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IN VITRO ANTIOXIDANT ACTIVITY OF C-PHYCOCYANIN PURIFIED FROM *Spirulina platensis* DRY BIOMASS

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Abstract. C-phycocyanin (C-PC) was a phycobiliprotein found exclusively in cyanobacteria as *Spirulina* and exhibited a variety of pharmacological properties, which were not yet studied for any purpose in commercial applications in the pharmaceutical and cosmetic industry in Vietnam. At present, there are no other sources of natural phycocyanin than *Spirulina*, it is the sole efficient source of natural phycocyanin production under photoautotrophic conditions. The present research was conducted to determine an efficient extraction method from C-PC from *Spirulina platensis* dry biomass. Antioxidant activity of purified C-PC was investigated. Extractions were carried out using distilled water and Na-phosphate buffer pH 7.0 as solvents and freeze/thaw and ultrasonication techniques were applied to optimize the extraction process. Extraction using the freeze/thaw method proved to be the most efficient method. Phycocyanin purification from crude extracts was carried out by a combination of methods such as ammonium sulfate $(NH_4)_2SO_4$ 65% precipitation, dialysis filter, and Sephadex G-100 gel filtration chromatography, to give 121.63 ± 0.03 mg/g C-PC content and 3.52 ± 0.04 purity. Antioxidant activity *in vitro* was determined by nitric oxide (NO) scavenging activity, percentage inhibition of NO by the C-PC in concentration 10 µg/mL to 100 µg/mL which significantly scavenged 55.89% to 91.54% of NO radicals, respectively. They exhibited strong NO radical scavenging capacity, the C-PC and vitamin C were able to reduce the stable NO radical to 50% reduction with EC_{50} of 4.53 and 3.66 µg/mL. Research results showed that *S. platenis* dry biomass provided by Dalitra was potential for obtaining C-phycocyanin (12.16% of dry biomass) and it was evaluated as an antioxidant *in vitro* able to scavenge nitric oxide. C-PC (12.16% of dry biomass) and was evaluated as an antioxidant *in vitro* able to scavenge nitric oxide.

Keywords: C-phycocyanin, antioxidant, *Spirulina*, pharmacological.

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1. Introduction

C-phycocyanin (C-PC) is a blue phycobiliprotein found in *Spirulina*, whose main function was to capture and transmit light energy to chlorophyll when chlorophyll had poor absorbability and transmit ability [1-3]. C-PC was used as a nutritional ingredient, a fluorescent marker, or as a natural colorant in food and cosmetics, and was known as an antioxidant [4, 5], anti-inflammatory [4, 6], anticancer, antifungal, and antiviral agent in nature [7]. C-PC also had a role in renal protection [4]. Among the multitude of biological activities, the antioxidant function of C-PC could be the most valuable. In fact, C-PC could protect living cells from "oxidative stress" by delaying or inhibiting lipid oxidation [8], as well as its ability to fight free radicals [5].

Several medical treatments using antioxidant foods have shown that consuming antioxidant food ingredients can maintain a balance between the antioxidant system and oxidative radical production (ROS) [8, 9]. Therefore, the human body can resist various diseases such as atherosclerosis, alzheimer, cancer, diabetes, rheumatoid arthritis, inflammatory diseases, and the aging process [10, 11].

In Vietnam, the research focused on extraction and purification of C-PC in *Spirulina*, its antioxidant activity requires further investigation [12, 13]. The objective of this study was to investigate the *in vitro* antioxidant activity of C-PC obtained from *Spirulina platensis* biomass and determine the optimal conditions for C-PC extraction and purification to enhance the antioxidant activity of C-PC.

2. Content

2.1. Method

**** Material***

Spirulina platensis biomass was provided by Dalitra Technology Co., Ltd (Nam Dinh, Vietnam). Microalgae were grown under controlled conditions and harvested by the company during log-phase growth. The biomass was then dried in a freeze-drying system. Biomass was stored under a vacuum.

Chemicals used in the study were from Merck, Germany, and Sigma Aldrich, USA.

**** Extraction of C-phycocyanin***

C-PC was extracted from the biomass of *Spirulina platenis* by the following methods: 500 mg of microalgae dry biomass was dissolved in 100 mL of different solvents: (DW) distilled water; (PB) sodium-phosphate buffer pH 7.0, 100 mM; (PBDW) sodium-phosphate buffer pH 7.0, 100 mM and distilled water at a ratio of 1:1 (v/v).

Cell disruption was accomplished by the following mechanical methods: Freeze - thaw: Biomass was frozen and thawed with 3 repeated cycles at -22 ^{o}C and 20 ^{o}C for 24 hours.

Ultrasonication: glass beads were added to biomass at a weight ratio of 1:1 (biomass : glass beads), then placed in an ultrasonic bath (50 kHz) for 40 minutes [14, 15].

The sample was centrifuged at 3500 rpm for 5 minutes, then supernatant of crude C-PC extract was collected and evaluated for content and purity.

**** Purification of C-phycocyanin***

C-PC purification was performed by direct precipitation with ammonium sulfate $(NH_4)_2SO_4$, at a concentration of 65%, overnight at 4°C. The blue precipitate was recovered by centrifugation at 4°C at 27000 rpm for 15 minutes and dissolved in 10 mL of pH 7.0, 100 mM Na-phosphate buffer.

The solute was dialyzed by membrane filtration (HiMedia, Mumbai, 12 - 14 kDa). Dialysed filtration was performed twice with 1000 mL extraction buffer at room temperature (1st time) and 4°C (2nd time) overnight. The extract was recovered from the dialysis membrane and filtered through a 0.45 mm membrane filter [16].

Dialysed phycocyanin was passed through a pre-equilibrated Sephadex G-100 (2.5 cm) column and eluted with 0.005 M Na-phosphate buffer (pH 7) at 1 mL/min. The fraction running through the blue column was collected and evaluated for purity.

**** Determine concentration and purity of phycocyanin***

The concentration and purity of phycocyanin were determined by spectrophotometric methods as previously described [1].

The optical absorbance and fluorescence of phycocyanin were at about 620 nm and 640 nm, respectively [17]. The optical absorbance at 620 nm showed the maximum C-PC absorption. The absorption band at 652 nm was characterized by allophycocyanin; while the absorption band at 280 nm was characterized by other proteins in solution [1].

Concentrations were calculated according to the formula below using absorbances at 620 nm (Abs_{620}) and 652 nm (Abs_{652}) using a 1900Yoke™ UV spectrophotometer.

$$CPC_{concentration}\left(\frac{mg}{ml}\right) = \frac{Abs_{620} - 0.474 \times Abs_{652}}{5.34},$$

$$CPC_{content}\left(\frac{mg}{ml}\right) = \frac{CPC_{concentration}\left(\frac{mg}{ml}\right) \times Volume}{Biomass}.$$

The purity of C-PC was assessed by absorption ratio Abs_{620}/Abs_{280}, purity 0.7 was accepted for food [1].

$$Purity = \frac{Abs_{620}}{Abs_{280}}.$$

**** Determination of antioxidant activity***

Nitric Oxide (NO) scavenging activity: Two substances were included for the assessment of antioxidant activity, which was purified C-PC in the study and vitamin C. The NO scavenging activity was measured by using Griess reagent: 50 µL, 100 µl, 150 µL, 200 µL and 250 µL of purified C-PC (121.63 mg/g, purity 3.52) and 500 µL of standard (vitamin C 1 mg/m µL) were taken and diluted up to 1.5 mL with distilled water in test tubes. Then, add 1.5 mL of 10 mM sodium nitroprusside to each tube and incubate at 25 °C for 150 minutes.

1.5 mL of the reaction mixture after incubation was transferred to new tubes and 1.5 mL of Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% NEDD) were added.

The samples were determined for optical absorbance at 545 nm [18, 19]. The percentage of NO scavenging activity was calculated according to the following formula:

$$NO_{scaveging}(\%) = \frac{(Abs_{control} - Abs_{sample}) \times 100}{Abs_{control}}.$$

In this experiment, vitamin C was used as a positive control to generate a standard curve. vitamin C antioxidant capacity of the sample was calculated from the vitamin C standard curve and. The EC_{50}, defined as the concentration of sample result in 50% reduction of the initial NO concentration, was calculated using the relationship between the percentage scavenging activity and the sample concentration.

**** Data processing***

Each experiment was repeated at least three times. Data are expressed as mean ± standard deviation (mean ± SD). Microsoft Excel 2006 software is used to calculate data and draw graphs. The data were statistically processed using Statistical Package for the Social Sciences 16.0 software. Mean values were analyzed by ANOVA according to Duncan's test. The p value < 0.05 indicated a statistically significant difference.

2.2. Results and discussion

2.2.1. Extraction of C-phycocyanin

The extraction method was key for maximum recovery of phycobilliprotein from algae [20]. The process of releasing proteins out of the cell was done by the breakdown of microalgae cells, *Spirulina* was small but the cell wall was composed of many layers with high resistance. Two solvents and two mechanical methods were used to disrupt the cell wall. The solvent used to evaluate the extraction efficiency of C-PC was distilled water and sodium-phosphate buffer at pH 7.0. The two methods of choice for implementation were freeze/thaw and ultrasonication. Figure 1a showed that the C-PC content was 57.93 - 74.32 mg/g of dry biomass when extracted by ultrasonication method in all three solvents gave the highest efficiency. The content of C-PC extracted by sodium-phosphate buffer (60.68 - 74.32 mg/g of dry biomass) was higher than that of the other two solvents in both freeze/thaw and ultrasonication. Compared to the same extraction method, Na-phosphate buffer gave the highest concentration of C-PC 60.68 mg/g, distilled water with C-PC of 59.67 mg/g of dry biomass, and the lowest C-PC was 46.08 mg/g dry biomass with Na phosphate-distilled water buffer (1:1, v/v) with the freeze/thaw method (Figure 1a).

For the purpose of obtaining C-PC for use in food, cosmetics, and pharmaceuticals, a buffered Na phosphate-distilled water (1:1, v/v) mixture was used for the study. However, the results have not yet achieved high efficiency, the highest C-PC content obtained with the ultrasonication method was 57.93 mg/g of dry biomass, and 46.08 with the freeze/thaw method. There was not a big difference in C-PC content when extracted with distilled water or Na-phosphate buffer in the same extraction method, the

difference ratio was 1.66% (C-PC content 59.67 mg/g and 60.68 mg/g dry biomass, respectively) with the freeze/thaw method; 10% (66.86 mg/g and 74.32 mg/g, respectively) with ultrasound. The results also showed that when cells were frozen, it was inevitable that intracellular ice formation, leading to cell damage, promotes better extraction of intracellular substances [21]. And the breaking phenomenon combined with the abrasive effect produced by the glass particles facilitated the extraction of intracellular proteins [14].

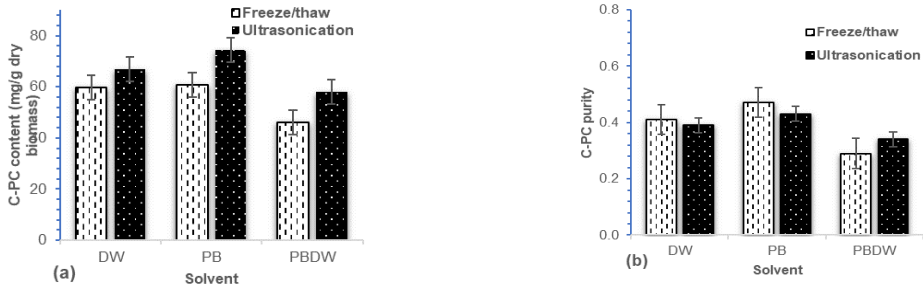

Figure 1. Content and purity of C-PC extracted by different methods and solvents from S. platensis

Ultrasonication had been shown to be the best; the ultrasonication method gave 10.67 - 18.35% higher extraction efficiency than freeze/thaw, but large-scale production would be difficult. The freeze/thaw method has a number of advantages such as simplicity, reproducibility, continuous extraction because it is independent of biomass, no abrasive materials and no significant loss of biological activity of proteins.

The concentration of C-PC extracted by freeze/thaw was 60.68 mg/g of dry biomass giving a purity of 0.47, while the ultrasonication method was 0.43 (Figure 1b). The C-PC crude extract extracted from dry biomass with distilled water and Na-phosphate buffer was statistically different in both C-PC content and purity. The difference was shown in the inverse correlation between the concentration and purity of C-PC with two independent solvents of distilled water and Na-phosphate buffer (Figure 1a and Figure 1b).

It could be seen that the extraction method without the solvent, had affected the C-PC content. The freeze/thaw method may be of more interest because of the higher purity phycocyanin content of 0.47.

2.2.2. Purification of C-phycocyanin

Purification is an important step to separate complex molecules. In this study, the crude C-PC extract was purified in three steps: precipitation with ammonium sulfate $(NH_4)_2SO_4$ at 65% concentration, filtration by dialysis and through Sephadex G-100 column chromatography. After each purification step, the concentration and purity of C-PC were checked. Table 1 showed the increase in purity after each step.

Table 1. Purity of C-PC extracted from S.platensis after different purification steps

Purification steps	C-PC content (mg/g)	Purity(A_{620}/A_{280})
Crude extract	60.68 ± 0.07	0.47 ± 0.02
Ammonium sulfate 65%	99.51 ± 0.13	0.89 ± 0.05
Dialysis	136.85 ± 0.09	1.45 ± 0.08
Sephadex G-100	121.63 ± 0.03	3.52 ± 0.04

Precipitation with 65% ammonium sulfate gave purity of 0.89 and obtained 3.52 after passing through a Sephadex G-100 chromatographic column. Dau Thi Nhung et al. purified phycocyanin in 2 steps (precipitated with ammonium sulfate 20, 50 % followed by LH20 column chromatography) and gave the purity of 1.2 [12]. The difference between results may be due to the steps in the process affected purity. In this study, the sample was filtered by dialysis before going through a Sephadex G-100 column chromatography.

Membrane dialysis in the second purification step was effective when the purity reaches 1.45 and the C-PC content is 2.25 times higher than the original crude extract (Table 1).

Kumar et al. purified phycocyanin from *S. platensis* by DEAE cellulose ion-exchange chromatography to phycocyanin purity of 4.5 [16]. The results of the presented study and literature review suggest that DEAE column chromatography may be a more efficient method than gel filtration chromatography for the purification of phycocyanin from *S. platensis*.

C-PC presented a final extraction content of 121.63 ± 0.03 mg/mL with a purity of 2.317 ± 0.08. The content of C-PC accounted for 12,163% of dry biomass, which is consistent with studies in the world on this microalgae and studies evaluating commercial *spirulina* products in Vietnam [12, 13].

2.2.3. Nitric oxide (NO) scavenging effect

Nitric oxide (NO) is an important chemical mediator produced by endothelial cells, macrophages, and neurons and is involved in the regulation of various physiological processes. Excessive NO levels are associated with a number of diseases. The reaction between oxygen with excess nitric oxide produces nitrite and peroxynitrite anions, which act as free radicals. In this study, purified C-PC was added to compete with oxygen in the reaction with nitric oxide and thereby inhibit the generation of anions.

Nitric oxide is made from sodium nitroprusside reacts with C-PC and vitamin C to form the nitrite anion. The nitrite ions are diazotized with acid sulphanilamide and naphthyl ethylenediamine. When C-PC and vitamin C transfer protons to nitrite radicals, the absorbance is reduced. The absorbance reduction was used to measure the extent of nitrite recovery. Table 2 showed the percentage of NO scavenging by C-PC at concentrations of 10 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL, NO removal at 55.89%, 78.25%, 85.67%, 91.54% and 91.54%. They exhibited strong NO radical scavenging capacity with valuable antioxidant content equivalent to vitamin C of 4.09 ±

0.12 µg/mL to 6.70 ± 0.02 µg/mL. The NO radical-scavenging activity increased in a concentration-dependent manner (Table 2).

Table 2. Antioxidant capacity of purified C-PC from S.platensis and vitamin C

Drug	Dose (µg/mL)	% NO scavenging	Antioxidant content equivalent to vitamin C (µg/mL)	EC_{50} (µg/mL)
Control	-	0.021±0.01		
C-PC	10	55.89 ± 0.35	4.09 ± 0.12	4.53
	25	78.25 ± 0.48	5.73 ± 0.06	
	50	85.67 ± 0.27	6.27 ± 0.04	
	100	91.54 ± 0.65	6.70 ± 0.02	
Vitamin C	25	94.42 ± 0.31		3.66
	50	98.53 ± 0.42		

Linear regression equation of vitamin C: y = 1.1011x + 45.961 (R^2 = 0.9879)

Linear regression equation of C-PC: y = 18.135x - 32.163 (R^2 = 0.9536)

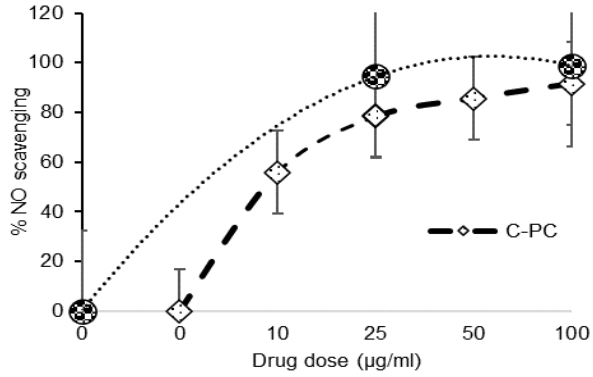

Figure 2. Antioxidant activity of C-PC extracted from S.platensis and vitamin C on nitric oxide radicals

Results of positive control with vitamin C showed high NO scavenging activity of C-CPC, which were 94.42% and 98.53% equivalent to vitamin C concentrations of 25 µg/mL and 50 µg/mL (Figure 2). The C-PC and vitamin C were able to reduce the stable NO radical to 50% reduction with EC_{50} of 4.53 µg/mL and 3.66 µg/mL. The antioxidant capacity of C-PC in the NO radicals assay was lower than the vitamin C standard.

Suresh et al. reported that C-PC has NO radicals scavenging activity with 92.58 % at 100 µg/mL concentrations [22]. While Wu et al. show ABTS and DPPH scavenging activity [23]. Therefore, research will continue to be expanded with investigations on different antioxidant activities.

3. Conclusions

For industry applications, the processes must achieve high extraction efficiency, and be environmentally friendly. In the study, mechanical methods can easily disrupt cells, compared to very expensive enzymatic methods or chemical methods, which will denature proteins. The freeze/thaw extraction method with Na-phosphate buffer has been shown to be the most effective.

To purify C-PC, different precipitates can be used such as PEG, ethanol, acetone, TCA and ammonium sulfate**.** The study chose ammonium sulfate 65% due to its easy precipitation, low dissolution temperature and bacteriostatic properties. C-PC after precipitation has a purity of 0.89; increased to 1.45 after being dialyzed through a membrane filter. Purified again through the Sephadex G-100 chromatographic column, the purity increased to 3.52 corresponding to the obtained C-PC content of 121.63 mg/g. The approximate purity achieved at the reaction level of C-PC is 3.9.

When it was evaluated as an antioxidant in vitro, it was able to scavenge nitric oxide. The NO radical-scavenging activity increased in a concentration-dependent manner. Antioxidant activity of purified C-PC was 55.89-91.54% NO scavenging. They exhibited strong NO radical scavenging capacity with valuable antioxidant content equivalent to vitamin C of 4.09 ± 0.12 µg/mL to 6.70 ± 0.02 µg/mL. The C-PC and vitamin C were able to reduce the stable NO radical to 50% with EC_{50} of 4.53 µg/mL and 3.66 µg/mL.

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