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Determination of bioactive properties of protein and pigments obtained from *Spirulina platensis*

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Abstract

Microalgae have a great potential for usage in functional foods or ingredients. The amount of the pigments obtained with different solvents from *Spirulina platensis* and their antioxidant activities, Angiotensin Converting Enzyme (ACE) inhibition of *S. platensis* and dried phycocyanin powder were investigated. The highest Chlorophyll-a and phycocyanin pigment contents were 9.26 mg chlorophyll-a/g dry spirulina and 78.57 mg phycocyanin/g dry spirulina using sequentially extraction, respectively. It was found that Chlorophyll extracts have higher antioxidant activity (14.62–16.19 mg $FeSO_4 \cdot 7H_2O$/g dry spirulina) than phycocyanin extracts (10.87–12.34 mg $FeSO_4 \cdot 7H_2O$/g dry spirulina). Single extraction was preferred due to increased pigment concentration and antioxidant activity. The percent ACE inhibition values of 10% *S. platensis* powder and phycocyanin powder suspensions were found as 92.40% and 89.50%, respectively. The necessary protein concentration of *S. platensis* and phycocyanin powder samples to inhibit 50% of the ACE activity (IC50 values) were 2.93 and 3.96 mg protein/ml, respectively. It was concluded that *S. platensis* suspension could be more effective than the pure phycocyanin on ACE inhibition.

Practical applications

Microalgae itself can be considered as functional food or food ingredient because of the bioactive contents such as polyunsaturated fatty acids, sterols, pigments, proteins and enzymes, vitamins, and other bioactive substances. In this study, the quantities and the antioxidant activities of phycocyanin and chlorophyll-a pigments obtained from *Spirulina platensis* by sequential extraction of methanol and aqueous sodium nitrate solutions were determined. Also, protein contents and ACE inhibition of residual protein rich fraction were determined after the pigment extraction. The results showed that pigment fractions and residual protein content after pigment extraction have antioxidative and antihypertensive health promoting potential.

1 | INTRODUCTION

Microalgae are rich source of chemical compounds such as polyunsaturated fatty acids (PUFA), sterols, pigments, proteins and enzymes, vitamins, and other bioactive substances (Raposo et al., 2013), and can be directly consumed as functional foods or used as raw material for many natural products that can fight against various diseases and used in human nutrition (Venugopal, 2009). The health benefit effects of microalgae due to the bioactive properties of above mentioned compounds were antiviral, anticancer, antidiabetic, antibiotic, antioxidant, prebiotic, probiotic, immune system enhancer, cardiovascular system protector, hypocholesterolemic, and antiallergic (Demiriz, 2008).

Microalgae (such as *Chlorella*, *Dunaliella*, and *Spirulina*) are also good sources of commercially important bioactive chemicals such as pigments. The most important pigments obtained

from microalgae are chlorophyll-a, β-carotene, astaxanthin, phycocyanin, xanthophyll, and phycoerythrin. While the produced pigments vary depending on the species, microalgea can generally produce pigments in the range of 0.5%–1.5% chlorophyll, 0.1%–0.2% carotenoid and 14%–20% phycobiliprotein on dry basis. These pigments can be used as natural colorants instead of synthetic pigments in industries of food, pharmaceutical, textile, and cosmetics. These pigments can be also used as nutraceuticals and pharmaceuticals due to their antioxidant, anticancer, anti-inflammatory, and cholesterol-lowering effects (Akoğlu & Çakmakçı, 2011; Duru & Yılmaz, 2013; Spolaore et al., 2006; Yılmaz et al., 2016).

In Recent years, the interest in obtaining bio-functional proteins and certain peptides from microalgae has increased. Comprehensive analyzes and nutritional studies have shown that algal proteins are of high quality. The functional and bioactive properties of proteins depend on mainly size and amino acid sequence of peptides. Bioactive peptides are released and can be activated by fermentation or enzymatic hydrolysis while they are inactive in the structure of the main native proteins. Bioactive peptides usually contain 2–20 amino acid units and have hormone-like structures. Bioactive peptides have many beneficial effects, including antihypertensive, antioxidative, anti-inflammatory, antithrombotic, hypocholesterolemic, opioid, antimicrobial, immunomodulatory, and cytomodulatory properties. Bioactive peptides can be derived as food proteins from animals and edible plants such as meat, eggs, fish, rice, soybeans, wheat, peas, broccoli, garlic, and algae proteins. Antihypertansive effect of peptides is due to the inhibition of Angitension Converting Enzyme which is responsible of high blood pressure health problems (Arslan, 2018; Becker, 2007; Bleakley & Hayes, 2017; FitzGerald & Meisel, 2000; Harnedy & Fitzgerald, 2011; Korhonen & Pihlanto, 2006; Mora et al., 2018; Rizzello et al., 2016; Samarakoon & Jeon, 2012; Sun et al., 2020; Tu et al., 2018).

In this study, antioxidative and antihypertensive bioactive properties of *Spirulina* powder, protein rich phycocyanin pigment and chlorophyll-a fractions obtained by sequential extraction from crude spirulina powder were compared. For these purposes the quantity and the antioxidant activities of phycocyanin and chlorophyll-a pigments obtained from *Spirulina platensis* by sequential extraction of methanol and aqueous sodium nitrate solutions were determined. Also, protein contents and ACE inhibition (antihypertensive activity) was determined of residual protein rich pellets were determined after the pigment extraction.

2 | MATERIAL AND METHODS

S. platensis powder obtained from CN Lab Nutrition Asian Group (Shaanxi, China) was used as the study material. Hippuric acid (HA), N-Hippuryl-HisLeu (HHL) and Angiotensin Converting Enzyme (ACE) from rabbit lung (0.25 unit) were purchased from Sigma-Aldrich. All the other chemicals were of analytical grade.

2.1 | Pigment extraction

In order to determine the amount of chlorophyll-a and phycocyanin in *S. platensis*, pigment analysis was performed by the ultrasonic-supported solvent extraction method (Aksay & Arslan, 2018). For chlorophyll-a, pure methanol as suggested by Pirinç (2014) and for phycocyanin, 1.5% sodium nitrate aqueous solutions as suggested by Oğuz (2008) were used and pigment extraction was performed as shown in Figure 1.

Sonication and sequential extraction were applied for obtaining one of the chlorophyll or phycocyanin pigments from *S. platensis* powder. In Figure 1a, sequential extraction of chlorophyll and then phycocyanin extraction were shown, respectively. *Spirulina* powder:solvent ratio of 1:10 (methanol for chlorophyll-a and $NaNO_3$ solution for phycocyanin) was used and after 30 min of sonication and 60 min of stirring on a magnetic stirrer, chlorophyll-a extract was obtained by centrifugation (J.P. Selecta Medifriger BL-S, Spain) at 5,000 rpm for 10 min. The residual precipitate was extracted several times by solvent extraction without sonication until no color was observed in the extract. Combined chlorophyll extracts (Chlorophyll-1.1) were stored over night at 4°C before quantity measurements. Then, chlorophyll-free residual precipitate (CF-1.1) was oven dried at 30°C for 4 hr and phycocyanin was extracted using 1.5% sodium nitrate aqueous solutions. Phycocyanin extracted from CF-1.1 by magnetic stirring for 60 min and extract was centrifuged at 5,000 rpm for 10 min. Phycocyanin was extracted by magnetic stirring and following centrifugation. Phycocyanin extraction was repeated until no more color observed in extract. Combined phycocyanin extracts (Phycocyanin-1.2) and residual pigment free precipitate were freeze dried to obtain Phycocyanin powder (PP-1.3) and Residual *Spirulina* Powder (CPFP-1.2), respectively. Phycocyanin and chlorophyll sequential extraction (Figure 1b) was carried out by the same method described above but first chlorophyll and then phycocyanin sequential extraction were applied. Similarly, Phycocyanin Residual Precipitate (PF-2.1) and Phcocyanin extracts (PP-2.3) were freeze dried. Precipitate (PCFP-2.2) obtained after phycocyanin and then chlorophyll sequential extraction was dried in an oven at 30°C for 4 hr.

The amount of chlorophyll-a and phycocyanin content of the extracts were calculated using the following equations. The absorbance value at 666 nm (Macías-Sánchez et al., 2005) and 620 nm wavelengths were measured (Boussiba & Richmond, 1979), respectively (Agilent Technologies, Cary 60-UV-Vis, Malesia).

$$\text{Chlorophyll} - \text{a}\ (\text{mg/g}) = 13.9 \times A_{666} \quad (1)$$

$$\text{Phycocyanin}\ (\text{mg/g}) = 137 \times A_{620} \quad (2)$$

2.2 | Antioxidant activity

Antioxidant activities of the extracts were measured according to FRAP (Ferric Reducing Ability of Plasma) method proposed by

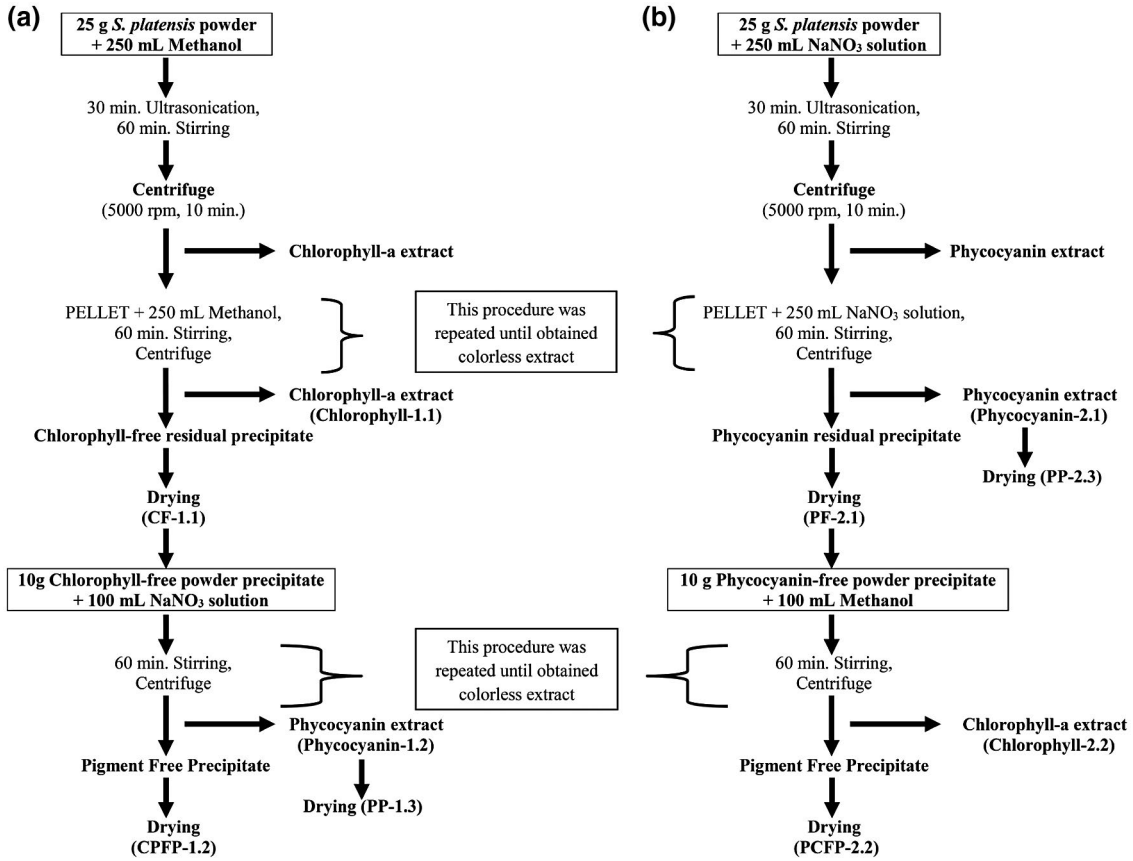

FIGURE 1 Pigment extraction scheme from *Spirulina* powder

Benzie and Strain (1996). Acetate buffer (0.3 M), TPTZ solution (pH 3.6) (prepared by dissolving 23.4 mg of 2,4,6-tripyridyl-s-triazine in 7.5 ml of 40 mM HCl) and 20 mM ferric solution (0.541 g $FeCl_3 \cdot 6H_2O$ weighed to 100 ml with distilled water) were mixed in a 10:1:1 ratio to obtain the FRAP solution. The FRAP solution was prepared on a daily basis during the determination of the antioxidant activity. For the analysis of antioxidant activity of appropriately proportioned pigment extracts, 200 µl of sample and 1.8 ml of FRAP solution were mixed and incubated in a 37°C water bath for 10 min. At the end of the incubation period, the absorbances of the samples were measured at a wavelength of 593 nm. The results were calculated with the standard graphics prepared with 0–100 ppm $FeSO_4 \cdot 7H_2O$.

2.3 | Protein analysis

The amount of protein was determined by modifying the Micro Kjeldahl analysis method AACC-Method No.46.12 (AACC, 2000). The method consists of three stages: burning, distillation, and titration. The principle of this method is based on the determination of the total amount of nitrogen in the sample. The conversion factor 6.25 was used for determining the total amount of nitrogen.

2.4 | Determination of ACE inhibition by HPLC

Angiotensin Converting Enzyme (ACE) inhibition effect was determined according to modified Wu and Ding (2002) method. The method is based on the determination of the amount of Hippuric Acid (HA) released from N-Hippuryl-Histidine-Leucine (HHL) due to the activity of ACE in the presence or absence of the inhibitor by HPLC. In the absence of the inhibitor, "Control Sample," ACE converts all HHL into HA which is taken as 100% activity. When inhibitor (*Spirulina* powder and PP-2.3) is used, "Inhibitor Sample," ACE activity decreases due to inhibition. The reduction in ACE activity compared to the control sample was measured by HPLC and inhibition percentage (INH%) of ACE was calculated using the following Equation (3).

$$\text{INH\%} = \left[1 - \left(\frac{HA_{\text{Inhibitor}}}{HA_{\text{Control}}}\right)\right] \times 100 \quad (3)$$

In this study, 10 g samples were weighed and 10% suspensions prepared in aqueous solution adjusted to pH 8.3 with 100 ml of 1 N NaOH were used. HA (5 mM), HHL (2 mM), and ACE (50 mU/ml) were dissolved in 0.1 M sodium-borate buffer (pH 8.3) containing 0.3 M NaCl. The assay was performed by mixing 40 µl of ACE, 40 µl of inhibitor samples (or borate buffer for control), and 200 µl of HHL.

The mixture was then incubated for 1 hr. Enzymatic activity was stopped by adding 100 μl of 1N HCl. In the control samples, it is assumed that ACE produces 100% HA.

The amount of HA occurring from HHL hydrolysis was measured by HPLC (Shimadzu-20AD, Japan) with UV/VIS detector. Samples (10 μl) were analyzed using a Perkin Elmer C18 column (4.0 × 150 mm, 5 μm) and HA were detected at 228 nm. The flow rate was adjusted to 0.7 ml/min with mobile phases as (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile. The gradient consisted 5%–60% acetonitrile for the first 10 min, maintained for 2 min at 60% acetonitrile, then returned to 5% acetonitrile in 1 min. This was followed by isocratic elution for 4 min at 5% acetonitrile. The IC50 value was calculated by diluting the samples with 2-150X dilution factor.

2.5 | Statistical analysis

All results were analyzed by one-way analysis of variance (ANOVA) using SPSS. Mean values were analyzed Tukey's test ($p < .05$).

3 | RESULTS AND DISCUSSION

3.1 | Pigment contents and antioxidant activity

Ultrasonic supported sequential solvent extraction by methanol and aqueous $NaNO_3$ solution was applied and the extraction procedures were repeated until a colorless extract was obtained. The amounts of Chlorophyll-1.1, Phycocyanin-1.2, Phycocyanin-2.1, and Chlorophyll-2.2 contents in the samples, were measured after eight, four, seven, and four times repeated extractions, respectively. The pigment contents and antioxidant activities of liquid extracts obtained at various stages of sequential pigment extraction process are given in Table 1 on dry basis.

While samples of sequential extraction of chlorophyll and then phycocyanin have about 9.26 mg chlorophyll-a/g dry spirulina (Chlorophyll-1.1) and 63.71 mg phycocyanin/g dry Spirulina (Phycocyanin-1.2), samples of sequential extraction of phycocyanin and then chlorophyll have about 78.57 mg phycocyanin/g dry spirulina (Phycocyanin-2.1) and 6.90 mg chlorophyll-a/mg dry spirulina (Chlorophyll-2.2), respectively. It was found that Chlorophyll-a content of Chlorophyll-1.1 was higher than Chlorophyll-2.2 at about 25.49% and Phycocyanin content of Phycocyanin-2.1 was higher than Phycocyanin-1.2 at about 18.91%. In sequential extraction processes, first extraction steps result in higher pigment concentration than the sequential steps for both pigments. After first extraction, Chlorophyll-a in methanol and Phycocyanin in aqueous sodium nitrate solution are shown in Figure 2. It was observed that pigment concentrations after the first step extraction were the highest compared to that after successive extractions steps. Phycocyanin pigment content was found about 7 to 11 times (depending on extraction sequence) higher than Chlorophyll-a content which was the dominant pigment in samples obtained by applying first extraction of phycocyanin and then chlorophyll (Figure 1a) or first extraction of chlorophyll and then phycocyanin pigments (Figure 1b).

Chlorophyll-a and phycocyanin contents of *S. platensis* were reported as 10–13 and 140–156 mg/g dry *Spirulina*, respectively (Koru, 2012; Soundarapandian & Vasanthi, 2008; Yılmaz & Duru, 2011; Kim, 2015). Soundarapandian and Vasanthi (2008) used liquid nitrogen, freezing and thawing, sonication and lysozyme application for pigment extraction from *S. platensis* culture CS-1. While the highest phycocyanin content was obtained as 110.20 mg/g

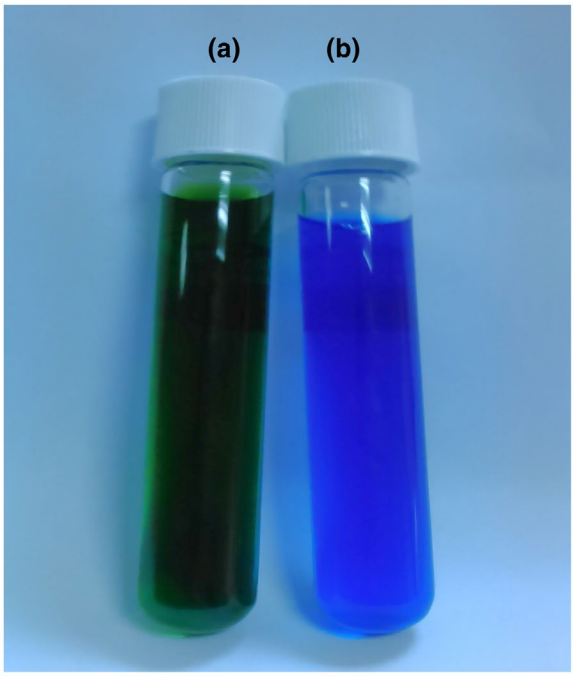

FIGURE 2 Chlorophyll-a (a) and phycocyanine (b) extracts

TABLE 1 Pigment content and antioxidant activity of sequential chlorophyll-a and phycocyanin extracts

Extract	Amount of pigment (mg/g dry spirulina)	Antioxidant activity (mg $FeSO_4 \cdot 7H_2O$/g dry spirulina)
Chlorophyll-1.1	$9.26 \pm 0.01^{c*}$	16.19 ± 0.82^{a}
Phycocyanin-1.2	63.71 ± 0.47^{b}	10.87 ± 0.25^{b}
Phycocyanin-2.1	78.57 ± 0.34^{a}	12.34 ± 0.33^{b}
Chlorophyll-2.2	6.90 ± 0.01^{d}	14.62 ± 0.05^{a}

*Mean values ± *SD* and different letters in the same column indicate significant difference ($p < .05$).

DW by liquid nitrogen method, and 82.20 mg/g DW by sonication method. Chlorophyll content was found to be 9.72 (mg/g DW). Patel et al. (2005) investigated phycocyanin content of three cyanobacteria (*Spirulina sp*., *Phormidium sp*. and *Lyngbya sp*.) species and they found that the highest rate of fhycocyanin in freeze-dried fresh cells was obtained from *Spirulina sp*. with 17.5% on dry basis. In this study, while chlorophyll-a content is similar to other reports with 6.90 to 9.26 mg/g dry spirulina, phycocyanin content with 63.71–78.57 mg/g dry spirulina is almost half of values reported by other works. Sarada et al. (1999) have reported that dried *Spirulina* samples contain about 50% less phycocyanin than the fresh samples and lower phycocyanin content in this study it can be explained by loss during drying process.

The antioxidant activity of Chlorophyll-1.1 and Chlorophyll-2.2 extracts obtained with methanol were found as 16.19 and 14.62 mg $FeSO_4{\cdot}7H_2O$/g dry spirulina, respectively. The antioxidant activities of Phycocyanin-1.2 and Phycocyanin-2.1 extracts obtained with 1.5% sodium nitrate aqueous solution were found as 12.34 and 10.87 mg $FeSO_4{\cdot}7H_2O$/g dry spirulina, respectively. Kuatrakul et al. (2017) investigated the antioxidant activity of the ethanol extracts after drying the fresh *Spirulina* sample in hot air and microwave vacuum. They found that antioxidant activity values of hot air and microwave vacuum dried samples as 5.17 and 9.65 (mg $FeSO_4{\cdot}7H_2O$/g DW), respectively. Chlorophyll-a pigment extracts have shown higher antioxidant potential than phycocyanin pigment extracts no matter the extraction sequence applied, first extraction of phycocyanin and then chlorophyll (Figure 1a) or first extraction of chlorophyll and then phycocyanin pigments (Figure 1b). It has been reported that the antioxidant activity values of the extracts obtained with organic solvents (hexane, petroleum ether, and ethanol) were found to be higher when compared with the antioxidant activity values of water extracts (Herrero et al., 2005).

TABLE 2 Protein content of *S. platensis* powder, the precipitates, and the dried phycocyanin powders

Sample	Protein (%)†
S. platensis	63.35 ± 0.05^{e*}
CF-1.1	62.38 ± 0.28^{e}
CPFP-1.2	66.17 ± 0.72^{d}
PP-1.3	80.26 ± 1.23^{b}
PF-2.1	69.28 ± 1.03^{c}
PCFP-2.2	59.35 ± 0.47^{f}
PP-2.3	83.69 ± 0.27^{a}

†On dry basis.

*Mean values ± *SD* and different letters in the same column indicate significant difference ($p < .05$).

3.2 | Protein content

Protein contents of dried residuals after pigment extraction are shown in Table 2. While *S. platensis* has a protein content of 63.35%, the highest protein contents found in dried phycocyanin precipitate PP-2.3 and PP-1.3 were 83.69% and 80.26%, respectively. Protein content of Chlorophyll extracted residual precipitates (CF-1.1) was found as 62.38% lower than that found in phycocyanin and chlorophyll extracted dried precipitates PF-2.1 (69.28%) and CPFP-1.2 (66.17%) except PCFP-2.2 (59.35%). High protein content in PF-2.1 and CPFP-1.2 can be explained as the relative increase of protein content in dry residual precipitate after aqueous extraction of phycocyanin by sodium nitrate.

3.3 | ACE inhibition

S. platensis and PP-2.3 samples were used for ACE inhibition and the obtained percent ACE inhibition (INH%) values are shown in Figure 3.

The highest ACE inhibition with 40 µl 10% suspension of *S. platensis* was observed as 92.40% while PP-2.3 extract sample showed as 89.50% inhibition (Figure 3a). For consideration of ACE inhibition potential of *Spirulina* powder and PP-2.3 extract, the suspensions were diluted in varying proportions between 2 and 150 times. Then the IC_{50} protein concentrations of the samples at which 50% inhibition of ACE activity occurs were calculated (Figure 3b). IC_{50} values of *S. platensis* and phycocyanin powder samples were calculated as 2.93 and 3.96 mg protein/ml, respectively. It can be concluded that *S. platensis* shows about %35 higher ACE inhibiton activity compared to that of the PP-2.3 extract, despite *Spirulina* having a lower protein content. Phycocyanin pigment extract shows lower ACE inhibition effect compared to *Spirulina* powder although having higher protein content (83.69%). Boschin et al. (2014), reported differences in ACE

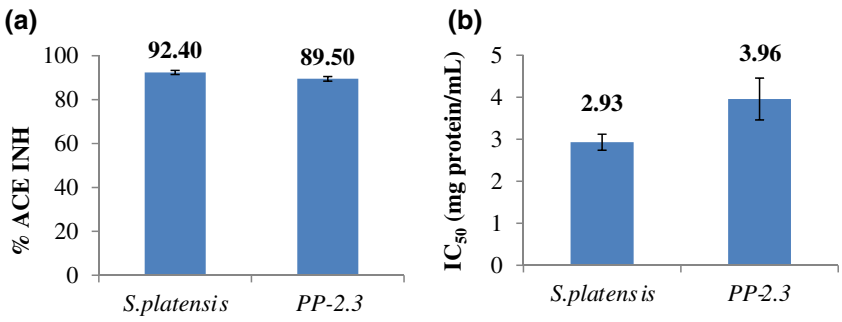

FIGURE 3 Percent ACE inhibition (a) and IC50 (b) values of *S. platensis* and PP-2.3 samples

inhibition activities depending on several reasons such as protein extraction method, parameters of hydrolysis process (substrate/enzyme ratio, pH, and temperature) and different analytical methods.

Mallikarjun Gouda et al. (2014) reported that IC_{50} ACE inhibition value of the extracts obtained with supercritical CO_2 was 274 μg/ml. *Spirulina* hydrolysates obtained using various proteases such as protamex, SM98011, alcalase, pepsin, and trypsin were ranging between 0.087 and 0.47 mg/ml (He et al., 2007; Liu et al., 2009; Lu et al., 2010; Suetsuna and Chen, 2001). In this study, the IC_{50} value of the *Spirulina* and PP-2.3 suspensions were found lower compared to the values mentioned in above researches.

4 | CONCLUSION

In this study, some bioactive properties such as ACE inhibition activity and antioxidant activity of *S. platensis* and its pigments were investigated. According to the results obtained it can be concluded that *Spirulina* can be used in many health beneficial functional foods due to the bioactive properties such as antioxidants and antihypertensive effects while high protein content pure phycocyanin has lower ACE inhibition compare to dried Spirulina. *S. platensis* is a well-known microalgae and can be recommended as a food or food ingredient because of high content of bioactive components besides high pigment and protein contents to promote health.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs.

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