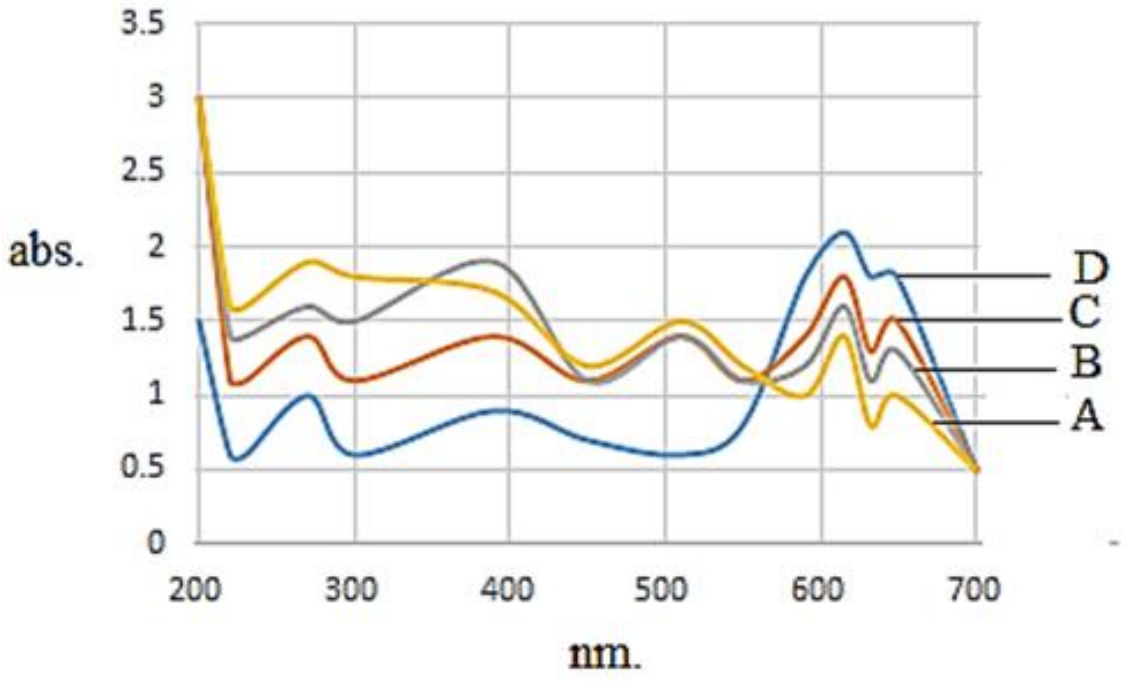


Figure 4. UV-spectrophotometer diagram of the enzymatic extraction process, A: extraction of crude, B: purification step with ammonium sulfate step, C: dialysis, D: standard phacocianine

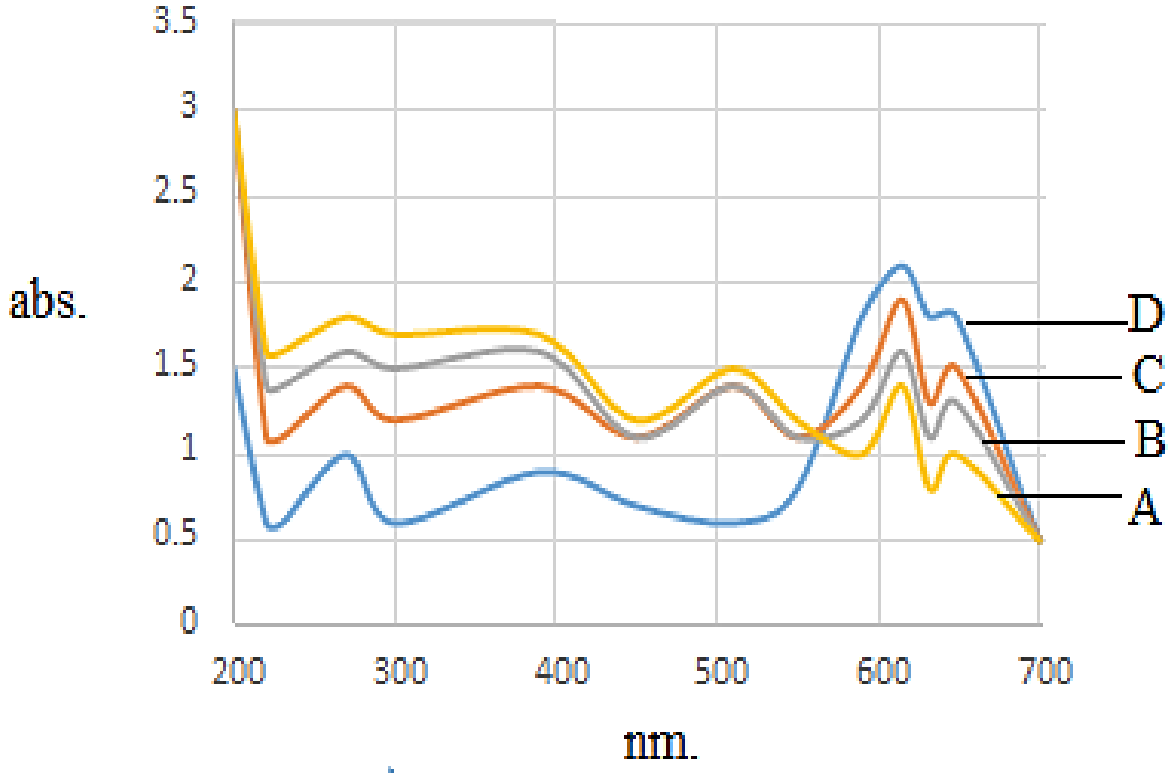


Figure 5. UV-Spectrophotometer diagram of the sonication extraction process, A: extraction of crude, B: purification step with ammonium sulfate step, C: dialysis, D: standard phacocianine

Table 3. Functional groups of blue pigment from phycocyanin

Wavenumber	Functional groups
1101.15	C-O stretching in alcohol groups
1459.85	C=C in aromatic groups
1638.23	C=C in Alkene groups
3465.46	N-H stretching vibrations presence of amine (proteins) groups, O-H in Alcohol or N-H in Amide groups

Purity was also confirmed by re-attendance from the single-unit bands of α-subunit and β–subunit during gel electrophoresis. Figure 10 has shown the scanned image from the SDS PAGE; (a: standard of phycocyanin, b: ladder or molecular weight markers, c: phycocynin obtained from sonication to 20 μl, d: samples of enzymatically to the 20 μl, e: samples obtained from sonication to the 10 μl, f: The sample 10 μl enzymatically method). Molecular weight of Phycocyanin was between 19–14 KD that estimated based on molecular markers.

DPPH is a stable violet color radical that turns to yellow or Brown close to yellow. When it reacts with

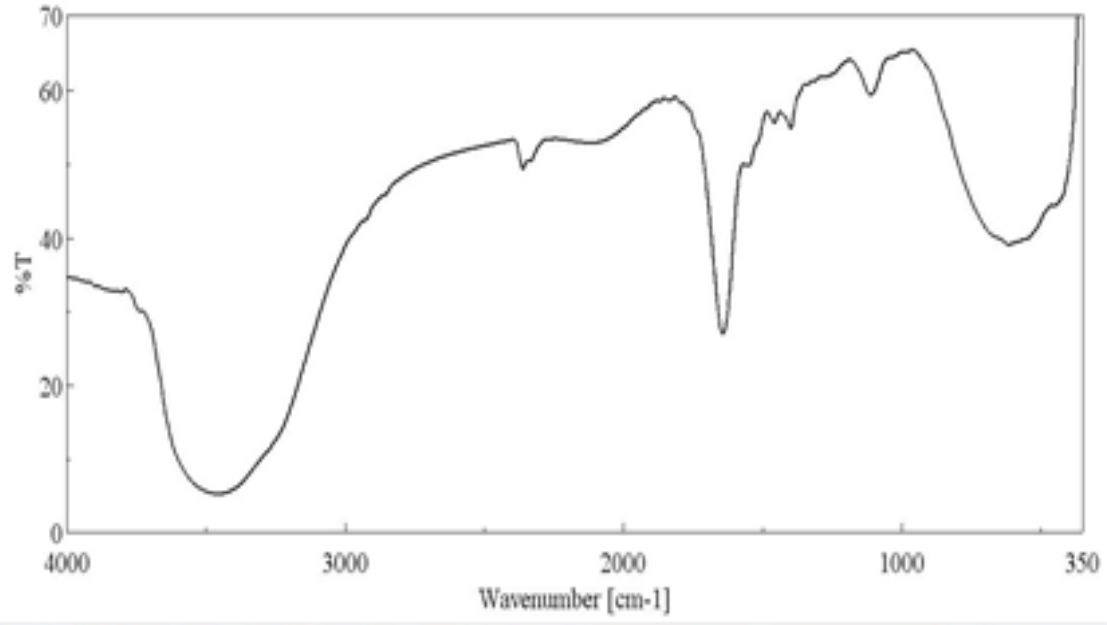


Figure 6. FT-IR charts of dialysis sample obtained from enzymatically

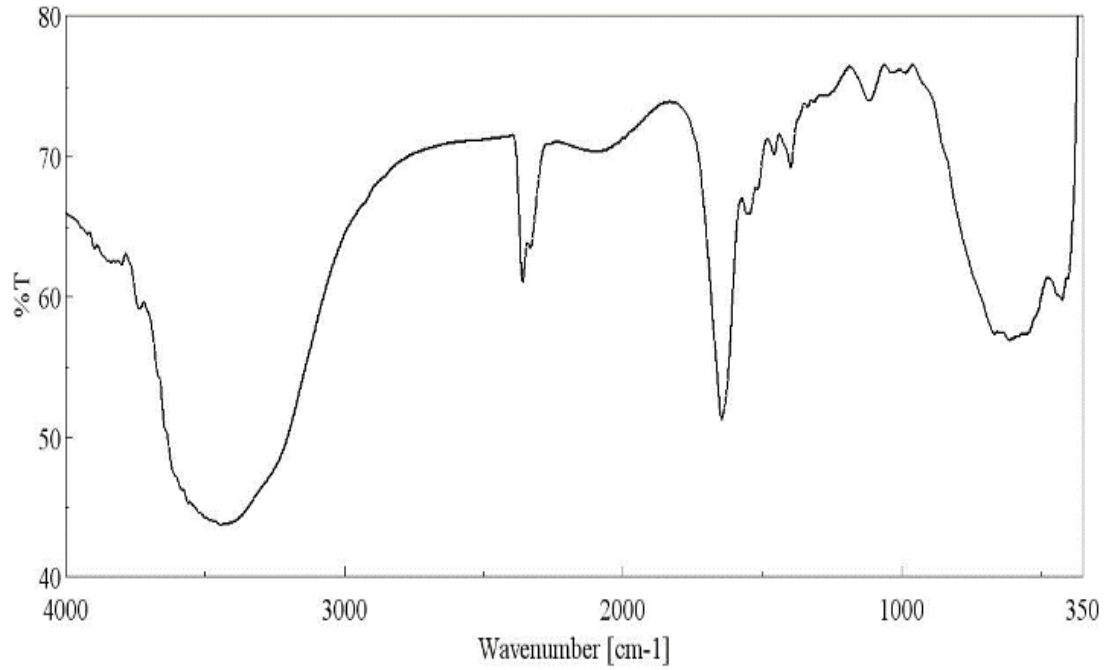


Figure 7. FT-IR diagram of dialysis sample obtained from sonication

antioxidants and the degree of color change indicates the scavenging potentials of antioxidant pigment. The scavenging activity was determined as compared with the standard vitamin C. The phycocynin showed radical scavenging activity of 23.51%. Therefore, this pigment is potent free radical scavenger.

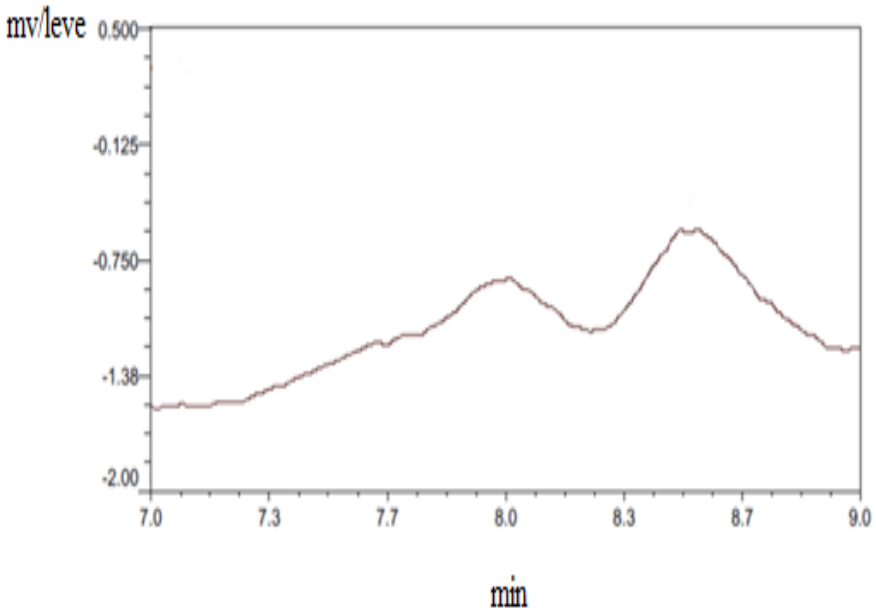


Figure 8. HPLC graph, for phycocyanin obtained from enzymatic method

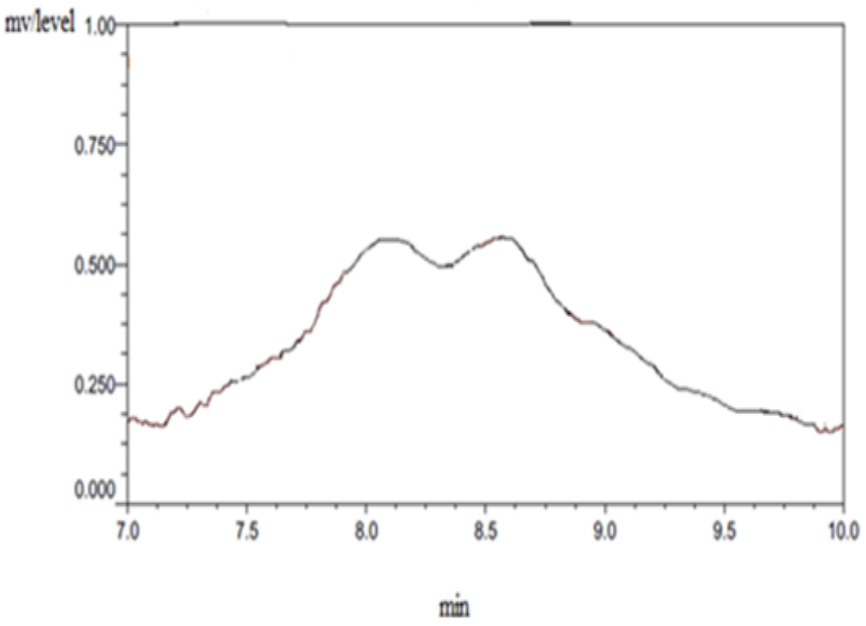


Figure 9. HPLC graph for phycocyanin obtained from sonication method

The antiinflammatory action of phycocyanin could be mediated at least in part by its capacity to remove HOCl. At high HOCl/protein levels, the whitening of the absorbance is total, indicating that all the bilin groups of phycocyanin are Completely disturbed. The scavengingactivity was determined and ascorbic acid (Vitamin C) was used as the control sample . The RSA for this test was calculated as 12.94.

Spirulina platensis with phycocyanin as a high-quality protein is widely having commercial and industrial applications. Phycocyanin is the main light harvesting pigment in Spirulina and include about 15% of the dry weight of this algae. Recent research has shown that is has medicinal properties and applications. One of the most important requirements for obtaining phycocyanin from spirulina dry is choice of extraction and purification method. So far, several methods have been used to extract phycocyanin. The extraction yield and purity of phycocyanin concentration depends on how the destruction of the cell coverage. The Phycocyanin located on the thylakoid.

In this study we have used two methods to extract phycocyanin, in the first method, the lysozyme and EDTA and sodium phosphate buffer were used that would destabilize the cell membrane. And in the second method, ultrasound used to destroy cell membrane of algae.

Phycocyanin was purified in several steps. First the PC was salted out with 50% ammonium sulphate which causes the pigment surface are neutralized and precipitated outside. Ammonium sulfate also prevents of phycocyanin denaturation. Then the crude was dialyzed against phosphate buffer to remove small contaminants. The concentration of the phycocyanin was measured by the UV absorption.

The UV visible absorption pattern of phycocyanin from spirulina platensis shows maximum absorbance at 652 and 615 nm. The purified phycocyanin has the highest absorption at 615 nm which represents a covalent bond that binds to prosthetic groups, which are the same as billins.

FT-IR is the most widely used to identify the chemical structure of the compounds which used in the medical and pharmaceutical fields. It is also a tool for screening chemical compounds that can be employed to characterize biomolecules in drug discovery. In this study FT-IR spectrum indicate the presence of ketone amide C=N and ester C–H bonds in phycocyanin [5].

HPLC, or high performance liquid chromatography was used to view the major peak of phycocyanin and calculated the concentrations of phycocyanin [24]. The concentrations obtained at for the enzymatically was 0.405 mg/mL and for sonication 0.422 mg/mL. According to concentrations obtained by the ultrasound method seems more efficient at extracting phycocyanin, and the procedure is far more economically affordable. SDS PAGE or protein electrophoresis was performed in vertical tanks SDS or sodium dodecyl sulfate was used and the material which is negatively charged proteins move to the gel under the influence of an electric current [25].

α and β subunits bands of phycocyanin was appeared and molecular weight of ς hycocyanin was estimated between 19–14 KD. The anti-inflammatory and antioxidant activity of phycocyanin estimated by reaction witch HOCl and DPPH. Antioxidants play a major role in protection of human body against damage of ROS. Epidemiology studies have shown that intake of natural antioxidants can reduced the risk of cancer, diabetes and other disease associated with oxidative stress. The

results showed that the extract had a protective effect on cell death due to DPPH-induced apoptosis. The extract can reduce its effect by removing free radicals, which causes the apoptotic pathway to be activated. The extract can action its effect by reducing oxidative damage due to free radicals. Phycocyanin reacts with DPPH, which is nitrogen centered radical with characteristic absorption at 517 nm and convert it to stable diamagnetic molecule 1, 1 dipenyl-picryl hydrazine, due to its hydrogen donating ability at rapid rate [5, 26]. The observed reaction of phycocyanin with HOCl can be due to the presence of methionine groups in this molecule. The Hypochlorite is generated by the activity of the NADPH oxidase in the human body [19].

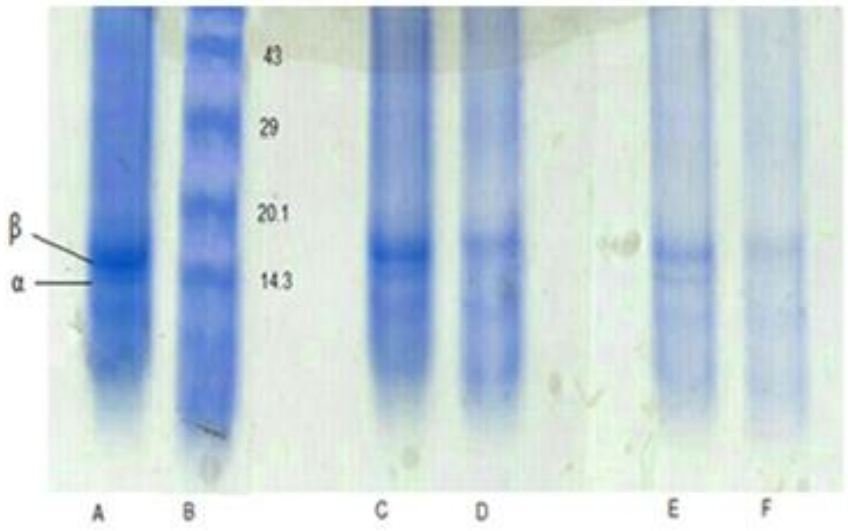


Figure 10. Image taken from SDS PAGE. a) Standard phycocyanin, b) Ladder or molecular weight markers, c) samples obtained from sonication to 20 µl, d) samples of enzymatically to the 20 μl, e) samples obtained from sonication to the 10 μl, f) The sample 10 µl enzymatically

Conclusion

Phycocyanin due to the structural similarity with biliverdin as a strong inhibitor of NADPH oxidase and the radicals produced in the process of inflammation in the human body. NADPH oxidase as the main source of reactive oxygen species (ROS) and free radicals, plays a crucial role in developing the related oxidative stress diseases. This illnesses as a result of an imbalance between the production of ROS and antioxidant and anti-inflammatory capabilities of a given system, which results in stimulation of degenerative signaling pathways, which often leads to indirect tissue growth, inflammation and physical immune responses. Therefore, trying to find specific inhibitors of NADPH oxidase, have been intensified in recent years. Most chemical inhibitors have side effects for the human body. However, the natural peptide inhibitors such as Phycocyanin, have the potential to target specific parts of the human body.

Acknowledgments

The authors appreciate the Payame Noor University of Isfahan for its financial support to carry out this research.

Disclosure statement

No potential conflict of interest was reported by the authors.

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How to cite this manuscript: Mahdieh Izadi*, Mohammad Fazilati. Extraction and purification of phycocyanin from spirulina platensis and evaluating its antioxidant and anti-inflammatory activity. *Asian Journal of Green Chemistry*, 2018, 2, 364-379. DOI: 10.22034/AJGC.2018.63597