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# Unraveling the unique bioactivities of highly purified C-phycocyanin and allophycocyanin

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## Abstract

**Background** The blue-green microalgae Spirulina, used in human nutrition for centuries, includes phycobiliproteins such as C-phycocyanin (CPC) and allophycocyanin (APC). Assessing their unique bioactivities separately is difficult as they have similar properties, such as molecular weight and isoelectric point. In the present study, we aimed to separate CPC and APC and to evaluate their bioactivities. CPC and APC were separated using a hydrophobic membrane and ammonium sulfate, which promotes reversible and specific protein binding to the membrane. Spectroscopic analysis, HPLC, and SDS-PAGE revealed a successful separation of CPC and APC. Their bioactivities were evaluated through CCK- 8 assays for anticancer activity, radical scavenging assays for antioxidant activity, and albumin denaturation assays for anti-inflammatory activity, respectively.

**Results** The results revealed that highly purified APC showed 40% higher anticancer activity than the control, whereas CPC increased the viability of cancer cells, resulting in a 30% decrease in anticancer activity compared to the control. In contrast, highly purified CPC showed approximately 25% higher antioxidant activity and twice as much anti-inflammatory activity as APCs; moreover, the presence of both showed higher antioxidant activity.

**Conclusion** This study provides important insights into the unique bioactivities of CPC and APC for their appropriate application as anticancer, antiphlogistic, and antioxidant agents.

**Keywords** C- phycocyanin, Allophycocyanin, Highly purified, Unique bioactivities

## Background

Phycobilisomes are elaborate antennae observed in cyanobacteria and red algae. This large protein complex captures incident sunlight and transfers the energy through a network of embedded pigment molecules called bilins to the photosynthetic reaction centers [1]. The most popular genus of cyanobacteria is *Spirulina*, which contains phycobilisomes comprised mainly of three phycobiliprotein classes (i.e., C-phycocyanin, allophycocyanin, and phycoerythrin) based on their long-wavelength absorption maxima [2]. C-phycocyanin (CPC) is the most abundant phycobiliprotein in cyanobacteria, and other phycobiliproteins are present in lower amounts [3]. Specifically, *Spirulina maxima* has the highest CPC content, followed by allophycocyanin (APC); whereas, phycoerythrin (PE) is almost absent [4]. These

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complexes play an important light-harvesting function in photosynthesis, and have important bioactivities such as anti-oxidation, anti-cancer, and anti-inflammatory effects, which are widely used in medicine, food, and other industries [5].

CPC extracted from cyanobacteria exhibits several beneficial bioactivities. It blocks tumor cell proliferation, inhibits the cell cycle, and induces apoptosis and autophagy of cancer cells [5]. Nishanth et al. [6] demonstrated a concentration-dependent reduction in the proliferation of HepG2 cells following CPC treatment at 1, 10, 25, 50, and 100 μM. A dose-dependent reduction in K562 cell proliferation was observed 48 h after CPC treatment (10, 25, 50, and 100 μM) [7]. CPC is an effective scavenger of reactive oxygen species (ROS), including the most prevalent free radicals such as hydroxyl radicals (•OH) and hydrogen peroxide ($H_2O_2$) [8]. Grover et al. [9] reported that CPC at 500 mg/kg and 1000 mg/kg resulted in significant enhancement of serum SOD and catalase activities, which were higher than those of vitamin E. Park et al. [10] reported that CPC extracted using sonication revealed 14.4–19.1 μmol Trolox/g and 42.5–108.3 μmol Trolox/g of DPPH and ABTS radical scavenging activities, respectively. The anti-inflammatory effects of CPC have also been demonstrated [11].

Comparing the bioactivities of CPC is difficult as different assays are used, which can lead to differences in mechanism, pH, solvent dependencies, and others [12]. Moreover, bioactivity measured using the same assay revealed significant variation. In terms of anticancer activity, the growth rate of HepG2 cells treated with 1000 μg/mL CPC is inhibited by only 8%, whereas 500 μg/mL CPC inhibits growth by 68%, revealing a significant difference [13, 14]. In terms of antioxidant activity, 500 μg/mL CPC inhibits 40–77% of ABTS radical, whereas DPPH assays showed a more pronounced difference, with a minimum of < 10% and a maximum of 82.4% inhibition at 100 μg/mL [15–21]. These differences in bioactivity may be attributed to variations in the species of *Spirulina* and the extraction and purification methods employed, which have resulted in differences in the type and purity of compounds present in the extracts and the contents of bioactive compounds (i.e., CPC) [10, 22]. Microalgae contain several complex mixtures such as carbohydrates, lipids, proteins, and fibers, as well as micronutrients, including minerals and trace elements, which interact with bioactivity compounds and hence affect their activity [10].

Various purification technologies have been used to achieve the desired purity of CPC as the purity of CPC and the type of compounds in the *Spirulina* extracts are affected by the design of the purification process. Purification methods such as fractional precipitation, membrane filtration, and chromatography have been applied for CPC purification, and the combination of these techniques is effective in obtaining highly pure CPC [8]. Although traditional purification processes are efficient in removing other proteins and impurities, separating CPC and APC is difficult because of their similar properties (e.g., structure, molecular weight, and isoelectric point) and amino acid sequences [23, 24]. Furthermore, APC is difficult to purify owing to its low yield and lack of effective purification methods [25, 26]. Although many studies have been conducted in the last decade to evaluate the bioactivity of CPC, the presence of impurities may have affected the results. To demonstrate the nutraceutical and analytical/diagnostic reagent application potential of CPC, understanding the contribution of CPC and APC to the bioactivities in *Spirulina* is imperative.

The objective of this study was to evaluate each bioactivity by obtaining highly purified CPC and APC from *Spirulina* extracts. The study employed a series of purification techniques, including ammonium sulfate precipitation, diafiltration, and anion-exchange chromatography, to eliminate impurities and retain only CPC and APC. Additionally, a novel technique combining precipitation with ammonium sulfate and membrane filtration was introduced for the separation of CPC and APC with high purity. The precipitated CPC was selectively and reversibly bound through hydrophobic interaction by filtration through a membrane with hydrophobic properties. The separated CPC and APC were characterized and their bioactivities (e.g., anticancer, antioxidant, and anti-inflammatory) were evaluated. This study provides a framework for better applications of phycobiliproteins by obtaining highly purified CPC and APC, each with unique bioactivities.

## Materials and methods

### Materials

Dried marine microalgae (*Spirulina maxima*) was provided by the Korea Institute of Oceanology Science and Technology (KIOST Jeju Center, Jeju, Republic of Korea). The materials used to extract highly purified CPC and APC are presented below. Ammonium sulfate used for precipitation was purchased from Duksan Co. (Republic of Korea). Pellicon 3 TFF cassettes with D-screen with 30 kD Ultracel® membranes (Merck Millipore, USA) and cellulose ester dialysis membranes (Spectra/Por® Biotech, USA) with molecular weight cut-offs of 20 kDa were used for desalting. The phycobiliprotein extract was purified using an Econo-Pac® chromatography column (14 × 1.5 cm I.D.) containing 10 cm (8 mL total volume) of the strong anion-exchange resin Q-Sepharose Fast Flow™ purchased from Cytiva (Uppsala, Sweden). The CPC and

APC separation procedure was performed using 0.45 µm hydrophilic PVDF Durapore® membranes (Merck Millipore, USA) using a vacuum filtration assembly.

Characterization of highly purified CPC and APC was conducted using a high-performance liquid chromatography system (Agilent, USA) with an auto-sampler consisting of 1260 Infinity II variable wavelength detector and an Eclipse XDB- C18 column (80 Å pore size, 4.6 ×250 mm, 5 µm, Agilent, USA). Acetonitrile (ACN; Honeywell, USA) and trifluoroacetic acid (TFA; Dae-Jung, Republic of Korea) were used as mobile phase. Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed using Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad Laboratories, Germany), Acrylamide/bis-acrylamide solution (Bio-Rad, CA), Laemmli 4× Sample Buffer (GenDEPOT, USA), and 10 ×Tris/glycine/SDS buffer (GenDEPOT, USA). Bio-Safe™ Coomassie blue stain (Bio-Rad Laboratories, Germany) was used to visualize protein bands, and ExcelBand™ Enhanced 3-color High Range Protein Marker (9–245 kDa) (SMOBIO, USA) was used to determine the molecular weight of the subunits. HepG2 human hepatoblastoma cells and K562 human leukemia cells were purchased from the Korea Cell Line Bank (KCLB, Republic of Korea) to measure anti-cancer activities. Cell viability was determined by performing WST- 8 assays using the Cell Counting Kit-8 (CCK- 8, Dojindo, Japan). LIVE/DEAD™ Viability/Cytotoxicity Kit (L3224, Invitrogen, USA) was used to evaluate the cytotoxicity. Hydroxyl radical and hydrogen peroxide scavenging activity was evaluated using hydrogen peroxide (Duksan Co., Republic of Korea), sodium salicylate (Duksan Co., Republic of Korea), and ferrous sulfate (Sigma-Aldrich Chemical Co, USA). 2,2′-Azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS), potassium persulfate, α,α-diphenyl-β-pricrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (USA) for ABTS and DPPH radical scavenging assays. Bovine serum albumin (BSA, Merck Millipore, USA) was used for albumin denaturation assays to evaluate in vitro anti-inflammatory activity.

### Purification process to remove other proteins from phycobiliprotein extracts

*Spirulina* powder (3 g) was added to 150 mL of sodium phosphate buffer (pH 7.5) and cell lysis was performed by stirring at 300 rpm. Cell debris was removed by centrifugation at 8000 ×*g* for 30 min and the phycobiliprotein extract was obtained. Three purification techniques were used to purify phycobiliproteins extracted from *S. maxima*, such as fractional precipitation with ammonium sulfate, diafiltration for desalting, and anion-exchange chromatography with stepwise elution. Using these techniques, phycobiliproteins were purified by removing other proteins.

#### Fractional precipitation with ammonium sulfate

Fractional precipitation with ammonium sulfate was performed. First, solid ammonium sulfate was added to the phycobiliprotein extract to reach a saturation concentration of 20% under constant stirring, followed by incubation at 4 °C for 4 h and centrifugation (15,000 ×*g*) at 20 °C for 20 min. For the second fraction, solid ammonium sulfate was added to the supernatant obtained in the previous step until a 60% saturation concentration was reached. The mixture was then centrifuged using the conditions mentioned previously. The precipitate was resuspended in 0.1 mol/L sodium phosphate buffer at pH 7.5.

#### Diafiltration

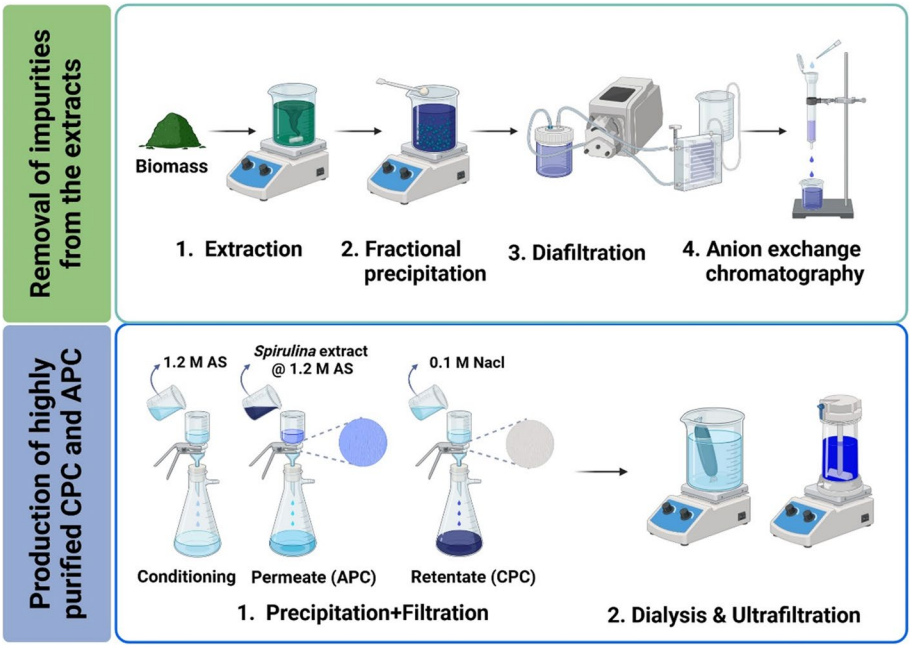
Diafiltration (DF) was performed at a feed flow rate of 51 mL/min, maintained using a Masterflex pµmp (Gelsenkirchen, Germany) at a transmembrane pressure of 1.3 bar. Before DF, the membrane was compacted with ultrapure water and conditioned with DF buffer at a constant permeate flux of 200 L $m^{-2}$ $h^{-1}$ (LMH) using the same operating parameters. DF was performed on six diavolumes to remove salt and exchange buffer using 0.1 M of sodium phosphate buffer at pH 7.0 as the DF buffer.

#### Anion-exchange chromatography stepwise elution

After equilibration with 0.025 mol/L Tris–HCl buffer pH 7.4, the phycobiliprotein extract was loaded onto a chromatography column containing a strong anion-exchange resin. The same equilibration buffer was used to wash the non-adsorbed proteins after the loading step. Elution mode was the stepwise elution with 0.1 M NaCl and 0.4 M NaCl. The phycobiliprotein solution purified up to anion-exchange chromatography (AEX) is referred to as the AEX sample (purified CPC and APC sample).

#### Separation procedure of CPC and APC

The separation of CPC and APC was performed using precipitation and membrane filtration technology. Membrane-based technologies have the advantage of being easier and economic to mass-produce, and hence can be used for processing large volumes of dilute streams or purification of large biomolecules such as proteins, antibodies, and nucleic acids [27]. The CPC and APC samples purified through AEX were dialyzed using 0.1 M NaCl before the separation procedure. Ammonium sulfate was added to the dialyzed sample to 1.2 M, and the mixture was stirred for 20 min to allow the ammonium sulfate to sufficiently dissolve and precipitate the target protein.




**Scheme 1** Production of highly purified CPC and APC for bioactivity measurements

A vacuum filtration device was assembled with a hydrophobic PVDF membrane. The membrane was conditioned with 30 mL of 1.2 M ammonium sulfate solution to impart hydrophobicity to the membrane. The phycobiliprotein extract was loaded onto the membrane and filtered, and the permeate was collected in a clean flask. The membrane was desorbed in a clean flask using a 0.1 M NaCl solution to recover the retentate. CPC penetrates to the permeate owing to its limited binding capacity and was removed using a vacuum filtration assembly equipped with a new membrane. This process was repeated until CPC was removed from the permeate. The CPC remaining in the permeate was easily removed by repeated filtration as the concentration of ammonium sulfate in the permeate remained constant at 1.2 M even after filtration. Finally, a retentate fraction with CPC and a permeate fraction with APC were obtained. The retentate and the permeate were dialyzed to 10 mM phosphate buffered saline (PBS) of pH 7.4 and concentrated using ultrafiltration (UF) to measure their bioactivities (Scheme 1).

## Characterization of highly purified CPC and APC

### *Spectrophotometric determination of CPC and APC*

The retentate and permeate were scanned at wavelengths between 250 and 700 nm in a UV–vis spectrophotometer. The concentration of CPC and APC in retentate and permeate was determined using Eqs. (1) and (2) as previously described [28, 29]. The purity of CPC and APC was measured using Eqs. (3) and (4). The separation factor between CPC and APC was calculated by the ratio between the absorption maximum of CPC and APC according to Eqs. (5) and (6). The separation factor was used to determine the separation of CPC and APC.

$$[CPC](mg/mL) = \frac{A_{620} - 0.474 \times A_{652}}{5.34} \tag{1}$$

$$[APC](mg/mL) = \frac{A_{652} - 0.208 \times A_{620}}{5.09} \tag{2}$$

$$P_{CPC} = \frac{A_{620}}{A_{280}} \tag{3}$$

$$P_{APC} = \frac{A_{652}}{A_{280}} \tag{4}$$

$$SF_{CPC} = \frac{A_{620}}{A_{652}} \tag{5}$$

$$SF_{APC} = \frac{A_{652}}{A_{620}} \tag{6}$$

where *A620*, *A652*, and *A280* are the absorbances at the CPC absorption maximum (620 nm), APC absorption maximum (652 nm), and total protein content (280 nm), respectively, $P_{CPC}$ and $P_{APC}$ are the purity of CPC and

APC, respectively, and $SF_{CPC}$ and $SF_{APC}$ are the separation factor of CPC and APC, respectively.

#### *High-performance liquid chromatography*

The α and β subunits of CPC and APC were analyzed using a high-performance liquid chromatography system. Absorbance was measured with a UV detector at 620 and 652 nm. The injection volume was 20 μL equilibrated with a mobile phase consisting of 80% buffer A (0.1% TFA in water) and 20% buffer B (0.1% TFA in ACN). The subunits of CPC and APC were eluted at a constant flow rate of 1 mL/min using the following program: from 0 to 80 min, linear gradient with 80% buffer B; from 60 to 65 min, linear gradient with 95% buffer B; from 65 to 70 min, linear gradient with 20% buffer A; from 70 to 75 min, 20% buffer B.

#### *SDS-PAGE*

SDS-PAGE was used to analyze the α and β subunits of CPC and APC and was performed by loading 25 μg of protein on a 1.5 mm thick, 5% (w/v) polyacrylamide slab gel containing 0.1% (w/v) SDS with a stacking gel of 4% acrylamide and 0.1% bis-acrylamide. Samples were pre-incubated with Laemmli sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 4.5% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue, and 0.06 M Tris (pH 6.8)) for 5 min at 95 °C. The gel was run at ambient temperature and stained with Coomassie blue to visualize protein bands. A molecular weight marker was used to determine the molecular weight of the subunits.

### Measurement of anticancer activities of highly purified CPC and APC

#### *Cell culture*

HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% $CO_2$. Cells were seeded on 90 ×20 mm cell culture dishes (20,101, SPL) at 4× $10^4$ cells/$cm^2$. Media were changed every 2–3 days. K562 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% $CO_2$. Cells were seeded on 90 ×20 mm cell culture dishes (20,101, SPL) at 1× $10^5$ cells/mL. Media were changed every 2–3 days.

#### *Cell viability test*

HepG2 and K562 cells were seeded in 96-well plates. After 24 h, the medium was replaced with media containing specific concentrations of CPC and APC, and the cells were incubated for 24 h. To remove the samples, HepG2 cells were washed with PBS once and fresh media was added. K562 cells were centrifuged at 180 ×$g$ for 5 min before replacing with fresh media. CCK- 8 reagent was added to the media and the culture was incubated further for 2 h. Absorbance at 450 nm was determined. Cell viability was calculated using Eq. (7).

$$\text{Cell viability (\%)} = \frac{Absorbance of the vibrational group}{Absorbance of the control group} \times 100(\%) \tag{7}$$

LIVE/DEAD viability/cytotoxicity assays were performed to evaluate the cytotoxicity of CPC and APC following the manufacturer's instructions. Both HepG2 and K562 cells were treated with 2 μM calcein AM and 4 μM EthD- 1 and incubated at 37 °C for 20 min in the dark. Fluorescent images were obtained using an Olympus CKX53 microscope equipped with a DP80 camera (Olympus, Japan).

### Determination of antioxidant activities of highly purified CPC and APC

The antioxidant activities of the highly purified CPC and APC were assessed using hydroxyl radical scavenging assays, hydrogen peroxide scavenging activity, ABTS radical scavenging assays, and DPPH radical scavenging assays. Scavenging activity on radicals was evaluated using microplate analytical assays.

Hydroxyl radical scavenging assays were performed as follows. Ferrous sulfate (50 μL, 1.5 mM) and 50 μL of hydrogen peroxide (0.01%) were combined with 100 μL of the CPC and APC samples, and 50 μL of 1,10-phenanthroline (1.5 mM) was added. Subsequently, the reaction mixture was incubated in the dark for 30 min at 37 °C, after which the absorbance at 536 nm was determined. The scavenging of hydrogen peroxide radicals was performed as follows. CPC and APC samples were combined with 0.6 mL of 40 mM hydrogen peroxide prepared in phosphate buffer at pH 7.4. Absorbance at 560 nm was determined using a UV spectrophotometer after incubation for 10 min at room temperature. The ABTS radical solution was prepared by mixing equal amounts of ABTS (7 mM) and potassium persulfate (2.45 mM). After 18 h in the dark, the stock solution was diluted with 10 mM PBS at pH 7.4 to obtain a working solution that exhibited an absorbance of 0.70 ± 0.05 at 734 nm. Aliquots of 180 μL of the diluted ABTS radical solution were mixed with 20 μL aliquots of the CPC and APC samples. The absorbance at 734 nm after incubation in the dark for 6 min at room temperature was determined. A volume of 20 μL of the CPC and APC samples was mixed with 180 μL of DPPH in methanol (0.06 mM). After incubation for 30 min in the dark at room temperature, the absorbance at 517 nm was determined. The

radical scavenging activities of the samples were evaluated according to Eq. (8).

$$radical\ scavenging\ activity\ (\%) = \frac{([A_{control} - A_{sample} - A_{sampleblank}])}{A_{control}} \times 100 \quad (8)$$

where $A_{control}$ is the absorbance of the radical solution with buffer, $A_{sample}$ is the absorbance of radical solution with sample, and $A_{sample\ blank}$ is the absorbance of sample with buffer.

#### Measurement of anti-inflammatory activities of highly purified CPC and APC

The in vitro anti-inflammatory activity of highly purified CPC and APC was estimated by inhibition of albumin denaturation. BSA solution (1%, w/v) was prepared in PBS. The CPC and APC samples were added to the solution containing 1% BSA. Negative control (PBS) was prepared in a similar manner. The solutions were incubated at 37 °C for 20 min and then heated at 70 °C for 10 min. The heated samples were cooled for 20 min at room temperature. The turbidity of the solutions was measured at 660 nm using a UV spectrophotometer. The inhibition of protein denaturation was evaluated using Eq. (9).

$$Inhibition\ of\ protein\ denaturation(\%) = \frac{([A_{control} - A_{sample} - A_{sampleblank}])}{A_{control}} \times 100 \quad (9)$$

## Results

#### Removal of impurities from phycobiliprotein extracts

CPC and APC were extracted from *S. maxima* powder and purified using various methods. Results of purity, separation factor, and recovery of CPC and APC for each process are presented in Table 1. To assess the reproducibility of the extraction process, the experiments were performed in multiple independent replicates (n ≥ 3). The results demonstrated consistent yields and purities across different batches, confirming the reliability of the method. Note that phycoerythrin was not detected in the phycobiliprotein extract and was excluded (Figure S1, supplementary information). The purity of the crude extract of phycobiliproteins showed a CPC of 0.9 and an APC of 0.4. The precipitation to the AEX process was used to remove impurities from the crude extract, leaving only phycobiliprotein. After fractional precipitation, the purity of CPC and APC in the extract increased from 0.9 to 1.5 and from 0.4 to 0.6, respectively. Fractional precipitation with ammonium sulfate was followed by DF for salt removal. The use of DF effectively removed ammonium sulfate and slightly increased the purity of both CPC and APC, resulting in a 1.2-fold increase in the purification factor. After the AEX step, the purity of CPC and APC in phycobiliprotein extract increased to 3.2 and 1.4, respectively, representing a 3.6-fold increase in CPC purity and a 3.5-fold increase in APC purity compared to crude extracts of phycobiliproteins.

The purification process of extraction, precipitation, and desalting followed by one or more chromatographic steps such as ion exchange, affinity, or adsorption chromatography can produce phycocyanin with a purity level of at least 3.0 [30, 31]. In addition, purified CPC from *S. platensis* by fractional precipitation with ammonium sulfate at 0–20%/20–50% saturation resulted in 83.8% recovery and 0.88 of purity, with a 1.7-fold purification factor (70% increase) [32]. This saturation range of ammonium sulfate was also used to increase the purity of CPC from 2.75 to 3.65, with a 2.1-fold purification factor (70% increase) [33]; UF followed by one step of DF improved the purity by approximately 1.4 times, from 0.53 to 0.76 [34]. A CPC purification designs also used ammonium sulfate precipitation before the DF/UF step, which increased the purity of the extract from 0.57 to 2.12, resulting in a purification factor of 3.7. [35]. Subsequently, ion exchange chromatography was employed

**Table 1** Purity, separation factor, and recovery of C-phycocyanin (CPC) and allophycocyanin (APC) from *Spirulina maxima* in each step of the purification process

| Purification step | Purity | | Separation factor | | Recovery (%) | |
|---|---|---|---|---|---|---|
| | CPC | APC | CPC | APC | CPC | APC |
| **Extraction** | 0.9 | 0.4 | 2.5 | 0.4 | 100 | 100 |
| **Fractional precipitation** | 1.5 | 0.6 | 2.6 | 0.4 | 74.3 | 60.6 |
| **Diafiltration** | 1.7 | 0.7 | 2.4 | 0.4 | 83.2 | 88.9 |
| **Anion-exchange chromatography** | 3.2 | 1.4 | 2.6 | 0.4 | 85.4 | 76.6 |

to achieve the desired level of purity. Ion exchange chromatography produces CPC with high purity, purification factor, and recovery [36, 37]. The elution by stepwise and NaCl gradients for the purification of C-PC extracted from the dry biomass of *S. platensis* LEB- 52 revealed a purification factor of 3.0-fold, being applied after a previous purification with ammonium sulfate precipitation and dialysis, resulting in CPC with purity of 4.0 and overall recovery of 44.0% [38].

Although the purity of CPC and APC in the extracts increased through a series of purification steps, the separation factor, which indicates the degree of separation of CPC and APC, remained almost constant, from 2.5 to 2.6 for CPC and 0.4 to 0.4 for APC, indicating that CPC and APC were not separated by the commonly used purification processes. Therefore, although highly purified CPC is used for the measurement of physiological activities, it cannot be assumed that the physiological activities are solely influenced by the unique properties of CPC if the CPC and APC are not separated. It appears that APC may also have an impact.

#### Production of highly purified CPC and APC

The process of separating CPC and APC was based on ammonium sulfate precipitation (salting out), hydrophobic interaction, and filtration. Salting out is achieved by adding a high concentration of salt to a solution, which increases the hydrophobicity of the protein by lowering the water activity of the solution and reducing the hydrogen bonds between the protein and the solution. During the salting out process, the solubility of proteins decreases, causing them to precipitate out of the solution. The precipitated solution is then filtered onto a hydrophobic membrane, resulting in selective and reversible binding of the target protein through hydrophobic interactions. It revealed that purification and separation of CPC from APC was achieved using selective binding through hydrophobic interactions [39].

The salt concentration and precipitation time for separating CPC and APC were determined experimentally. To separate CPC and APC, we evaluated the purity and separation factor of CPC and APC at different concentrations of ammonium sulfate as presented in Figure S2. The highest purity and separation factors were observed for both CPC and APC at a salt concentration of 1.2 M. At salt concentrations below 1.2 M, CPC was only partially bound to the membrane. At salt concentrations above 1.2 M (i.e., 1.4 M, 1.6 M, and 1.8 M), both CPC and APC were bound to the membrane (Figure S3), which is consistent with the previous study that the concentration of ammonium sulfate for the separation of CPC was approximately 1.1 M [39]. Additionally, the purity and separation factors of CPC and APC were evaluated as a function of precipitation time (Figure S4). After complete dissolution of ammonium sulfate for 20 min, both CPC and APC revealed similar purity and separation factors for the 0-, 20-, 40-, and 60-min conditions. The precipitation time was insignificant for the separation and next filtration was performed immediately after the precipitation. The filtration was repeated to separate CPC and APC from the extract, as the maximum binding capacity of CPC was 1.2 mg (Figure S5). Repeated filtration decreased the amount of CPC in the phycobiliprotein extract and the membrane-bound CPC. Highly purified CPC and APC were obtained by desalting and concentrating after separating them using a method that combines precipitation and filtration.

As presented in Fig. 1(a), the purity of CPC and APC increased by 20% and 53%, respectively, compared to purified CPC and APC (referred to as AEX sample), achieving a purity of approximately 4.0 and 3.0, respectively. Additionally, the separation factor of CPC and APC increased by 35% and 69%, resulting in a separation factor of 4.0 and 1.3, respectively, as presented in Fig. 1(b). The recovery using this technology was low owing to the loss of protein caused by multiple filtrations (CPC: 78.8% APC: 33.6%). However, this can be solved by using a membrane with a large effective area to increase the binding capacity and minimize the number of procedures or by recovering the protein through washing.

Highly purified CPC, obtained from retentate and APC, obtained from permeate samples were analyzed by UV–vis spectrophotometry, RP-HPLC, and SDS-PAGE. As presented in Fig. 1(c), the absorbance spectra of the AEX sample revealed a broad peak containing the characteristic absorption peaks of CPC and APC. However, after the separation of CPC and APC, the retentate sample showed only the characteristic absorption peak of CPC near 620 nm, whereas the permeate sample revealed the characteristic absorption peak of APC near 650 nm and a shoulder at 620 nm [40, 41]. CPC and APC in *S. maxima* consists of basic monomer components obtained from the association of two homologous α- and β-subunits [42, 43]. The highly purified retentate and permeate were analyzed using the RP-HPLC system for confirmation that they contained CPC and APC, respectively, and the results are presented in Fig. 1(d). The highly purified retentate sample revealed two major peaks referred to as the α- and β-subunit of purified CPC, which is consistent with a previous study [44]. The highly purified permeate sample revealed two major peaks referred to as β—and α -subunit of purified APC, consistent with a previous study [45]. The AEX sample showed three broad peaks including the characteristic peaks of CPC and APC. SDS-PAGE was performed to more clearly confirm the proteins present in each sample. Two intense bands were

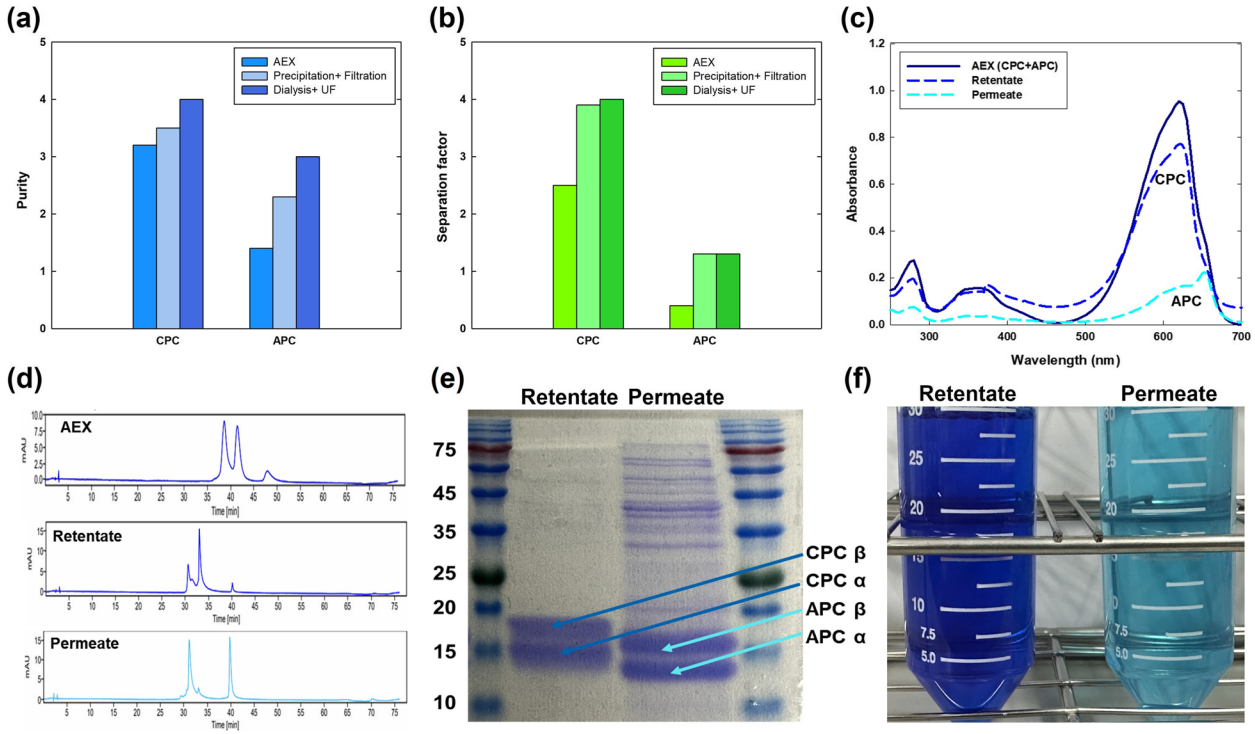



**Fig. 1** **a** Purity of highly purified CPC and APC from *Spirulina maxima* after the separation process. **b** Separation factor of highly purified CPC and APC from *S. maxima* after the separation process. **c** Absorption spectra of the purified CPC and APC (AEX), highly purified CPC (retentate), and APC (permeate) samples. **d** HPLC chromatograms of the purified CPC and APC (AEX), highly purified CPC (retentate), and APC (permeate) samples. **e** Denaturing gel electrophoresis (SDS-PAGE) of highly purified CPC (retentate) and APC (permeate) samples. **f** Color of highly purified CPC (retentate) and APC (permeate) samples from *S. maxima* after the purification and separation procedure

visible both in the purified retentate and permeate samples (Fig. 1(e)). Two bands of retentate showed apparent molecular weights of 19 and 21 kDa, corresponding to the α- and β-subunits of CPC, respectively. The two bands appearing between 11 and 17 kDa in the permeate corresponded to the α and β subunits of APC. The purity of samples was further demonstrated using SDS-PAGE. As presented in Fig. 1(a), the purity of the permeate sample was 3.0, which was less purified than the retentate sample. As presented in Fig. 1(f), the retentate exhibited the characteristic dark blue color of CPC and the permeate represented the blue-green color of APC, demonstrating good separation of CPC and APC.

### Anti-cancer activity

Anti-cancer activity was assessed using CCK- 8 assays to determine cancer cell viability. The highly purified CPC and APC samples were prepared by diluting each sample to 400 μg/mL. The AEX sample was prepared so that the sum of the CPC and APC concentrations in the sample was 400 μg/mL, and the ratio of CPC to APC was approximately 4:1. The three samples were used to treat HepG2 cells. As presented in Fig. 2(a), the viability of the cells treated with APC only decreased to approximately 30% compared to the control. In contrast, the cell viability of the cells treated with CPC only increased by more than 40% compared to the control. This suggests that APC had a stronger anti-cancer effect than CPC, with CPC revealing cell proliferation at 400 μg/mL. These results are also consistent with those of the live-dead assays (Fig. 2(b)). However, the viability of cells treated with the AEX sample without performing the CPC and APC separation process was 106.7%, which likely resulted from the presence of both CPC and APC at an approximately 4:1 ratio. The effect of sample concentration on anticancer activity was determined by treating HepG2 cells with 300 μg/mL of the sample. As presented in Fig. 2(c), cell viability increased with decreasing sample concentration; however, only APC-treated cells revealed a reduction in cell viability of approximately 8%. In addition, as the concentration of CPC decreased, no effect on cell proliferation was observed. The human chronic myeloid leukemia K562 cells treated with the same APC concentration (300 μg/mL) also revealed anticancer activity, whereas CPC only slightly inhibited cell proliferation (Fig. 2(d)).

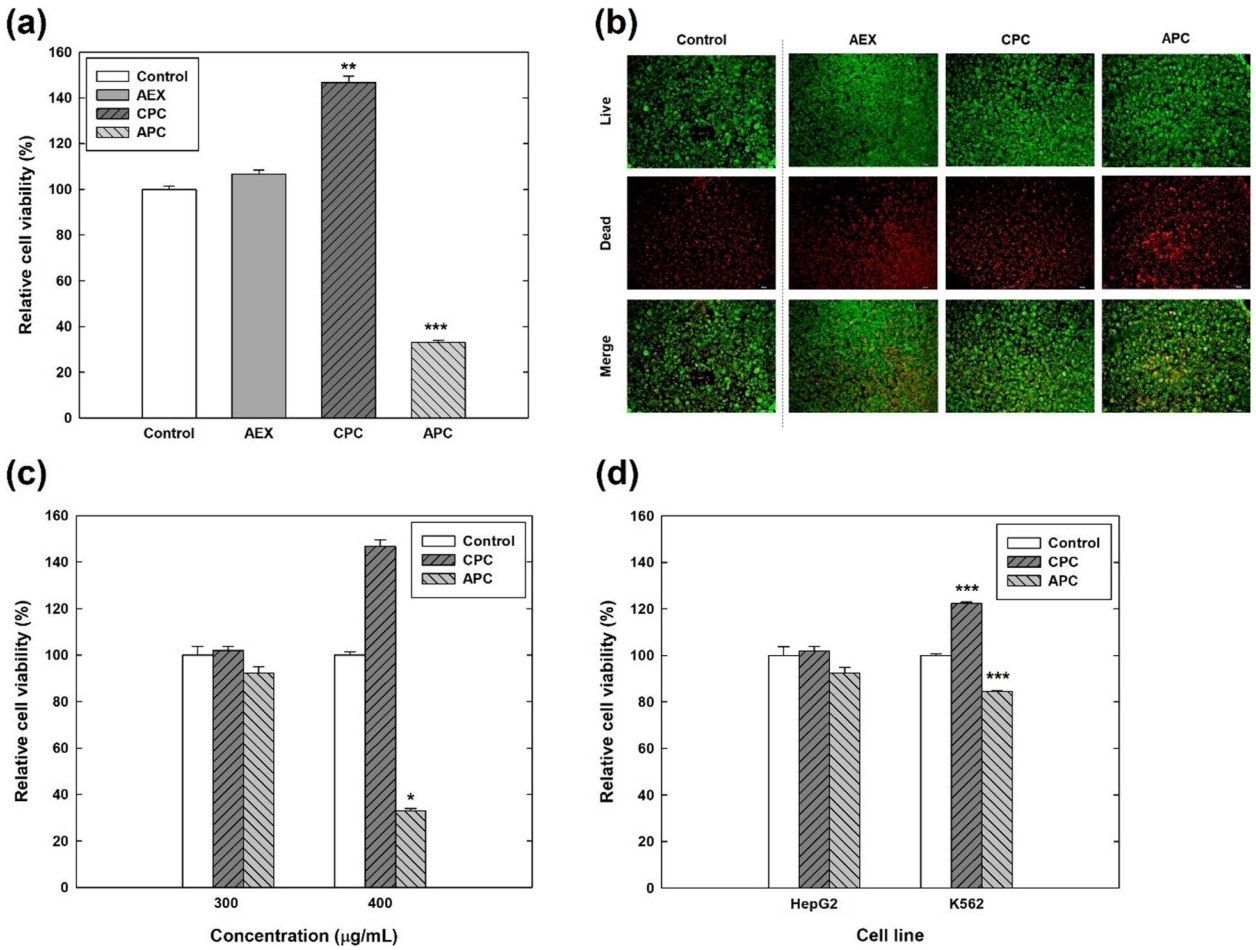



**Fig. 2** Relative cell viability of HepG2 cells treated with the purified CPC and APC (AEX) highly purified CPC and APC samples by (**a**) CCK- 8 assay and (**b**) Live-dead assay at 400 μg/mL. **c** Relative cell viability of HepG2 cells as a function of the concentration of the highly purified CPC and APC samples. **d** Relative cell viability as a function of human cancer cell lines at 300 μg/mL of the highly purified CPC and APC samples. Experiments were performed in triplicate ($n = 3$), statistical analysis was performed using a t-test, and a $p$-value < 0.05 was considered statistically significant. Significance markers are indicated: *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$)

### In vitroantioxidant and anti-inflammatory activities

The antioxidant activity was evaluated by hydroxyl and hydrogen peroxide scavenging assays. Hydroxyl and hydrogen peroxide radical scavenging activity was measured to determine the effect of CPC and APC on ROS. As presented in Fig. 3(a), CPC had a higher hydroxyl radical scavenging activity (37.4%) than APC (11.5%). The AEX sample revealed the highest hydroxyl radical scavenging activity among the three samples (38.7%). The hydrogen peroxide radical scavenging activities of the CPC, APC, and AEX samples at 400 μg/mL are depicted in Fig. 3(a). The hydrogen peroxide scavenging activity of the CPC, APC, and AEX samples was 24.5%, 11.4%, and 28.3%, respectively, showing a tendency comparable to that of hydroxyl radical scavenging activity. Additionally, the antioxidant activity of the highly purified CPC and APC samples was measured using ABTS and DPPH radical scavenging assays, which are frequently used to measure the antioxidant capacity of natural compounds (Figure S6). The data revealed a consistent pattern of AEX having the highest radical scavenging activity, followed by CPC, and then APC. The results showed that APC also had significant radical scavenging activity, resulting in a higher scavenging capacity for AEX than for CPC and APC separately. Furthermore, ABTS assays showed approximately 2.5 times higher radical scavenging activity than DPPH assays at the same concentration. These results are consistent with those of previous reports suggesting that pigmented and highly hydrophilic antioxidants are better assayed with ABTS than with DPPH assays [46].

The higher antioxidant capacity of CPC compared to APC and the higher anticancer activity of APC compared to CPC may be owing to their different antioxidant capacities, which influences their anticancer activity. Generally,

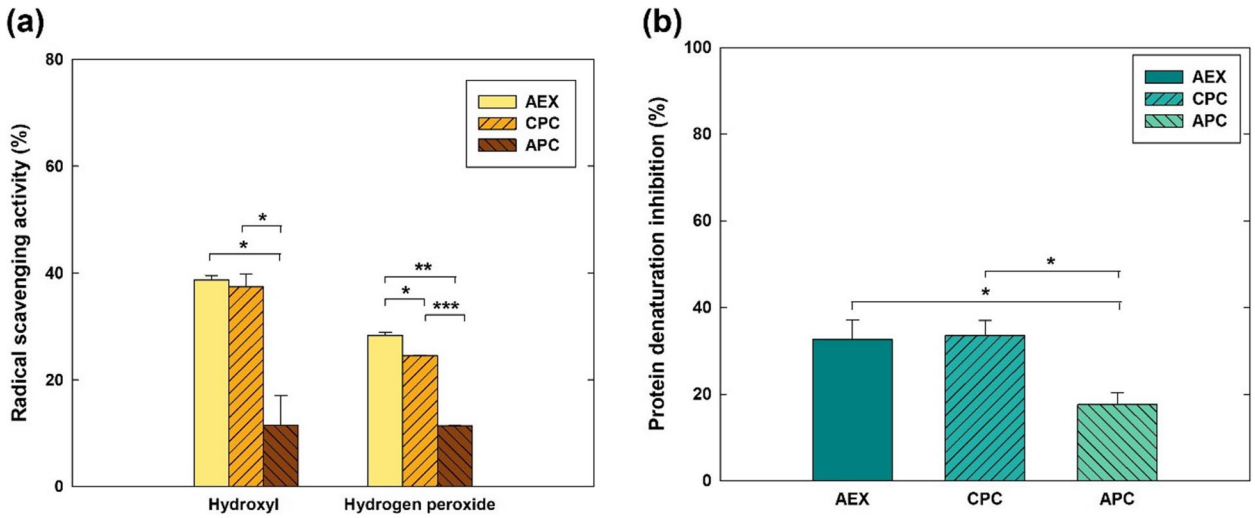



**Fig. 3** **a** Antioxidant activity of the purified CPC and APC (AEX) sample and highly purified CPC and APC samples determined by hydroxyl and hydrogen peroxide radical scavenging assays at 400 μg/mL. **b** In vitro anti-inflammatory activity of purified CPC and APC (AEX) samples and highly purified CPC and APC samples was determined using albumin denaturation assays at 400 μg/mL. The experiments were performed in triplicate ($n = 3$), statistical analysis was performed using a t-test, and a $p$-value < 0.05 was considered statistically significant. Significance markers are indicated: *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$)

CPC alters the mitochondrial membrane potential, which promotes the release of cytochrome c and stimulates the formation of ROS, which eventually induces cancer cell apoptosis [13]. However, CPC is an effective antioxidant and inhibits ROS when its concentration increases [47]. Therefore, CPC may not be able to effectively exert the damaging effects resulting from free radicals owing to its relatively high antioxidant capacity. In contrast, APC has a relatively lower antioxidant capacity than CPC, and thus APC may have a higher anti-cancer effect owing to increased ROS release. Some antioxidants inhibit ROS release, thereby reducing their anticancer effects [48, 49]. However, the mechanisms of anti-cancer effects of CPC and APC are diverse, and further research is required on this aspect.

External stresses such as heat or exposure to organic solvents can induce protein denaturation [50]. Denaturation of protein causes the production of autoantigens in diseases such as rheumatic arthritis, cancer, and diabetes, which are conditions with inflammation. Therefore, to investigate anti-inflammatory activity, the ability of the isolated samples to inhibit heat-induced denaturation of albumin was assessed. As depicted in Fig. 3(b), highly purified CPC and APC revealed 33.5% and 17.6% inhibition, respectively, at 400 μg/mL, with CPC having approximately twofold higher inhibition activity than APC. The AEX sample revealed a 32.6% inhibition of protein denaturation. In addition to antioxidant activity, CPC had a higher anti-inflammatory activity than APC. The antioxidative and oxygen-free radical scavenging properties may contribute, at least in part, to its anti-inflammatory activity [51, 52]. Additionally, highly purified CPC and APC did not reveal antimicrobial activity against four bacterial strains (*Micrococcus luteus, Staphylococcus aureus, Bacillus subtilis,* and *Escherichia coli*) (Figure S7).

## Discussion

Over the past few decades, many studies on the bioactivities of CPC have been conducted without considering the removal of APC from CPC extracts. For easy comparison with our results, we restricted the anti-cancer activity to the treatment of HepG2 cells and reported only the results of experiments using ABTS assays for antioxidant activity and albumin denaturation assays for anticancer activity as the studies on the bioactivities of phycocyanin were performed under different experimental conditions and methods [6, 13–15, 17, 20, 21, 53, 54]. The values reported in the literature and the data from this study were compared by plotting them in Figs. 4 and Figure S8. Figure 4 depicts reports on the anticancer activity of CPC previously. Black symbols are located between the star and X symbols. This phenomenon is attributed to the overlapping effect of CPC, which reveals a cell proliferation effect with increasing concentration, in addition to APC, which shows a strong decrease in cell viability with increasing concentration. Despite some differences in bioactivity depending on the species of microalgae used in the experiments and the extraction and purification method, highly purified APC isolated from CPC in the

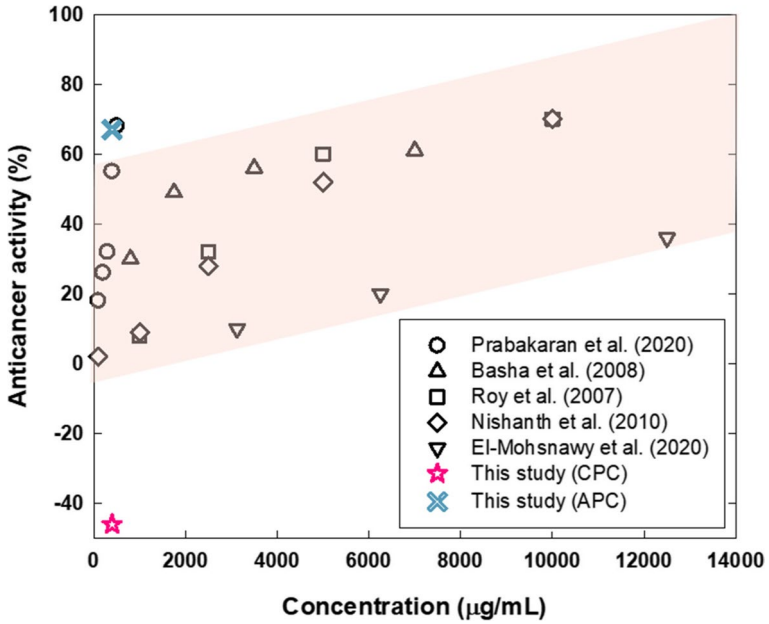



**Fig. 4** Comparison of anticancer activity of phycocyanin in this work with previous results

present study revealed high anticancer activity compared to previously described compounds at similar concentrations. Therefore, it is considered essential to separate CPC and APC in *Spirulina* extracts for their anticancer function. When the anticancer activity is measured in the presence of CPC and APC simultaneously, the overall anticancer effect will be reduced owing to cell proliferation of CPC above a certain concentration. On the contrary, all black symbols are located above the star and X symbols in Figure S8, which is expected to result from the synergistic effect of the physiological activities of each protein, as both CPC and APC showed significant antioxidant and anti-inflammatory effects (Fig. 3). Consequently, the utilization of *Spirulina* extracts as an antioxidant and anti-inflammatory agent does not require additional purification processes to separate the two proteins.

Additionally, to determine the effects of other proteins on the antioxidant capacity of *Spirulina* extracts, we performed a separation procedure immediately after extraction and measured the ABTS radical scavenging capacity of low-purity CPC (purity: 90%) and APC (purity: 40%) obtained using the same method. The overall ABTS radical scavenging capacity increased by approximately 10% or more (Figure S9). In addition, APC samples containing 60% of other proteins showed higher antioxidant capacity than CPC, in contrast to the results presented in Figure S6(a), indicating that other proteins present in *Spirulina* extracts can have a significant impact on their antioxidant capacity. This is consistent with a previous study that demonstrated that a protein fraction that did not contain phycobiliprotein also had a radical scavenging effect of approximately 15% [55]. The results suggest that the isolated CPC had significant radical scavenging activity and is a potential antioxidant agent. However, APC and other proteins of *S. maxima* also have radical scavenging activity. The bioactivities of *Spirulina* extracts are influenced by a complex interaction between CPC, APC, and other proteins. The underlying molecular mechanisms behind these bioactivities have not yet been fully elucidated. Understanding the interactions between CPC, APC, and cellular pathways could provide deeper insights into their biological effects. Future studies should focus on identifying key molecular targets and signaling pathways involved in their bioactivity, contributing to a more comprehensive understanding of their therapeutic potential.

## Conclusion

CPC, derived from *Spirulina*, exhibits several beneficial bioactivities. However, it is challenging to remove APC, a phycobiliprotein with properties similar to CPC, from phycobiliprotein extract through conventional purification processes. Despite the extensive research conducted over the past decade on the bioactivities of CPC, existing studies have possibly been conducted without the elimination of APC from phycobiliprotein extracts. To demonstrate the potential application of phycobiliproteins, it is essential to assess the bioactivity of both CPC and APC. Therefore, we aimed to separate CPC and APC

by selective binding to a membrane through hydrophobic interactions. We introduced a technology into CPC purification to separate CPC and APC and evaluate their respective bioactivities. The results revealed that CPC and APC were obtained with over 90% purity while achieving a separation factor of approximately 4.0. The measurements of the bioactivities of purified CPC and APC showed that CPC had 25% higher antioxidant activity and twice as much anti-inflammatory activity than APC; however, APC also revealed significant antioxidant and anti-inflammatory activities. Conversely, APC contributed more to the anticancer activity than CPC. Cell proliferation occurred in CPC-treated cells with increasing concentrations, resulting in a 40% increase in cell viability compared to the control, whereas APC showed a 30% decrease in cell viability compared to the control. In addition, other proteins present in the phycobiliprotein extract are expected to have a significant effect on bioactivities. Our results showed that CPC, APC, and other protein fractions have distinct bioactivities. Our results showed that CPC, APC, and other protein fractions have distinct bioactivities. This study contributes to the identification of their respective bioactivities to utilize phycobiliproteins as potential therapeutic agents.

**Abbreviations**

| | |
|---|---|
| CPC | C-phycocyanin |
| APC | Allophycocyanin |
| PE | Phycoerythrin |
| HPLC | High performance liquid chromatography |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| CCK- 8 assay | Cell counting kit- 8 assay |
| TFF | Tangential flow filtration |
| ABTS | 2,2′-Azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid |
| DPPH | Potassium persulfate, α,α-diphenyl-β-pricrylhydrazyl |
| PVDF | Polyvinylidene fluoride |
| ACN | Acetonitrile |
| TFA | Trifluoroacetic acid |
| BSA | Bovine serum albumin |
| DF | Diafiltration |
| LMH | L $m^{-2}$ $h.^{-1}$ |
| AEX | Anion exchange chromatography |
| RP-HPLC | Reverse phase high performance liquid chromatography |
| ROS | Reactive oxygen species |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13036-025-00496-x.

Supplementary Material 1.

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Not applicable.

**Authors' contributions**
Jimin Na: Conceptualization, Investigation, Data curation, Writing-original draft & editing. Soobin Jang: Data curation, Writing-review & editing. Myeongkwan Song: Data curation, Writing-review & editing. SeungEun Nam: Writing-review & editing. Woonyong Choi: Writing-review & editing. Hwasung Shin: Writing-review & editing. Soonjo Kwon: Writing-review & editing. Youngbin Baek: Supervision, Conceptualization, Methodology, Writing-review & editing

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**Data availability**
No datasets were generated or analysed during the current study.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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