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Extraction and purification of C-phycocyanin from dry *Spirulina* powder and evaluating its antioxidant, anticoagulation and prevention of DNA damage activity

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ABSTRACT

C-Phycocyanin (C-PC) is a phycobiliprotein found in blue green algae, such as *Spirulina platensis*, is often used as a dietary nutritional supplement and exhibit a variety of pharmacological properties. In this regard, extraction, partial purification and antioxidant, anticoagulation and prevention of DNA damage activity of C-PC was investigated. In the present study, a simple and efficient method to extract C-PC from *Spirulina platensis* dry powder is reported. The extractions were carried out using two different methods: cold maceration and sonication method. The extraction using cold maceration method proved to be the most efficient method. Obtained crud C-PC was purified by ammonium sulphate precipitation, dialysis and gel filtration and presented a final extraction yield of 3.27±0.09 mg/ml with a purity ratio of 2.317±0.08. When it was evaluated as an antioxidant *in vitro*, it was able to scavenge nitric oxide. C-PC showed significant anticoagulation and prevention of DNA damage activity.

INTRODUCTION

The Cyanobacterium (blue-green alga) *Spirulina platensis* has been commercialized in several countries for its use as a health food and for therapeutic purposes due to its valuable constituents, particularly proteins and vitamins (McCarty, 2007). Cyanobacteria and algae possess a wide range of colored compounds, including chlorophyll, carotenoids and phycobilliproteins (Raja *et a.l*, 2008).

Among the protein present in *Spirulina*, Phycobiliproteins are accessory photosynthetic pigments that participate in an extremely efficient energy transfer chain in photosynthesis. It is a hydrophilic, brilliantly colored and stable fluorescent pigment protein that can be classified into three main groups: Phycocyanin (deep blue), Phycoerythrin (deep red) and Allophycocyanin (bluish green) depending on the inherent color and absorbance properties (Raja *et a.l*, 2008). The phycobilliproteins such as C-phycocyanin (C-PC), allo-phycocyanin, and phycoerythrin, are made up of dissimilar α and β polypeptide subunits ((Raja *et a.l*, 2008). C-Phycocyanin (C-PC) could be extracted from cyanobacteria such as *Spirulina platensis*, which has been widely used in commercial applications in the food and cosmetic industry as a natural blue dye.

Recent studies have demonstrated the role of C-PC in hepatoprotective (Ferreira *et al*., 2010), anti-inflammatory (Ferreira *et al*., 2010; Deng and Chow, 2010) and antioxidant (Ferreira *et al*., 2010; Gantar *et al*., 2012) as well as being a free radical scavenger (Gantar *et al*., 2012).

Each microorganism has particular characteristics referring to the location of intracellularly produced proteins. Hence the extraction protocol could vary according to the desired protein. In general the extraction method is the key for maximum recovery of phycobilliproteins in the natural state from algae (Moares *et al*., 2010). The extraction of phycobilliproteins involves cell rupture and release of these proteins from within the cell. By considering various significant biological applications of C-PC, the aim of this study was extraction and purification of C-phycocyanin from the dry *Spirulina* powder using various

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methods and to evaluate its biological activities like antioxidant, anticoagulant and DNA scavenging activity.

MATERIALS AND METHODS

Materials

Spirulina platensis dry powder was purchased from NB Laboratories Pvt. Ltd. (Uppalwadi, Nagpur, India). Protein marker (broad range) was obtained from Bangalore GeNei, India.

Protein extracts preparation- (C-PC isolation from *Spirulina*)

Two different extraction methods i.e. cold maceration and sonication methods were used to optimize the isolation of C-phycocyanin (C-PC). Briefly, C-PC was extracted from *Spirulina platensis dry* powder 1:25 (w/v) in distilled water at 4^0C for 24 hr (cold maceration). In sonication method, 1:25 (w/v) *Spirulina* powder in distilled water was irradiated at 40 kHz for 40 min. The resultant slurry from both the methods was centrifuged at 10,000g for 15 min at 4°C to remove the cell debris. The precipitate was discarded and the supernatant crud extract was collected. The pH of the crud extract was adjusted to pH 7.0 for the following steps.

Purification of C-PC

Ammonium sulfate precipitation

Ammonium sulfate was gradually added in 100 ml crude extracts to achieve 25% and 50% saturation with continuous stirring. Resulting solution was kept for 2h and centrifuged at 12,000g for 30 min. The obtained blue precipitate was dissolved in 0.005 M Na-phosphate buffer (pH- 7.0) (Silva *et al.*, 2009). At each extraction step, the C-PC concentration was calculated by the method of Boussiba and Richmond (Boussiba and Richmond, 1979) and purity was calculated by the method of Bennett and Bogorad (Bennett and Bogorad, 1973).

Dialysis and Gel filtration

The obtained crude C-PC was dialyzed against 1000 volumes of 0.005 M Na-phosphate buffer (pH- 7.0) overnight at 4^0C. Dialyzed sample was further purified by passing through Sephadex G-25 column (12 × 2 cm). The column was pre-equilibrated and eluted with same buffer. Fractions were collected at a 0.5 ml/min flow rate (Liao et *al.*, 2012). Then, the purity of all fractions was checked by equation and fractions showing maximum purity was selected for SDS-PAGE.

Electrophoresis in polyacrylamide gel (SDS-PAGE)

Electrophoresis of dialyzed sample as well as gel filtration fractions in polyacrylamide gel was carried out in a vertical chamber using 12% polyacrylamide gel with SDS (SDS-PAGE) (Laemmli, 1970). Our molecular markers was protein marker broad range, (Myosin 205 kDa, Phosphorylase B 97.4 kDa Bovine serum albumin 66 kDa, Ovalbumin 43 kDa, Carbonic anhydrase 29 kDa, Soyabin trypsin inhibitor 20.1 kDa, Lysozyme 14.3 kDa, Aprotinin 6.5 kDa, Insulin 3.5 kDa.) obtained from Bangalore GeNei, India. Following electrophoresis, the gel was stained by 0.1% Coomassie Brilliant G250 solution.

Determination of anticoagulation activity

The anticoagulation activity of purified C-PC was investigated using the method of USA pharmacopia (1985) as follow: In each tubes, 0.8 ml of extract solution (1%), 0.8 ml of standard heparin sodium solution (0.5 U.S.P unit/0.8 ml), or 0.8 ml saline solution was added. Then, 1 ml plasma and 0.2 ml of calcium chloride solution (1%) were added in each tube. The tubes were stopped immediately, and inverted three times to mixed the contents and the entire inner surface of the tube became wet. The time required for clotting was recorded.

Determination of Anti-oxidant Activity

Nitric Oxide (NO) scavenging activity

The nitric oxide radical scavenging activity was measured by using Griess reagent. 50, 100, 150, 200 and 250 µl of purified C-PC (2.295 mg/ml, Purity 1.41) and 500 µl of standard (Vit.C 1 mg/ml) were taken and diluted up to 1.5 ml with distilled water in test tubes. Then, 1.5 ml of 10 mM Sodium Nitroprusside was added to all tubes and incubated for 150 min at 25^oC. After incubation, 1.5 ml of the reaction mixture was transferred to the new tubes and 1.5 ml Griess reagent (1% Sulphanilamide, 2% Orthophosphoric acid, 0.1% NEDD) was added to all tubes. O.D. was taken at 545 nm (Green *et al.*, 1982; Kalim *et al.*, 2010). Vit.C was used as positive control and decrease in absorbance indicated higher number scavenging activity. The experiments were performed in triplicate and percent scavenging activity was calculated as follows;

$$\text{Scavenging \%} = \frac{\text{Abs of control} - \text{Abs of test} \times 100}{\text{Abs of control}}$$

Prevention of oxidative DNA damage by C-PC

Prevention of oxidative DNA damage was determined as described by Kalim et al. (Kalim *et al.*, 2010; Singh *et al.*, 2010). Plasmid DNA pUC19 (250ng) was treated with $FeSO_4$ and Phosphate buffer (pH 7.4) in a final concentration of 0.5mM and 50mM, respectively, and the test extracts at different concentrations (0-6 µg/ml). The total reaction volume was set to 12µl and the mixture was incubated at 37°C for 1Hr. After incubation, the extent of DNA damage and the preventive effect of the *Spirulina* (C- PC) were analyzed on 0.8% agarose gel at 80V at room temperature. Vitamin C (1mM) was used as positive control.

RESULTS

Extraction of C-PC

One of the most important requirements for obtaining phycobiliproteins from *Spirulina* dry powder is selection of extraction protocol. In the present study, we used cold maceration method (using water and 0.1M sodium phosphate buffer, pH 7.0) and Sonication method for extraction of C-PC and its concentration and purity was assessed. (Table 1.).

Purification of C-PC

For further purification of C-PC, phosphate buffer crude extract showing maximum purity and concentration was selected.

The purification steps involved fractional precipitation with 25% and 50% ammonium sulphate, dialysis and finally sephadex G-25. At each purification step, the concentration and purity of C-PC was checked (Table 2).It was found to be increased after each purification step.

Table. 1: Comparison of different methods for extraction of C-PC from *Spirulina.* (mean values ±SD).

Extraction methods		C-PC (mg/ml)	Purity Ratio ($A_{615}/_{280}$)
Cold maceration	Distilled water	0.57±0.05	0.149±0.07
	Sodium phosphate buffer	0.606±0.03	0.161±0.05
Sonication		0.26±0.05	0.07±0.02

Table. 2: Determination of spectrophotometric purity of C-PC from *Spirulina* after different steps of purification. (mean values ±SD).

Steps of purification		C-PC (mg/ml)	Purity Ratio ($A_{620}/_{280}$)
Crude extract		0.606±0.04	0.161±0.01
Ammonium sulphate precipitation	25%	4.176±0.05	0.248±0.03
	50%	4.55±0.08	0.628±0.03
Dialysis		5.674±0.20	1.08±0.07
Sephadex G-25		3.27±0.09	2.317±0.08

Molecular weight of C-PC by SDS-PAGE

The extracted and purified C-PC was further used to determine the molecular weight by SDS-PAGE (Singh *et al.*, 2010). After running the purified fractions at each step, the result showed the presence of C-PC (Fig. 1).

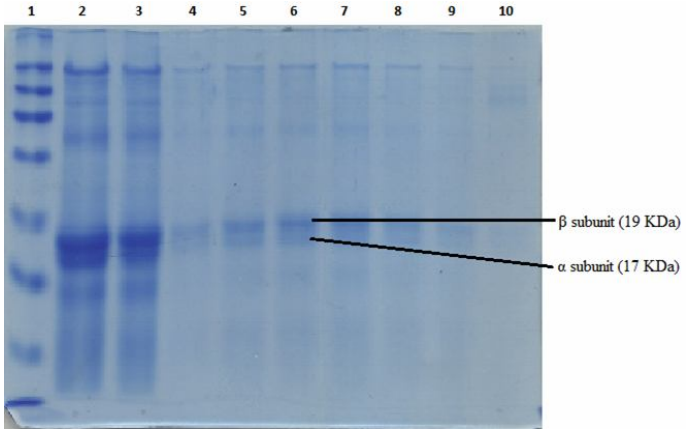

Fig. 1: 12% polyacrylamide gel electrophoresis (SDS-PAGE) of different fractions of C-PC**.** (**1**- Protein marker (Myosin 205 KDa, Phosphorylase b 97.4 KDa Bovine serum albumin 66 KDa, Ovalbumin 43 KDa, Carbonic anhydrase 29 KDa, Soyabin trypsin inhibitor 20.1 KDa, Lysozyme 14.3 KDa, Aprotinin 6.5 KDa, Insulin 3.5 KDa.), **2**- 50% precipitation sample, **3**- Dialyzed sample, **4**- Fraction no. 19, **5**- Fr. No.20, **6**- Fr. No.21, **7**- Fr. No.22, **8**- Fr. No.23, **9**- Fr. No.24, **10**- Fr. No.25.).

Anticoagulation activity of C-PC

The obtained results for anticoagulation activity of extracted C-PC (Fig. 2) showed that it possesses great anticoagulating efficiency (expressed by clotting time assay) compared with that of the standard anticoagulant heparin (sulfate glucouronic acid) to be 12 and 14 minutes respectively.

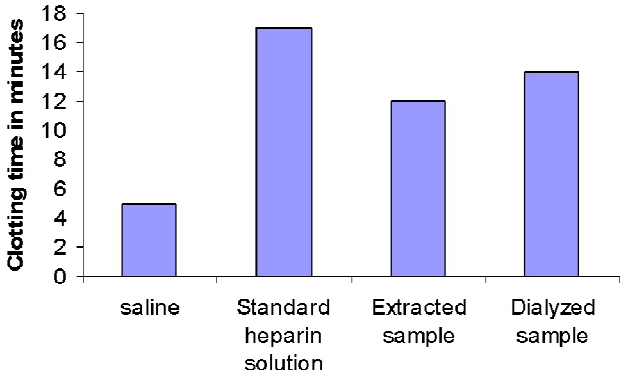

Fig. 2: Anticoagulation activity (clotting time) of purified C-PC from *Spirulina*.

Nitric oxide radical scavenging effect

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals.

In the present study, the purified C-PC competes with oxygen to react with nitric oxide and thus inhibits generation of the anions. Table-3 illustrates the percentage inhibition of nitric oxide generation by the C-PC in concentration 10, 25, 50 and 100µg/ml which significantly scavenged 64.6%, 86.65%, 90.17% and 92.4% of the nitric oxide radicals respectively.

Table. 3: Effect of purified C-PC from *Spirulina* on Nitric oxide scavenging activity.

Drug	Dose (µg/ml)	Nitric oxide scavenging	% inhibition
control	--	0.032	-
C-PC	10	0.116	64.6
	25	0.295	86.65
	50	0.517	90.17
	100	0.778	92.58
Vitamin C	25	0.691	96.2
	50	0.919	97.1

Prevention of oxidative DNA damage by C-PC

To asses the prevention of oxidative DNA damage by the purified C-PC, the preventive effect was evaluated over Fenton-induced damage of plasmid DNA, pUC19. Control pUC19 showed two bands, one of is circular DNA, which was clearly visible, and one of supercoiled DNA.

The treatment with $FeSO_4$ in the absence of C-PC leads to the formation of open circular DNA by strand scission of the supercoiled DNA. Whereas, the C-PC at different concentrations showing optimum activity and prevention of strand scission. The maximum prevention of DNA damage was shown at 4µg/ml concentration (Fig. 3).

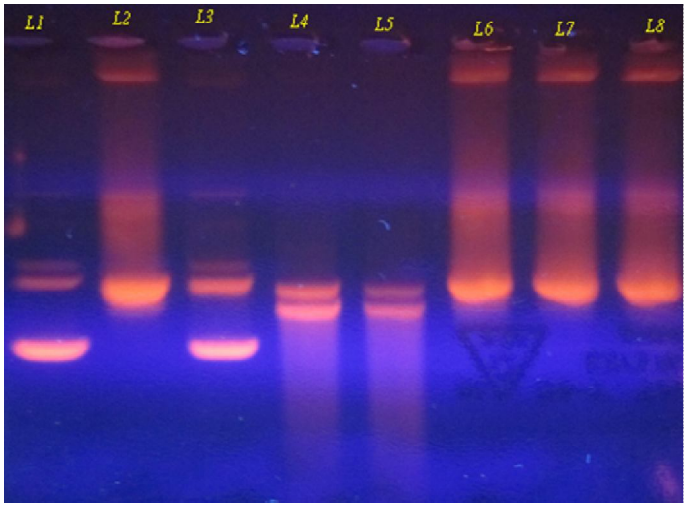

Fig. 3: Electrophoresis pattern of plasmid pUC19 DNA breaks by OH generated from the Fenton reaction and prevented by C-PC. Lane 1: untreated control DNA (250ng); lane 2: DNA (250ng) + $FeSO_4$ (0.5mM); lane 3: DNA (250ng) in presence of Vit. C (1mM); lane 4: DNA (250ng) + $FeSO_4$ (0.5mM) in presence of Vit.C (1mM); lane 5: DNA (250ng) + $FeSO_4$ (0.5mM) in presence of Vit.C (1mM) + C-PC (2µg/ml) lanes 6-8: DNA (250ng) + $FeSO_4$ (0.5mM) + C-PC (2µg/ml); DNA (250ng) + $FeSO_4$ (0.5mM+ C-PC (4µg/ml); DNA (250ng) + $FeSO_4$ (0.5mM+ C-PC (6µg/ml).

DISCUSSION

One of the most important requirements for obtaining phycobiliproteins from *Spirulina* dry powder is selection of extraction and purification protocol. A purification procedure that works well for a phycobiliprotein from one organism may not be the method of choice for the corresponding phycobiliprotein from another organism. For, these reason the phycobiliprotein extraction methods were standardized in present investigation. During comparison of various methods for extraction of crude C-PC, distilled water yields 0.57 mg/ml, 0.606mg/ml for sodium phosphate buffer and 0.26mg/ml for sonication method. Thus, the result obtained shows that extraction of C-PC using sodium phosphate buffer, pH 7.0 gave maximum yield then extraction by water and sonication methods and it was used for further purification.

For purifying C-PC various precipitating agents can be used such as PEG, ethanol, acetone, TCA and ammonium sulfate. Among these, we have selected ammonium sulfate because it is cheap, best and reliable method as it precipitates readily and also prevent denaturation of protein due to its low heat of solublization and bacteriostatic effect (Rito-Palmares *et al.*, 2001).

The efficiency of extraction methods was determined by calculating concentration and purity ratio of isolated C-PC. The purity of C-PC plays a significant role in commercial applications and is generally evaluated using the absorbance ratio of A_{615}/A_{280} where A_{615} represents maximum peak height for phycocyanin and A_{280} indicates contamination of aromatic amino acid rich proteins. A purity of 0.7 is considered as food grade, 3.9 as reactive grade and greater than 4.0 as analytical grade (Majdoub *et al.*, 2009).

The purity of crude *Spirulina* phycocyanin came to be 0.161±0.05 with a yield of 0.606±0.03 mg/ml. The purification of crude extract involves fractional precipitation with 25% and 50% ammonium sulfate which is particularly useful in salting out unwanted proteins and at the same time to concentrate phycocyanin. Supplementation of 25 % and 50% ammonium sulphate saturation gave 0.248±0.03 and 0.628±0.03 purity ratio respectively with 4.176±0.05 and 5.674±0.20 mg/ml yield, respectively. Different workers have reported different purity values ranging from 1.26-3.1 at 50% ammonium sulfate saturation in *Calothrix*, *Phormidium*, *Oscillatoria*, *Lyngbya*, *Aphanozomenon-flos-aquae* and *Arthronema africanum* strains. For further purification, removal of phycocyanin is compulsory and for this dialysis was carried out and improved the purity value up to 1.08±0.07 that comes under food grade pigment category. It was further purified by passing through Sephadex G-25 column. Result showed increased purity ratios to 2.317±0.08.The successive purified fractions from each step were further used for SDS PAGE.

SDS-PAGE result shows that 40% ammonium sulphate precipitated sample and dialyzed sample shows two bands of C-PC i.e. α and β with 17 and 19kDa respectively along with other proteins (Fig.1, Lane 2&3). When these samples were further purified by chromatography on Sephadex G-25 and loaded on SDS PAGE, result showed that contaminating proteins were minimized and showing the two subunits of C-PC.

Anticoagulation activity results for purified C-PC showed similarity with those recorded by Majdoub et al (Majdoub *et al.*, 2009) on *S. maxima*. The activity shown by C-PC may be due to sulphate polysaccharides and phenolic compounds and depend on the molecular size, type of sugar, sulphate content and position of the active components Majdoub *et al.*, 2009). Therefore, in future, C-PC extract from alga can be used as anticoagulant/antithrombotic agent in medical purposes, replacig the known heparin which was extracted from internal organs of higher animals and exhibited haemorrhagic like side effects.

Further purified C-PC was used to evaluate its antioxidant activity. The nitric oxide ($NO^{\bullet}$) scavenging activity of a compound is of potential health interest as it has been proposed that $NO^{\bullet}$ plays an important role in the progression of many diseases and pathological conditions such as septic shock, atherosclerosis, ischemia reperfusion, neurodegenerative disorders like Alzheimer's and Parkinson's diseases, cancer and diabetes (Wojcik *et al.*, 2010).

Purified C-PC was checked for its role in prevention of oxidative DNA damage**.** The Fenton reaction is a major physiological source of $^{\bullet}OH$, which is produced near DNA molecules in the presence of transition metal ions such as iron and copper (Powers and Jackson, 2008). As previous reports suggest, polyphenol-rich diets may decrease the risk of chronic diseases by reducing oxidative stress (Hollman *et al.*, 2010). The Fenton reaction is prevented by hydroxyl radical scavenging flavonoids. Here the capacity of C-PC to protect against DNA strand scission by DNA damage were checked against DNA strand scission by $^{\bullet}$ OH generated in Fenton reaction on pUC19. We concluded that a

significant contributor to DNA damage prevention is the scavenging of ⁻OH by C-PC.

CONCLUSION

We concluded that C-PC from the dry *Spirulina platensis* powder showed significant antioxidant activity in vitro by scavenging nitric oxide. It also showed anticoagulation activity and thus can be used as anticoagulant/antithrombotic agent in medical purposes replacig the known heparin. Finally, C-PC is significant contributor to DNA damage prevention by scavenging of ⁻OH.

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