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Antioxidant and antibacterial activities of C-phycocyanin from common name *Spirulina platensis*

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Abstract

In this study, the antibacterial and antioxidant properties of C-phycocyanin (C-PC) from *Spirulina platensis* were evaluated. The extraction and purification of C-PC were carried out using lyzosyme and ammonium sulphate precipitation, respectively. DPPH radical-scavenging activity, ferric reducing antioxidant power (FRAP) and Fe^{2+}-chelating activity were used for evaluation of antioxidant properties of C-PC. Antibacterial activity was also performed using agar well diffusion and microdilution [minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)]. The selected bacteria were *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Streptococcus iniae* and *Yersinia ruckeri*. The results showed that the biomass production was 1120 mg L^{-1} and C-PC concentration in crude extracts and purified by $(NH_4)_2SO_4$ were also 1.815 and 3.75 mg ml^{-1}, respectively. The results of DPPH, FRAP and Fe^{2+}-chelating activities of C-PC was 45.75%, 0.051 mg TAE g^{-1} and 40.23% at zero time and 41.56%, 0.046 mg TAE g^{-1} and 36.56% after 60 days at -18°C, respectively. The results of agar well diffusion indicated that *L. monocytogenes* and *S. iniae* were the most sensitive and resistant, respectively among examined bacteria in different concentrations of C-PC (0- 25 µg ml^{-1}) and the mean of inhibition zones were also 7.50-22.11mm. The results of MIC and MBC of C-PC (as µg ml^{-1}) were 50-500 and 100-500, respectively. As a conclusion, C-PC from *S. platensis* had high potential of antioxidant activity *in vitro* and it can be used as natural antioxidants in variety of foods. Antibacterial activity of C-PC was lower than other algal pigments (such as astaxanthin and C-PC in other cyanobacteria).

Keywords: Antibacterial properties, Antioxidant activity, C-phycocyanin, *Spirulina platensis*

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Introduction

Microalgae are a very various group of organisms that include both prokaryotic and eukaryotic forms. Microalgae are photosynthetic microorganisms that transform sunlight, water, and CO_2 to algal biomass. Some of microalgae biomass contains rich source of some nutrients such as proteins, minerals, carbohydrates, and other essential nutrients. The quality of these proximate factors depends on the type of algae, the environmental conditions and artificial culture media (Muthulakshmi *et al.,* 2012). Marine cyanobacteria are considered as emerging source of several compounds including fatty acids, carotenoids, polysaccharides and pigmented proteins showing various biological activities. Specifically, proteins and peptides of marine origin are widely studied and found to have potential biomedical applications (Lordan *et al.,* 2011). Spirulina is a photosynthetic blue green microalga, filamentous and spiral in shape and is a predominant species, commercially cultivated in many countries. It is one of the most nutritive microalgae food sources available in the market. Chemical composition of spirulina includes proteins (55-70%), carbohydrates (15-25%), essential fatty acids (18%) vitamins, minerals and pigments like carotenes, chlorophyll-a, and phycobiliproteins (phycocyanin, phycoerythrin and allophycocyanin). The two most important species of *Spirulina* are *S. maxima* and *S. platensis* (Xalxo *et al.,* 2013).

Phycobiliproteins (PBPs) are found in very high abundances (around 60% of the total protein content and 20% of the dry cell weight) in cyanobacteria. They have been considered as a potent pharmacological and medicinal agent due to their antioxidant capacity (Soni *et al.,* 2008; Sonani *et al.,* 2014). Phycocyanin (C-PC), which is present in many cyanobacteria and some red algae, is a photosynthetic pigment of the phycobiliprotein family. It is blue in color, fluorescent water soluble and is placed in the photosynthetic lamella in the cytoplasm membrane. When the envelope is broken, thylakoid membrane together with C-PC is released (Stec *et al.,* 1999; Pizarro and Sauer, 2001; Minkova *et al.,* 2003).

The purity of C-PC is generally evaluated using the absorbance ratio of A620/A280, and a purity of 0.7 is considered as food grade, 3.9 as reactive grade, and more than 4.0 as analytical grade. The most known C-PC extracted from *Spirulina* was first marketed in 1980 by Dainippon Ink and Chemicals under the brand name “Lina Blue–A” (Minkova *et al.,* 2003; Muthulakshmi *et al.,* 2012). C-PC is known to be hepato-protective, antioxidant, radical scavenger, anti-arthritic, anti-inflammatory, antitumor and immunity boosting activities and fluorescent markers in biomedical research. C-PC has been used as natural colorants in foods and cosmetics such as chewing gum, ice sherbets, soft drinks, candies, lipsticks, eyeliners (Eriksen, 2008) and also aquatic feed diets (Hagh Nejat *et al.,* 2005; Ansarifard *et al.,* 2018).

It is well known that microbial pigments are highly responsible for the

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health benefits and plays a key role as antioxidant due to the presence of hydroxyl substituent and their aromatic structures which enable them to scavenge free radicals. Antioxidants are substances that protect the cells from harm caused by instable molecules known as free radicals. Antioxidants include carotenoids: beta carotene, phenolic compounds: gallic acid, flavanoids, quercetin, alkanoids, capsaicin, hydroxytoulene, etc., (Bhat and Madyastha, 2001). C-PC can scavenge alkyl, hydroxyl and peroxyl radicals. Many diseases are formed due to exceeding formation of reactive oxygen species (ROS). ROS can also promote lipid oxidation that adversely affects the texture, color and flavor of food products, resulting in the discharge of remarkable volume of food and economic loss (Min and Ahn, 2005). Patel *et al.* (2005) studied the antioxidant properties of C-PC isolated from cyanobacteria species. The results showed that *Lyngbya*, *Phormidium* and *Spirulina* sp. were able to scavenge peroxyl radicals. However, antioxidant potential in *Lyngbya* sp. was higher than two other species. Jerle and Prabu (2015) surveyed antioxidant activities of C-PC from *S. platensis*. The C-PC showed the presence of antioxidant potency which is one of the important health components for functional foods. C-PC as natural antioxidant can be also used for the treatment of some neurodegenerative disturbances such as alzheimer, Parkinson and Huntington diseases and as biological preservative in different foods (Romay *et al.,* 2003; Xalxo *et al.*, 2013).

The Center for Disease Control and Prevention (CDC) assessments that approximately 48 million foodborne diseases occur annually in the United States and about 9.4 million of them have been caused by seven pathogens including *Salmonella*, *norovirus*, *Campylobacter*, *Toxoplasma*, *E. coli* O157:H7, *Listeria* and *Clostridium perfringens* (CDC, 2013). A variety of different chemical and synthetic compounds are used as antimicrobial agents to inhibit the growth of pathogenic microorganisms, but they can cause a variety of negative side-effects as well. Compounds derived from natural sources such as marine algae can be used as safe antimicrobial agents (Demirel *et al.,* 2009; Dashtiannasab *et al.,* 2012). C-PC is efficient against many bacterial and viral infections mainly through promoting the biological defense activity at relatively low concentration. The recent report indicates the C-PC from cyanobacterium *Westiellopsis* sp. has exhibited the antibacterial activity against *Pseudomonas* sp., *B. subtilis* and *Xanthomonas* sp. However, an antimicrobial property of C-PC from spirulina is rarely studied (Li *et al.,* 2005; Li *et al.,* 2006; Madhyastha *et al.,* 2006). Therefore, the aim of this study was to evaluate antioxidant and antibacterial activity of C-PC from *Spirulina platensis in vitro* experiments.

Material and methods

Growth of Spirulina platensis

The primary stock of *Spirulina platensis* was obtained from Phycology

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Laboratory, Biology Department, Tarbiat Modares University, Noor, Iran. The alga was cultivated in a 1000 ml conical flask containing 500 ml of modified Zarrouk's medium with pH 8.5, under sterile condition. Zarrouk's medium consists of $NaHCO_3$ (8 g), K_2HPO_4 (0.50 g, $NaNO_3$ (2.50 g), K_2SO_4 (0.50 g), NaCl (2.00 g), $MgSO_4{\cdot}7H_2O$ (0.20 g), $FeSO_4{\cdot}2H_2O$ (0.05 g) and urea (0.20 g). The Growth of the algae culture was done in an illuminated (3500 lux) growth room at 29±2°C under 12/12 hour light- dark cycles for 16 days. Manual shaking of cultures was done 3 times daily. After 16 days, it was viewed for its morphological structure under microscope and the biomass was collected, filtered and dried at 45°C for 48 hours (Kamble *et al.,* 2013; Prabakaran and Ravindran, 2013).

Extraction of phycocyanin

The C-PC extraction was performed according to Jerle and Prabu (2015) with minor modification. 2 g of dried *Spirulina* was added to 40ml of 0.1M sodium phosphate buffer (PBS) pH 7.0. Afterward, lysozyme (40 mg g^{-1} DW) and 100 mM EDTA were also added. This mixture was placed in 44°C shaker water bath for 4 hours to decompose the cell walls of the algae by enzymatic digestion process. After treating with enzyme, the mixture was centrifuged for 25 min at 10000 rpm in 4°C. This gives a clear blue colour supernatant which is further purified and the cell reminder is discarded.

Partial purification of phycocyanin using ammonium sulphate precipitation

Solid ammonium sulfate was gradually added into the beaker containing crude extracts of phycocyanin to obtain 40% saturation with continuous stirring for 1h. This solution was stored overnight at 4°C in dark condition and the precipitation was collected by centrifugation at 15,000 g for 15 min at 4°C. The colourless, clear supernatant was discarded and blue precipitate was dissolved in small volume of 0.1M PBS pH 7 and stored at 4°C in brown bottle until the examination time (Kumar *et al.,* 2013; Prabakaran and Ravindran, 2013; Kumar *et al.,* 2014).

Calculation of phycocyanin Concentration

The C-PC concentration in mg ml^{-1} of the supernatant was calculated by measuring the absorbance at 620 and 652 nm using the following equation (Antelo *et al.,* 2010):

$$\text{C}-\text{PC mg ml}^{-1} = \frac{A620 - 0.474 \times A652}{5.34}$$

Where A620 is absorbance at 620 nm, A652 is absorbance at 652 nm and 5.34 is constant factor.

Phycocyanin extract purity

The purity of the C-PC extract was monitored according to the OD620/OD280 ratio. The optical density at 620 nm indicates maximum absorption of the C-PC, while OD at 280 nm shows concentration of proteins in the solution (Liu *et al.,* 2005).

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Extraction yield

The extraction yield was calculated using the following equation (Silveira *et al.* 2007):

$$\text{Yield} = \frac{\text{C-PC} - V}{DB}$$

Where Yield is the extraction yield of phycocyanin in mg of C-PC/dry biomass (g), V is the solvent volume (ml) and DB is the dry biomass (g).

Determination of antioxidative activities

DPPH radical-scavenging activity

DPPH radical-scavenging activity of C-PC was determined as described by Jerley and Prabu (2015) with slight modification. Briefly, 23.5 mg of DPPH (1,1-diphenyl-2-picrylhydrazyl,Sigma–Aldrich, teinheim, Germany) was dissolved in 100 ml of ethanol and stored at 4°C until being ready for using. This solution was diluted 1:10 in ethanol for the direct assay. One hundred microliters of each algal extract was added to 3.9 ml of diluted DPPH solution in 15 ml screw-cap tubes. Due to the coloration of the extracts, it was necessary to prepare a background blank, which consisted of 100µl of C-PC added to 3.9 ml of ethanol (without DPPH). The primary mixture was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was read at 517 nm using a UV-1601 spectrophotometer (Cecil CE1020, England). DPPH radical-scavenging activity was calculated according to the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = A_{\text{Blank}} - \frac{A_{\text{Sample}}}{A_{\text{Blank}}} \times 100$$

Where A_{Blank} is the absorbance of C-PC and ethanol solutions without DPPH, and A_{Sample} is the absorbance of the C-PC and DPPH solutions at 517 nm.

Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) of C-PC was determined as described by Zhu *et al.* (2002) with some modifications. The sample solution (0.5 ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50 °C for 20 min, and then the tubes were permitted to adjust to room temperature. An aliquot (2.5 ml) of 10% trichloroacetic acid was added to the mixture, followed by 2.5 ml of 0.1% $FeCl_3$ and mixed and incubated for 5 min. Final solution (2.5 ml) was mixed with 2.5 ml of distilled water. Absorbance of the resulting solution was read at 700 nm. The reducing power was calculated as the ΔOD/mg DW. Increased absorbance of the reaction mixture indicates increasing ferric reducing antioxidant power. FRAP was stated as mg TAE g^{-1} of DW.

Fe^{2+}-chelating activity (%)

The ability of C-PC to chelate Fe^{2+} was determined using the method described by Ismaiel *et al.* (2016). Briefly, 150 µl of freshly prepared 500 µM $FeSO_4$ were added to a reaction mixture containing 168µl of 0.1 M Tris–HCl (pH 7.4) and

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218 µl of C-PC. The reaction mixture was incubated for 5 minutes at room temperature before the addition of 13 µl of 0.25% 1, 10-phenanthroline. The absorbance was subsequently measured at 510 nm. The chelating activity was calculated as: A_{blank} is the absorbance of the ferrous solution alone, and A_{sample} is the absorbance of the sample within the ferrous solution at 510 nm. All tests were carried out in triplicate.

The chelating activity was calculated as:

$$\text{Chelating activity (\%)} = \frac{A_{Blank} - A_{Sample}}{A_{Blank}} \times 100$$

Where A_{Blank} is the absorbance of the ferrous solution alone, and A_{Sample} is the absorbance of the C-PC within the ferrous solution at 510 nm.

Antibacterial activity of phycocyanin

Bacterial strains and culture conditions

Lyophilized foodborne bacterial pathogens consist of *Escherichia coli PTCC 1330*, *Staphylococcus aureus PTCC 1113* and *Listeria monocytogenes PTCC 1165* which were obtained from Persian Type Culture Collection of Iranian Research Organization for Science and Technology. Two fish bacterial pathogens including *Streptococcus iniae* and *Yersinia ruckeri* were isolated from rainbow trout (*Oncorhynchus mykiss*) in Caspian Sea Ecology Research Institute. Antibacterial assay was carried out according to Sarada *et al.* (2011) with some modifications. The bacterial strains were inoculated in the Brain Heat Infusion (BHI) broth (Merck, Germany) and incubated at 35 °C for *E. coli*, *Staph.aureus* and *L. monocytogenes* and at 30 °C for *Strep iniae* and *Y. ruckeri* in a shaking incubator at 150 rpm for 18 h. The turbidity of bacterial cells suspension was adjusted to 0.5 McFarland at 580 nm (1.5×10^8 CFU ml^{-1}). Final dilution of bacterial suspension for culture on Muller Hinton agar (Merck, Germany) was 10^6 CFU ml^{-1}.

Agar well-diffusion assay

The conventional well-diffusion method was tested according to Zgoda and Porter (2000). Each strain was spread uniforml1y onto Mueller Hinton Agar (MHA) plates using sterile cotton swabs. Wells of 6-mm diameter were made on MHA using gel puncture. Aliquots (50 µl) of C-PC from different concentrations (50, 100, 200, 400 and 500 µg ml^{-1}) were transferred onto each well so that the final concentrations were 2.5, 5, 10, 20 and 25 µg ml^{-1}. After incubation at 35 ˚C and 30 ˚C for 24 hrs, the inhibition zones diameter (mm) were measured using a transparent ruler. The sterilized distilled water as negative control and Tetracycline (30 mg disc) and Amikacin (30 mg disc) were used as positive controls for $gram^-$ and $gram^+$ bacteria, respectively, and Doxycycline (30 mg disc) was also used for both groups. All tests were performed in triplicate (Sitohy *et al.,* 2015; Khezri *et al.,* 2016).

Microdilution assay

The minimal inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined

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by serial dilution assay. Serial dilutions of C-PC were adjusted to final concentrations of 50, 100, 200, 400 and 500 µg ml^{-1} of BHI and 1 ml of each dilution was transferred into 5 ml test tube. To each tube, 100 µl of the 24 hrs old culture bacterial was inoculated and incubated for 24 hrs at 37˚C and 30˚C. The turbidity was determined at 600 nm using UV/VIS Spectrophotometer after 24 hrs. At the end of the incubation time, MIC was identified as the lowest concentration of C-PC which inhibits the visible growth. The MBC was defined as the lowest concentration of C-PC that completely kills inoculated bacteria. All tests were performed in triplicate. The sterilized distilled water and Doxycycline were used as negative and positive control, respectively (Sitohy *et al.,* 2015).

Statistical analysis

All data were analyzed by SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA), using one-way analysis of variance (ANOVA). The normality was tested by Kolmogorov-Smirnov Test. Duncan test was used for comparing the mean values of different treatments. P values less than or equal to 0.05 were considered statistically significant.

Results

Algal growth and biomass production

S. platensis was successfully cultured in modified Zarrouk's media for 16 days and their growth and biomass production were evaluated. The growth curve of the algae showed lag, log and stationary phases. The lag-phase continued for the first two days of culturing followed by the exponential phase (log-phase) (Fig. 1) and lasted until the 16th day of growth when the stationary phase began. There was a change in the appearance of culture from light green to dark green proportionate to the increasing cell mass. The light microscope observation of *Spirulina* appeared as blue green filament composed of cylindrical cells arranged in unbranched helicoidal trichome. Value of the biomass production was 1120 mg L^{-1} of culture.

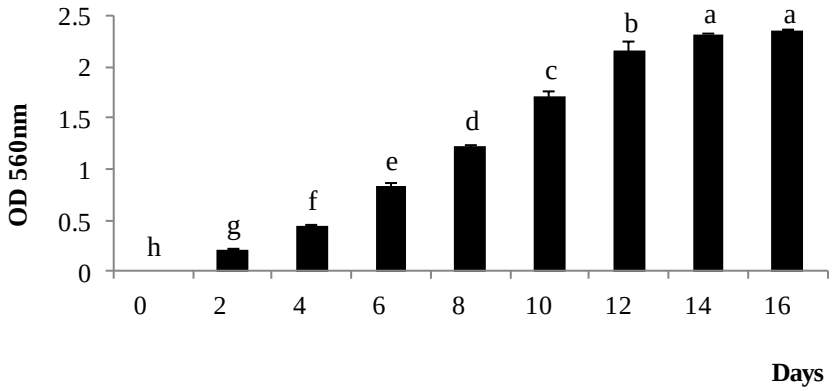

Figure 1: The growth curves of *Spirulina platensis* at different times. Each point represents the mean±SD of three replications. The different letters present significant differences at $p<0.05$.

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Phycocyanin concentration, purity and extraction yield

In this study, one step purification process was done to relatively get purified C-PC. The blue pigment was saturated by 40% of ammonium sulphate. The purity of C-PC was increased after purification by saturated ammonium sulphate. The optical density (OD) of crude C-PC and treated C-PC were read and the purity of the samples were calculated (Table 1). The final amount of phycocyanin was 62.34±0.34 mg g^{-1} DW.

Table 1: Purity and concentration of phycocyanin from *Spirulina platensis.*

Purification Step	Purity(A620/A280)	C-PC concentration (mg ml^{-1})
Crude Extract	0.825 ± 0.04^{b}	1.815 ± 0.06^{b}
40% $(NH_4)_2SO_4$ Precipitation	1.135 ± 0.08^{a}	3.751 ± 0.05^{a}

Different small superscript letters within each column, represent significant differences ($p<0.05$).

Antioxidant activity

The results of DPPH radical-scavenging activities FRAP and Fe^{2+} chelating potential of C-PC in zero time and 60 days stored at -18 °C are shown in Table 2. Mean (±SD) of antioxidant activity of C-PC in zero time was more than that of 60 days. There was no significant difference between two time periods, except FRAP.

Table 2: Antioxidant activity of C-PC from *Spirulina platensis* (DPPH, FRAP and Fe^{2+}-chelating activity in zero and 60 days).

C-PC	DPPH (%)	FRAP (mg TAE/g)	Fe^{2+}-chelating activity (%)
C-PC in zero time	45.75 ± 2.16^{a}	0.051 ± 0.01^{a}	40.23 ± 1.45^{a}
C-PC in 60 days	41.56 ± 1.83^{a}	0.046 ± 0.01^{b}	36.56 ± 2.37^{a}

Different small superscript letters within each column, represent significant differences ($p<0.05$).

Antibacterial properties

Agar well-diffusion assay

The antibacterial activity of C-PC (in two times) was examined against three gram positive ($gram^{+}$) bacteria (*L. monocytogenes*, *Staph. aureus* and *Strep. iniae*) as well as *Y. ruckeri* and *E. coli* as gram negative ($gram^{-}$) bacteria by measuring the area of the inhibition zones. The used bacterial strains were shown different results and, the diameter of the inhibition zone has increased by increasing the amount of C-PC. Among gram-negative bacteria, the maximum and minimum inhibition zones were observed in *L .monocytogenes* (22±1.25 mm) and *Strep.iniae* (10.76±0.41), respectively. *Y. ruckeri* as a $gram^{-}$ (14.50±0.32 mm clear zone) was more sensitive than *E. coli* (12.22±0.24) in 25µg ml^{-1} of C-PC (Table 3). The inhibitory actions of C-PC against the $gram^{+}$ bacteria were always higher than $gram^{-}$ bacteria except *Strep. iniae*. The diameter of inhibition zones in 60 days was significantly lower than zero time in all bacteria ($p<0.05$).

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Table 3: Inhibition zone diameters (mm) in agar well diffusion assays of phycocyanin from *Spirulina platensis* against dominant gram$^-$ and gram$^+$ bacteria.

Strain	Time (day)	Zone of inhibition (mm)								
		C-PC(µg ml^{-1})						Positive control		
		0	2.5	5	10	20	25	Amikacin	Tetracycline	Doxycycline
L. monocytogenes	zero	0	10.0±0.11^{eA}	13.0±0.41^{dA}	17.20±1.14^{cA}	19.50±1.41^{bA}	22.11±1.25^{aA}	-	33.0±1.56	41.0±1.14
	60	0	8.22±0.31^{cB}	9.0±0.55^{cB}	13.41±1.22^{bB}	14.30±1.32^{bB}	17.6±1.45^{aB}			
Staph. aureus	zero	0	R*	R	11.45±1.11^{bA}	15.44±1.30^{aA}	16.5±1.27^{aA}	-	24.0±0.32	35.45±1.21
	60	0	R	R	8.50±0.26^{bB}	12.50±0.70^{aB}	13.68±1.11^{aB}			
Strep. Iniae	zero	0	R	R	R	10.55±0.47^{aA}	10.76±0.41^{aA}	-	19.0±0.44	27.22±1.46
	60	0	R	R	R	8.50±0.33^{bB}	9.50±0.32^{aA}			
Y. ruckeri	zero	0	R	R	10.43±0.51^{cA}	12.34±0.24^{bA}	14.50±0.32^{aA}	16.0±0.35	-	32.11±1.28
	60	0	R	R	8.11±0.15a^{bB}	9.66±0.21^{aB}	10.20±0.17^{aB}			
E.coli	zero	0	R	R	9.26±0.30b^{cA}	10.44±0.50^{bA}	12.22±0.24^{aA}	17.50±0.60	-	36.55±2.17
	60	0	R	R	7.50±0.22^{bB}	9.11±0.25^{aB}	9.70±0.43^{aB}			

R*: resistance to C-PC, Different small and capital superscript letters within each row and column respectively, represent significant differences (p<0.05).

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of C-PC were shown in Table 4. The MIC and MBC values for C-PC were found in the range of 50- 400 µg ml^{-1} and 100-500 µg ml^{-1}, respectively.

Table 4: Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) (µg ml^{-1}) of phycocyanin from *Spirulina platensis.*

Strain	Time (day)	MIC (µg ml^{-1})	MBC (µg ml^{-1})
L. monocytogenes	zero	50	100
	60	100	200
Staph. Aureus	zero	100	200
	60	200	400
Strep. Iniae	zero	400	500
	60	500	-
Y. ruckeri	zero	200	400
	60	200	500
E.coli	zero	200	400
	60	200	500

Discussion

S. platensis is an edible alga with valuable sources of phycobiliproteins, which helps production of blue pigment phycocyanin. This alga can grow abundantly under suitable environmental conditions and with sufficient nutrients. After 16 days of cultivation, the dried biomass was found to be 1120 mg L^{-1}. Several studies have been conducted regarding the optimum of *spirulina* biomass (Sharma *et al.*, 2014). Jerle and Prabu (2015) used modified Zarrouk's medium with pH 9 for 9 days that final algal biomass was 280 mg L^{-1}. Biomass product aforementioned was lower than present study (4 fold). It may be due to range of pH and composition of medium, time and temperature of storage as well as light periods. Ismaiel *et al.* (2016) recorded that the highest value of the biomass production of *spirulina* was obtained at pH of 9.0 (66 mg DW 50ml^{-1}) after 14 days which was similar to the current study.

The extraction efficiency, purity and concentration of the C-PC mainly

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depend on the cell envelope disruption. In present study, lysozyme with EDTA and PBS were used for the extraction of C-PC from *Spirulina*. The cell envelope was broken and the C-PC placed on the thylakoid cell membrane was released outside by the lytic enzyme lysozyme. EDTA and buffer chelate the mg^{2+} cations and destroy the cell membrane and release the C-PC. Several extraction methods such enzyme, ultrasound, homogenization, freezing and thawing as well as organic and inorganic solvents were used for the extraction of C-PC from *spirulina* (Duangsee *et al.*, 2009; Kumar *et al.*, 2013; Sivasankari *et al.*, 2014; Kuddus *et al.*, 2015; Yu *et al.*, 2016) and also other algae (*Pseudoanabaena* sp. and *Lyngbya* sp.). Lysozyme is one of the most important enzymes for the extraction of the algal pigments particularly C-PC (Jerle and Prabu, 2015). In the current study, the partial purification of C-PC was done with aggregation of 40% ammonium sulphate. C-PC concentration in the extract and purified ammonium sulphate was 1.81 and 3.75 (mg ml^{-1}), respectively, that was more than Jerle and Prabu (2015) report, and was similar to the study by Ismaiel *et al.* (2016). The composition of the culture media, time and temperature of incubation and so on are some important factors that influence on the growth of *Spirulina* and subsequently C-PC production.

The combination of three techniques such as ammonium sulphate precipitation, dialysis and ion exchange chromatography provides a simple and rapid way to obtain large amount of blue pigment phycocyanin (Jerle and Prabu, 2015; Yu *et al.*, 2016; Moraes *et al.*, 2010). When the measurement of A620/A280 was greater ≥4, the phycocyanin was considered to be highly pure (as pharmaceutical and analytical agents). The purity of 0.7 and 3.9 are considered as food or cosmetic and reactive grades, respectively (Muthulakshmi *et al.*, 2012). The purity of C-PC in the present study was between cosmetic and reactive grades (1.135).

DPPH method is widely used to measure the ability of antioxidant compounds to act as free radical scavengers (Shon *et al.*, 2003; Ismaiel *et al.*, 2014). In this study, the antioxidant activity of phycocyanin in zero and 60 days later was 45.75 and 41.56, respectively. These results confirm that the C-PC is potent free radical scavenger and inhibits lipid peroxidations in both temperatures. However, an antioxidant property of C-PC decreased in 60-day time at -18 °C. It is probably due to the nature of the C-PC protein that is changed when stored in freezing temperature. In the study conducted by Jerle and Prabu (2015), the antioxidant activity of phycocyanin was lower than (25.21%) that of the present study. The results of Ismaiel *et al.* (2016) showed that *S. platensis* had stronger antioxidant activity than the positive control (2.5µg BHT) at a wide range of pH levels from 7.5 to 11.0. The radical scavenging activity, reducing power and chelating activities showed the highest value at pH of 8.5-9.0. Phycocyanin and other pigments

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such as chlorophyll and carotenoids as well as phenolic compounds of *S. platensis* have antioxidant properties. The most antioxidant activity of the C-PC in this study was at pH of 8.5 which was similar to Ismaiel *et al.* (2016). In another study, water and methanolic extracts of *Spirulina* sp., *Lyngbya* sp. and *Pseudanabaena* sp. were studied and determined that antioxidant efficiency of water and methanolic extracts of *Lyngbya* was more than two other algae (Paliwal *et al.*, 2015). The C-PC is one of the most water soluble substances in blue-green algae (Marx and Adir, 2013).

FRAP assay is often used to measure the ability of an antioxidant to donate an electron. The extract causes the reduction of Fe^{3+}/ferricyanide to the ferrous (Fe^{2+}) form and therefore, the Fe^{2+} complex can be monitored by measuring absorbance at 700 nm (Hossain *et al.*, 2016). In this study, the ferric reducing antioxidant power of C-PC ranged from 0.046 to 0.051 mg TAE g^{-1}that its content in zero time was more than 60 days. Hossain *et al.* (2016) studied the DPPH and FRAP values of four cyanobacteria including *Oscillatoria* sp., *Spirulina* sp., *Lyngbya* sp. and *Microcystis* sp. The results showed that FRAP value in *Spirulina* was more than other algae except *Oscillatoria;* and DPPH value of *Spirulina* was also more than *Microcystis* sp. and lower than *Lyngbya* sp. and *Oscillatoria* sp. The phycocyanin, phycoerythrin and allophycocyanin values in *Spirulina* were lower than other algae. However, its antioxidant activity was remarkable. The results of the present study was higher than Hossain *et al.* (2016) and Sharathchandra and Rajashekhar, (2013) results.

In the metal chelating assay, ferrozine can quantitatively form complexes with Fe^{2+}. Algal extracts can inhibit the formation of ferrozine- Fe^{2+} complex, resulting in a decrease of color development (Cho *et al.*, 2007). Our results showed that C-PC has a good metal chelating activity (36.56-40.23%). However, metal-chelating activity in zero time was more than 60 days later at -18 °C. Bermejo *et al.* (2008) reported that the antioxidant activity of C-PC may arise from both radical-scavenging and metal chelation and it was an indicator of the metal-chelating activity. The results of Ismaiel *et al.* (2014) showed that *S. platensis* had the highest radical scavenging activity and reducing power whereas chelating activity in *Nostoc linkia* was more than *S. platensis*, *Nodularia* sp. and *Anabaena flosaquae*. These abilities may be attributed to the antioxidant-specific properties of *S. platensis* because of cell contents such as proteins, polysaccharides, vitamins, carotenoid compounds, phycocyanins, and minerals (Singh *et al.*, 2005; Suhail *et al.*, 2011).

Food-born and spoilage bacteria (*Staph. aureus, E. coli and L. monocytogenes*) and also bacterial fish disease (like *Y. ruckeri and Strep iniae*) were used for antibacterial property assay. Generally, chemical preservatives were utilized for decreasing the spoilage and pathogen microorganisms and increasing the

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shelf life of food. However, most chemical preservatives have severe side effects and are carcinogenic and teratogenic (Anand and Sati, 2013; Inetianbor *et al.*, 2015). In aquaculture, antibiotics and chemical substances were applied such as malachite green for controlling the microbial infections in fish. These materials cause antibiotic resistance in different microorganisms and the distribution of resistant strains in aquatic ecosystems. In addition, reports have been indicated that the residues of these materials in fish tissues cause various problems in the consumers in the long time (USEPA, 2009; Pham *et al.*, 2015; Chuah *et al.*, 2016). Therefore, the use of natural preservatives such as bacterial metabolites (bacteriocins, organic acids) and algal extracts (phenolic compounds and pigments) in food appears to be necessary. The results of antibacterial activity of the this study showed that *Listeria* and *Streptococcus* were the most sensitive and resistant isolates against C-PC, respectively. Few studies were done on the antimicrobial effects of C-PC of *Spirulina*. However, several studies have evaluated the antimicrobial activity of phycocyanin from other cyanophytes. It seems that the lower concentration of C-PC of *Spirulina* had no significant effect on the studied bacteria. Antibacterial effects of the commercial antibiotics were more than C-PC (p<0.05). In the study carried out by Sitohy *et al.* (2015), antibacterial effects of C-PC from *Anabaena* sp. on *Klebsiella* sp., *Escherichia coli*, *S. aureus*, and *Bacillus cereus* have been examined. The results indicated that the inhibitory property of C-PC on *Staph. aureus* was more than other isolates. Antibacterial characterization also promoted with increasing C-PC concentration from 5 to 30 µg $disc^{-1}$. The overall results of Sitohy *et al.* (2015) confirm the current study. However, the antibacterial potential of C-PC from *Anabaena* was more than C-PC from *Spirulina*. Mohite *et al.* (2015) reported that the decline percentages of the studied bacteria against C-PC from *Spirulina* were 66.34%, 60%, 58.5%, 85% and 20% for *E. coli*, *B. cereus*, *B. subtilis*, *S. aureus* and *Salmonella typhi*, respectively. With increasing C-PC value from 0.34 to 1.74 µg, the antibacterial effects significantly incremented. The results of the study by Mohite *et al.* (2015) are similar to the present study. The results of the study by Sarada *et al.* (2011), regarding the antibacterial activity of C-PC (100 µg), both for agar well diffusion and micro-dilution methods, were slightly different from the current study. The range of MIC of C-PC was 50-50 µg ml^{-1} while in this study it was 50-400 µg ml^{-1}. In the study by Sarada, *Acinetobacter* and *Enterococcoccus* were also resistant to C-PC in all concentrations. Muthulakshmi *et al.* (2012) studied the inhibitory effects of C-PC from *Spirulina* on *E. coli*, *Streptococcus* sp., *Pseudomonas* sp., *Bacillus* sp., and *S. aureus*. The results indicated that the antibacterial property of C-PC has increased in higher concentrations (400µl $disk^{-1}$). These results were compatible with the present study. Among the used bacterial strains, *S. aureus* and *Streptococcus* sp.

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were the most resistant and sensitive isolates which were incompatible with this research respectively. It was probably due to bacterial resistance of these strains to C-PC that varied from one species to another.

As a conclusion, C-PC from *S. platensis* had high potential of antioxidant activity *in vitro* and it can be used as a natural antioxidant in a variety of foods, especially foods with high lipids. According to the purity of C-PC in this study, it can be applied in different types of cosmetics. Antibacterial activity of C-PC was lower than other algal pigments (astaxanthin from *Haematococcus pluvialis*) and C-PC from *Anabaena* and *Lyngbya*. Both antioxidants and antibacterial activities of C-PC reduced with long time storage at -18°C. It is probably due to the effect of freezing temperature on protein structure of C-PC.

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