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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

A comparative study on antioxidant and cytotoxic effects of *Oscillatoria amphibia* and *Spirulina platensis* C-phycocyanin and crude extracts

Oscillatoria amphibia ve *Spirulina platensis'in* C-Fikosiyanin ve ham e**kstraktlarının** antioksidan ve sitotoksik etkileri üzerine k**arşılaştırmalı çalışma**

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Özet: Bu çalışmada *Oscillatoria amphibia* (Ege Macc-14, Acıgöl-Denizli, Türkiye) anti oksidan ve sitotoksik etkileri, *Spirulina platensis'*in ham ekstrakt ve fikosiyanini ile karşılaştırılmıştır. *S. Platensis*'in tek ticari C-fikosiyanin kaynağı olarak görülmesinden dolayı bu çalışma yeni fikosiyanin kaynakları araştırma ve umut vaat eden sitoksik bileşik araştırmalarında yeni veriler sağlayabilir. Her iki tür de laboratuar koşulları altında kültüre alınarak ham ekstrakt ve C-fikosiyanin ekstraksiyonları yapılmıştır. *O. amphibia*'nın sitokisik bileşikler üretebilme potansiyelinden dolayı biyomasın toksisitesini araştırmak üzere sulu *O. amphibia* ekstreleri hazırlanmış ve artemialara direkt olarak uygulanmıştır. Sulu ham ekstraktlar en yüksek konsantrasyonda (10 $mg.ml^{-1}$) bile toksik bulunmamıştır. Ekstraktların sitotoksisitesini belirlemek amacıyla insan karaciğer karsinoma (HEP-3B) hücre hattı kullanılmıştır. *S. platensis* C-fikosiyanini, *O. amphibia* C-fikosiyaninleri için IC_{50} değerleri sırasıyla 13,27 µg/ml ve 4,19 µg/ml olarak hesaplanmış ve bu değerler ham ekstratlar içinde yine sırasıyla 16,34 µg/ml ve 12,94 µg/ml olarak bulunmuştur. Ham ekstrakt ve C-fikosiyaninlerin antioksidan potansiyelleri ise DPPH serbest radikal ve hidroksil süpürme aktivite testleriyle belirlenmiştir. Sonuç olarak her iki siyanobakterinin saf C-fikosiyaninleri sitotoksik ve antioksidan aktivite göstermeleriyle birlikte en yüksek değerler *O. amphibia* ekstraktları için bulunmuştur.

Anahtar kelimeler: C-fikosiyanin, Sitotoksisite, *Oscillatoria amphibia*, *Spirulina platensis*.

Abstract: In this study, antioxidant and cytotoxic effects of *Oscillatoria amphibia* Ege-Macc-14 (Lake Acıgöl-Denizli, Turkey) C-phycocyanin and the crude extract were compared with *Spirulina platensis*. Since *S. platensis* is seen as the sole economical source of C-phycocyanin this comparison study may provide novel information for investigating new sources of C-PC and besides new promising cytotoxic compounds. Both species are cultivated under laboratory conditions and both the crude extracts and the C-PCs were obtained. Since O*. amphibia* has the possibility to produce toxic compounds, in order to figure out the toxicity of the biomass, the aqueous crude extracts of *O. amphibia* were tested against the brine shrimp directly. The aqueous extracts were found as non-toxic even at the highest concentrations (10 $mg.ml^{-1}$). Human hepatocellular carcinoma (HEP-3B) cell line was used to investigate the cytotoxic effect of the extracts. The determined IC_{50} values of *S. platensis* C-PC, *O. amphibia* C-PC were 13.27 µg/ml, 4.19 µg/ml respectively and for *S. platensis* crude extract and *O. amphibia* crude extract the values were 16.34 µg/ml and 12.94 µg/ml, respectively. The potential antioxidant activity of the crude extracts and the pure C-PCs were evaluated by DPPH free radical, hydroxyl radical scavenging activity assays. As a result, although the pure C-PC of both cyanobacteria showed cytotoxic and antioxidant activities, the highest activity levels were obtained from *O. amphibia* extracts.

Keywords: C- phycocyanin, Cytotoxicity, *Oscillatoria amphibia*, *Spirulina platensis*.

INTRODUCTION

Cyanobacteria are prokaryotic microorganisms and have many beneficial features; they are highly nutritive and have therapeutic effects such as immuno enhancing (Hayashi and Katoh, 1994; Qureshi and Ali, 1996a, 1996b), antioxidant, antiviral (Gustafson *et al.*, 1989), and anti-inflammatory effects (Liu *et al.*, 2000; Romay *et al.*, 1998). The antioxidant activity is particularly related to the phycobiliprotein content of the cyanobacteria. *S. platensis* and *O. amphibia* are both members of filamentous cyanobacteria, which are in rich of phycobiliproteins, especially C-PC. Generally, under standard conditions, the C-PC content can constitute up to 15% of the proteins in *Spirulina platensis* (Jaouen *et al.*, 1999).

C-phycocyanin is a water soluble, non-toxic, blue colored pigment as being one of the major phycobiliprotein in cyanobacteria. It plays an important role in photosynthesis, and phycobiliproteins are protein-pigment complexes that are functional in photosynthetic energy transfer (Li *et al.*, 2001; Reis *et al.*, 1998). C-phycocyanin is composed of two different sized subunits called α and β protein, and carries an apoprotein and an open-chain tetrapyrole prosthetic group

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called phycocyanobilin (Grossman *et al.*, 1993). The chemical structure of phycocyanobilins is very close to that of bilary pigment bilirubin (Strocker *et al.*, 1990; Benedetti *et al.*, 2004). The bilirubin pigment acts as a powerful scavenger, in vivo. This resemblance could allow to be thought that C-PC is an antioxidant agent. Beside these features, it is commonly used in food industry as a natural food colorant (Yoshida *et al.*, 1996), cosmetics and biomedical research as a biochemical tracer in immunoassays (Glazer, 1994).It is well documented that C-PC has many therapeutic properties such as cyclooxygenase inhibition (Reddy *et al.*, 2003), antioxidant and anti-inflammatory effects (Benedetti *et al.*, 2004; Romay *et al.*, 2003) and anticancer effects (Pardhasaradhi *et al.*, 2003).

In this study, the C-PC content of O. amphibia, isolated from Lake Acigol in Turkey, was determined as an alternative C-PC source against *S. platensis*. In a previous study carried out with brine shrimp toxicity assay O. amphibia was found non toxic, in vitro (Conk-Dalay *et al.*, 2005). After cultivation of biomass until stationary phase, in batch culture, crude extracts and pure C-PCs were obtained from both species. Antioxidant activity was tested by DPPH (1,1-diphenyl-2-picryl hydrozyl) and hydroxyl radical scavenging methods and the cytotoxic activity was investigated by MTT assay with HEP-3B cell line, in vitro.

MATERIALS AND METHOD

O. amphibia was harvested from the lake Acıgöl (Denizli, Turkey). The isolation was done by plating onto Erdshreiber medium (EM) containing 1.5% agar (Schreiber, 1927). Individual cells were picked up and were inoculated into the liquid medium. O. amphibia was allowed to grow at a density of 1.5-1.7 mg.L^{-1} at 22 ºC. Continuous illumination was provided by fluorescent lamps (40 µmol photons m^{-2} s^{-1}). The taxonomy of the species is given below:

Taxonomic position (Komarek, 2010); Cyanophyceae, Oscillatoriales, Oscillatoriaceae, Oscillatorioideae

S. platensis (Ege-Macc 31) was obtained from the culture collection of Ege University Microalgae Culture Collection, Ege-Macc, Turkey. The cell culture was grown in Zarrouk's medium under the same conditions with *O. amphibian* (Zarrouk, 1966).

Taxonomic position (Komarek, 2010); Cyanophyceae, Oscillatoriales, Phormidiaceae, Phormidioideae

O. amphibia (Ege-Macc 14) cells were harvested by centrifugation at 5000 rpm, for 5 min while *S. platensis* cells were filtered with Whatman No:1. The harvested cells were stored at -20 °C and were lyophilized (Cipsy 4, Alpha Q).

The quantitative analysis was performed by the low temperature method (Lorenz, 1998). Briefly, the cells were dried in an oven at 45 ºC for six hours. After removing the excess moisture, 40 mg of powder was weighed. The cells were disrupted by freeze-thawing cycles in 10 ml of 100 mM saline PBS. After centrifugation, the spectrophotometric absorbance was read at 620 nm (UV-Visible spectrophotometers, Helios λ, Cambridge, UK). The hycocyanin content was determined using the equation below:

$$CPC\% = \frac{A_{620} x 10 x 100}{7.3 x (\text{mg sample}) x (\text{dry weight}\%)}$$

The lyophilized *O. amphibia* sample, prepared by 2% (w/v) sea water infusion, was tested for its cytotoxic activity using a rapid brine shrimp lethality bioassay. The bioassay against *Artemia salina* Leach, brine shrimp nauplii, was performed as described previously (Meyer *et al.*, 1982; Laughlin, 1991). The cytotoxicity test was evaluated in ranges of 1000:100:10. The sample was dissolved in the sea water (20 mg. 2 ml^{-1}), then 1000, 100 and 10 ppm solutions were prepared by dilution and were placed into vials. The number of survivors was counted after 24 hours of incubation. LC_{50} (Lethal Concentration) values after 24 h exposure and 95% confidence intervals were determined by using the probit analysis method. Colchicine and umbelliferone, having concentrations of 500, 50 and 5 ppm, were used as reference compounds.

Crude extraction; one gram of freeze-dried algae was added to 10 ml of 0.01 M PBS (pH 6.7, 0.15 M NaCl), then it was kept at -86 °C (Sanyo, VIP Series -86 °C, Japan). The frozen suspension was thawed and heated at 30 °C for 1 h in the ultrasonic bath and was kept at 4 °C overnight. The slurry was then centrifuged for 1 h at 9000 rpm and filtered throught Whatman #1 filter paper (Whatman International Ltd., England). The supernatant were evaporated at freeze-drying.

C-PC purification was performed by the method described by Minkova *et al.* (2003). Briefly, 800 mg lyophilized algae was suspended in 0.01 M PBS (pH 6.7, 0.15 M NaCl), then it was kept at -86 °C. The frozen suspension was thawed and heated at 30 °C for 1 h in the ultrasonic bath and was kept at 4 °C overnight. The slurry was then centrifuged for 1 h at 9000 rpm to remove the cell debris. 10 ml of 0.3% (w/v) aqueous rivanol solution was added to the crude extract obtained by the same procedure. The mixture was continuously stirred at 4 ºC overnight, and then was recovered by centrifugation at 9000 rpm for 45 min. The supernatant was exposed to rivanol treatment repeatedly. Finally, after the last centrifugation, the blue supernatant was saturated with 40% ammonium sulphate ($(NH_4)_2.SO_4$) and it was dialyzed against ultra pure water for 12 h and the sample was centrifuged. The precipitate was dissolved in 30 ml of PBS, and was saturated with 70% with ammonium sulphate solution and then it was dialyzed at 4 °C overnight in the same buffer. Finally, Sephadex G-100 (Sigma-Aldrich, USA) was pre-equilibrated and the extract was eluted with the buffer. The absorbance ratio A620/A280, an index for C-PC purity, (Ganapathi *et al.*, 2006) was over 4.00. The carbohydrate and protein contents of the extracts were determined by the phenol-sulfuric acid (Dubois *et al.*, 1956) and modified Lowry methods (Lowry *et al.*, 1951), respectively.

The purity of the extracts was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which was carried out according to the method of Okadjima *et al.* (1993) using a 12.5% polyacrylamide gel and Brilliant Blue R (Sigma-Aldrich) to visualize the proteins. For calibration, marker proteins SM1811 (Fermantas, Canada) 18-250 kDa from were used.

Human hepatocellular carcinoma (HEP-3B) cells were purchased from the Germany Collection of Microorganisms and Cell Cultures (DSMZ). The HEP-3B cells were maintained in RPMI 1640 (Biochrom, Germany) medium supplemented with 10% heat–inactivated fetal bovine serum, 1% L-glutamine (Biochrom, Germany) and 1% gentamycine (Biochrom, Germany) in a humidified atmosphere with 5% CO_2, at 37 °C. The cells were subcultured twice weekly.

The screening of crude extract and C-PC cytotoxicity were done by using a modified MTT assay as described by Mosmann (Mosmann, 1983; Surakka *et al.*, 2005) which affects the mitochondrial activity of viable cells. HEP-3B cell line in 96 well microplates with 6 x 104 cells/ml initial concentration was cultivated for 24 h. Then, the cultures were incubated with different concentration of the extracts for 48 h and the viable cells were determined by MTT. The growth inhibition was estimated as the 50% effective concentration (IC50).The assay is based on cleavage of the yellow tetrazolium salt, MTT, which forms water-insoluble, dark blue formazan crystals. This cleavage only takes place in living cells by the mitochondrial enzyme succinate-dehydrogenase. The water-insoluble, dark blue formazan crystals solubilized using dimethyl sulfoxide. The optical density of the dissolved material is measured at 570 nm (reference filter, 690 nm). Cytotoxicity was expressed as mean percentage increase relative to the unexposed control. Control values were set at 0% cytotoxicity. Cytotoxicity data (where appropriate) was fitted to a sigmoidal curve and a four parameter logistic model used to calculate the IC50, which was the concentration of nanomaterial which caused a 50% inhibition in comparison to untreated controls. The mean IC50 was the concentration of agent that reduces cell growth by 50% under the experimental conditions and was the average from at least three independent determinations that were reproducible and statistically significant. The IC50 values were reported ±95% confidence intervals (±95% CI). This analysis was performed with GraphPad Prism (San Diego, CA). The morphological studies of the cells were done with inverted microscope (Olympus, Japan) comparing with the control group 48 h after treatment.

The DPPH radical is considered to be a model of a stable lipophilic radical. A chain reaction in lipophilic radicals was initiated by the lipid oxidation (Xu *et al.*, 2005; Velioğlu *et al.*, 1998). The crude extracts and C-PC were tested by measuring of bleaching purple colored methanol solution of DPPH (1,1-diphenyl-2-picryl hydrozyl) radical, spectrophotometrically. 1 ml of the extracts was added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min of incubation period at room temperature in the dark, the absorbance was read against a blank at 515 nm. Every sample was done in triplicate, and the percentage of DPPH scavenging activity was calculated with the following calculation. BHT (butylated hydroxytoluen) and α-tocopherol were used as positive control.

$$\text{DPPH}\cdot \text{ Scavenging effect}(\%)=[(\text{AControl}-\text{ASample}/\ \text{AControl})\times 100]$$

where AControl is the initial concentration of the stable DPPH radical without the test compound and ASample is absorbence of the remaining concentration of DPPH· in the presence of extracts.

Hydroxyl radical scavenging activity assay was carried out by measuring the rate of competition between deoxyribose and the extract for hydroxyl radicals (Sreemantula *et al.*, 2005; Singh *et al.*, 2007). The attack of the hydroxyl radical on deoxyribose leads to thiobarbituric acid reactive substances (TBARS). As a result of reaction between TBARS and TBA, pinkish red chromogens are formed in acidic conditions. 28 mM deoxyribose, 20mM phosphate buffer (pH 7.4), 10 mM $FeCl_3$, 1 mM EDTA, 1 mM H_2O_2 and 1 mM L-ascorbic acid and different concentrations of the extracts were prepared all having a final volume of 1 ml and were incubated at 37 °C for 1 h. Lipid peroxidation was monitored by the formation of TBARS. 1 ml of 0.5% TBA (thiobarbituric acid) was added to 1 ml of 2.8% TCA (trichloroacetic acid). The test tubes were left in 90 ºC water bath for 10 min to allow the colour development then were cooled and the absorbance was measured at 532 nm (UV-Visible, Varian Cary 300 bio) against blank. α-tocopherol and BHT were taken as positive controls. Percentage of inhibition (AA) of deoxyribose degradation was calculated using the following equation:

$$AA=\left[\left(A_0-\frac{A}{A_0}\right)x100\right]$$

where A_0 is the absorbance of the control reaction and A is the absorbance of the crude extract and C-PC.

RESULTS

In this study, *O. amphibia* was isolated from Acıgöl Lake located in Denizli, Turkey. This lake is a shallow salt lake and its crystallized water contains 55.9% (w/v) sodium sulphate. The C-phycocyanin content and efficiency of *O. amphibia* were compared with *S.platensis* which is a well known C-PC source. For this purpose, pharmacological and toxicological activities of *O. amphibia* crude extracts and the C-PC reliability of the cells were investigated by brine shrimp toxicity test and were confirmed to be safe. The results for the cytotoxicity evaluation of the infusion 2% of *O. amphibia* are summarized in Table 1. According to the previously described method, the LC_{50} value more than 1000 ppm was not considered as toxic.

In this study, the phycocyanin content determined in *O. amphibia* was approximately the same with *S. platensis.* The C-PC content of *O. amphibia* was found to be 55.56% when cultured, while the content of C-PC was 56% in *S. platensis*. The protein content of *O. amphibia* for both crude extract and C-PC values were 270.52 mg.ml^{-1} and 140 mg.ml^{-1}, respectively. The extracts from each step were confirmed by SDS-PAGE (Figure 1). $Abs_{620/280}$ ratios of the extracts increased up to 3.8 for *S. platensis* and 4.1 for *O. amphibia* (Table 2). The total carbohydrate, protein and the absorbance ratio of the crude extracts and C-PCs were given in Table 2. Total carbohydrate content in *S. platensis* crude extract and C-PC values were 214.46 mg.ml^{-1} and 29.86 mg.ml^{-1}, respectively. Treatment with SDS under reduced condition caused C-PC to dissociate into two subunits (Figure 1). The results are in conformity with earlier reports on the molecular masses of α and β subunits of phycocyanin from blue-green algae.

Table 1. *In vivo*- brine shrimp cytotoxicity of *O. amphibia*

Extract	Concentration (ppm)	LC_{50}
Sample	1000:100:10	>1000
Colchicine	500:50:5	0.0009
Umbelliferone	500:50:5	377.02

Table 2. Analysis of carbohydrate, protein and absorbance values for *S. platensis* and *O. amphibian*

	S. platensis crude extract	*O. amphibia crude extract*	*S. platensis C-phycocyanin*	*O. amphibia C-phycocyanin*
Total Carbohydrate (mg.ml^{-1})	214.46	126.35	29.86	20.4
Total Protein (mg.ml^{-1})	422.36	270.52	71.35	140
$Abs_{620/280}$ ratio	0.85	0.95	3.8	4.1

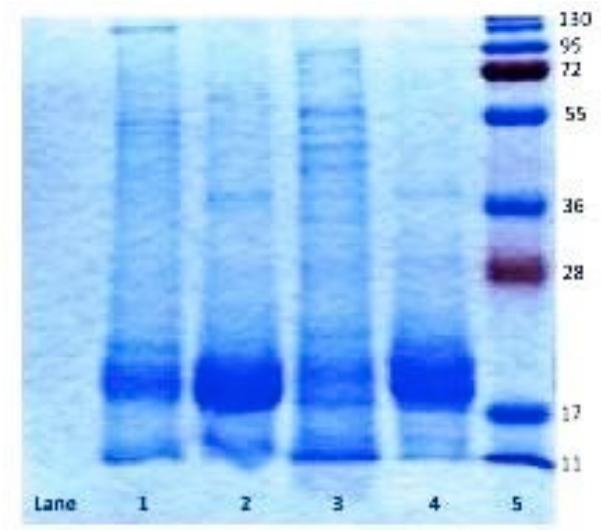


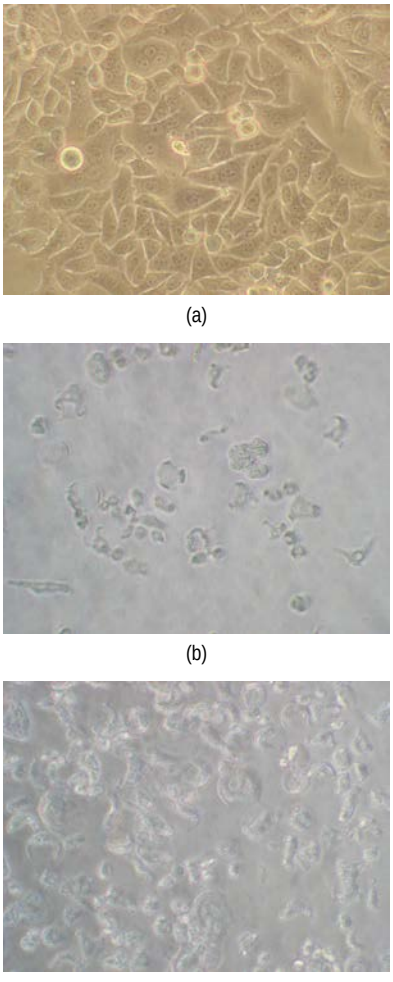
Figure 1. SDS-PAGE of crude extracts and C-PC. Lane (1-5): Lane 1. *O. amphibia* crude extracts, Lane 2. *O. amphibia* C-phycocyanin, Lane 3. *S. platensis* crude extracts, Lane 4. *S. platensis* C-phycocyanin, Lane 5. Marker (kDa).

The cytotoxic effect of *S. platensis* and *O. amphibia* extracts were investigated on HEP–3B cell lines and the calculated IC_{50} values are summarized in Table 3.

The MTT assay results showed that the extracts inhibit cell proliferation in a dose-dependent manner. Cytotoxicity level increases gradually at higher concentrations of the extracts. In this part of the study, the morphological changes were obtained for HEP–3B cells which were growing in logarithmic phase throughout the treatment with extracts. After the 48 h post treatment of extracts, an increased number of rounded cells and growth inhibition were observed when compared with the untreated control cells (Figure 2).

Table 3. Estimated IC50 of crude extracts and C-PC of *S. platensis* and *O. amphibia*

	IC_{50} (μg.ml^{-1})	
	S. platensis	*O. amphibia*
Crude extracts	16.34	12.94
C-phycocyanin	13.27	4.19



(a) (b) (c)

Figure 2. Cytotoxicity in HEP-3B cell line (10X magnification) a 48-h exposure to C-phycocyanin: (a) untreated cells, (b) treated with *O. amphibia* 4.19 μg.ml^{-1} of C-phycocyanin, (c) treated with *S. platensis* 13.27 μg.ml^{-1} of C-phycocyanin

DPPH is a stable free radical donor, which has been widely used to test the free radical scavenging effect of natural antioxidants. The percentage of crude extracts, C-PC and, positive control effects on free radical generation *in vitro* are given in Table 4. In this study *Spirulina* crude extract was found to be most potent hydroxyl radical scavenger, whereas C-PC was determined as least potent hydroxyl scavenger.

Table 4. Antioxidant activity[a] of *S. platensis* and *O. amphibia* crude extracts and C-PC

	S. platensis crude extract	*O. amphibia* crude extract	*S. platensis* C-phycocyanin	*O. amphibia* C-phycocyanin	BHT	α-tocopherol
DPPH (%)	34.5	27	26.2	38.7	90	82
Hydroxyl (%)	49.6	30.88	20.6	33	94	90

[a] Antioxidant activity was assayed at a concentration of 3 mg.ml^{-1}. BHT and α-tocopherol was used at a concentration of 0.2 mg.ml^{-1}. $p<0.01$
BHT: Buthylated hydroxyl toluene

DISCUSSION

The number of species of microalgae is estimated at 22.000 to 26.000, however only a little part of these organisms have been studied in detail with regard to their biochemistry and ecophysiology (Callegari, 1989). All these aquatic microorganisms have some beneficial properties as being a good source of high value chemicals such as polysaccharides, polyunsaturated fatty acids, and pigments, which are widely used in medicinal research area.

Cyanobacteria play an important role for being the only source of the blue pigment phycocyanin, and since Spirulina is a very rich source of valuable chemicals, particularly C-PC, commercialized for its use for medical purpose. As a result of many research, C-PC has found to be anti-inflammatory, antiviral, hepatoprotective, anticancer and antioxidant activities (Reddy *et al.*, 2003). Antioxidant compounds play important role for human health and prevention of diseases such as, coronary heart diseases, radio-protective effects, antitumor activity, inflammation and mutagenesis leading to carcinogenesis (Hsiao *et al.*, 2005; Romay *et al.*, 1998).

In this study, *O. amphibia* isolated from Lake Acıgöl, Denizli, Turkey, was thought to be an alternative source of C-PC. To investigate its convenience, first of all the cells had to be confirmed to be safe on the basis of the brine shrimp toxicity test. *O. amphibia* crude extract were safe either at the highest concentrations (10 mg.ml^{-1}) (Conk Dalay *et al.*, 2005).

S. platensis, is an excellent source of phycocyanin, because its protein fraction contains up to 20% of phycocyanin (Jaouen *et al.*, 1999). Conk-Dalay *et al.* (2005) determined and compared the C-PC content of *O. amphibia* and *S. platensis* according to the low temperature method (Lorenz, 1998). There haven't been found a significant difference between the C-PC content of both species. Boussiba and Richmond (1979) have used 50% and 30% (w/v) ammonium sulfate solutions, respectively for precipitation, following by hydroxylapatite, DEAE-cellulose and Sephadex A-50 columns. This method offered an improved separation of C-PC (Boussiba and Richmond, 1979; Yi Ming and Fheng, 1999). In this study, phycocyanin was obtained by ammonium sulfate precipitation. 40% (w/v) ammonium sulfate solution was used prior to precipitate other proteins except C-PC, and then 70% ammonium sulfate solution was added to obtain C-PC. Anamika *et al.* (2005), purified C-PC from *Spirulina, Phormidium* and *Lyngbya* spp. and they determined the molecular weights as 112, 131, and 81 kDa, respectively. In this study it has been shown that all the β subunits (24.4 kDa) were same for all the three species, whereas the molecular weights of α subunits were different for all (17 kDa *Spirulina* sp., 19.1 kDa *Phormidim* sp., and 15.2 kDa for *Lyngbya* sp.). However, in a study reported by Madhyastha *et al.* (2006) the α subunit of *S. fusiformis* showed 59% homology with that of *Oscillatoria* sp., β subunit differed largely with only 6% homology between the two species. The β subunit of *Oscillatoria* sp. differed from all of the genera selected, in its amino acid composition, except for positions of Thr^{6} and Asp^{13}. This result may explain the differences between the cell viability and the antioxidant activity data.

S. platensis C-PC showed 0.0725 mg.ml^{-1} as Li *et al.* (2001) and 50 µM IC_{50} as Subhashini *et al.* (2004). On the other hand, there is no literature cited on the cytotoxicity of *O. amphibia.* The *O. amphibia* C-PC extracts showed the highest cytotoxic effect on the tested tumor cell lines with IC_{50} values unlike *S. platensis* extracts. This result indicated that the *O. amphibia* C-PC was shown higher cytotoxic effect than the *S. platensis* C-PC on HEP–3B cells. There are many studies about the effect of C-PC on various cancer cell lines cited in literature. However, it is little known on the action of mechanism of C-PC; Subhashini *et al.* (2004) showed that this molecule acts as a cyclooxygenase-2 (COX-2) inhibitor, additionally induces apoptosis in mouse macrophage cell line RAW 264.7 stimulated with lipopolysaccharide (Reddy *et al.*, 2003) and in rat histiocytoma cell line, AK5 (Pardhasaradhi, *et al.*, 2003), proliferation and apoptosis effect on chronic myeloid leukemia cells, K 562 (Subhashini *et al.*, 2004) were also studied to evaluate the mechanism.

Phycocyanin is shown to possess radical scavenging and antioxidant properties (Romay *et al.*, 1998; Romay and Gonzalez, 2000; Gonzalez *et al.*, 1999). The lineer tetrapyrrol prosthetic group commonly called as 'bilins' of phycocyanin are able to scavenge the reactive oxygen species (ROS) and the molecule can act as an antioxidant (Romay *et al.*, 2003). DPPH radical scavenging activity among all the extracts and the radical scavenging activities of water extracts were as high

as the activity of α-tocopherol, which scavenged the highest amount of DPPH radicals with respect to the two commercial antioxidants (BHT and α-tocopherol) tested. The scavenging activities of crude extracts and C-PC on DPPH radical are shown in observed that the radical scavenging activity increased with the increase of phenolic content. Phycocyanin was also reported to have a positive correlation between DPPH radical scavenging activities and total polyphenolics. Phenols are particularly effective antioxidants for polyunsaturated fatty acids; in fact they easily transfer a hydrogen atom to lipid peroxyl radicals and form the aryloxyl, which being incapable of acting as a chain carrier, couples with another radical thus quenching the radical propagation process (Velioğlu *et al*., 1998). Hydroxyl radicals are the major reactive oxygen species causing enormous biological damage and initiate lipid peroxidation. Hydroxyl radicals are generated in the Fenton system (Fe^{2+}/H_2O_2).

Our study has distinctly demonstrated that C-PC and the crude extracts of *O. amphibia* and *S. platensis* may be promising in formation or proliferation of the studied cell line, HEP-3B, by their cytotoxic effects. Also, antioxidant activity was detected in both C-PC and crude extracts. Since most of the cyanobacteria have not been previously screened against the cell line spread in the present study, the present data are novel. An integration of biochemical studies will doubtlessly help to extend this knowledge to therapeutic approaches.

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REFERENCES

Anamika, P., Sandhya, M., Richa, P. Ghosh, P.K., 2005. Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. *Protein Expression and Purify*, 40(2):248-255. doi:10.1016/j.pep.2004.10.028

Benedetti, S., Benvenuti, F., Pagliarani, S. Francogli, S. Scoglio, S. Canestrari, F., 2004. Antioxidant properties of a novel phycocyanin extract from the blue-green alga *Aphanizomenon flos-aquae*. *Life Sciences*, 75(19):2353-2362. doi:10.1016/j.lfs.2004.06.004

Boussiba, S., Richmond, A., 1979. Isolation and characterization of phycocyanins from the blue-green alga *Spirulina platensis*. *Archives of Microbiology*, 120:155-159. doi:10.1007/BF00409102

Callegari, J.P., 1989. Feu Vert Pour Les Microalgues. *Biofuture*, 76:25-40.

Conk-Dalay, M., Hatipoğlu, S., Sağlam, H., 2005. Phycocyanine content and toxicity of *Oscillatoria amphibia* agardh isolated from Acı Lake, Turkey. *Tropical and Subtropical Cyanoprokaryota Workshop*. Gran Canaria, Las Palmas, Spain.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.S., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28:350–356. doi:10.1021/ac60111a017

Gustafson, K.R., Cardellina II, J.H., Fuller, R.W., Weislow, O.S., Kiser, R.F. Snader, K.M., Patterson, G.M.L., Boyd, M.R., 1989. AIDS-Antiviral sulfolipids from cyanobacteria (blue-green algae). *Journal of the National Cancer Institute*, 81(16):1254-1258. doi:10.1093/jnci/81.16.1254

Ganapathi, P., Chethana, S., Sridevi, A.S., Raghavarao, K.S.M.S., 2006. Method to obtain C-phycocyanin of high purity. *Journal of Chromatography A,* 1127(-2):76–81. doi:10.1016/j.chroma.2006.05.073

Glazer, A.N., 1994. Phycobiliproteins-a family of valuable, widely used fluorophores. *Journal* of *Applied Physiology*, 6:105-112.

Gonzalez, R., Rodriguez, S., Romay, C., Ancheta, O., Gonzalez, A., Armesto, J., Remirez, D., Merino, N., 1999. Anti-inflammatory activity of phycocyanin extract in acetic acid-induced colitis in rats. *Pharmacological Research*, 39(1):55–59. doi:10.1006/phrs.1998.0409

Grossman, A.R., Schaefer, M.R., Chiang, G.G., Collier, J.L., 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. *Microbiology Reviews*, 5:725–749.

Hayashi, O., Katoh, T., 1994. Enhancement of antibody production in mice by dietary *Spirulina platensis*. *Journal of Nutritional Science and Vitaminology*. 40:431-441. doi:10.3177/jnsv.40.431

Hsiao, G., Chou, P.H., Shen, M.Y., Chou, D.S., Lin, C.H., Sheu, J.R., 2005. C-Phycocyanin, a Very Potent and Novel Platelet Aggregation Inhibitor from Spirulina platensis. *Journal of Agricalturel and Food Chemistry*, 53:7734-7740. doi:10.1021/jf051352y

Jaouen, P., Lepine, B., Rossignol, N., Royer, R., Quéméneur, F., 1999. Clarification and concentration with membrane technology of a phycocyanin solution extracted from Spirulina platensis. *Biotechnology Techniques*, 13:877-881. doi:10.1023/A:1008980424219

Komarek, J., 2010. Recent changes (2008) in cyanobacteria taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). *Hydrobiologia*, 639:245-259. doi:10.1007/s10750-009-0031-3

Laughlin, J.L., 1991. Assay for. Bioactivity, In: *Methods in Plant Biochemistry*, K. Hostetman (Ed.), *Academic Press*, London. Vol. 6:1-31.

Li, Y., Zhang, J.P., Xie, J., Zhao, J.Q., Jiang, L.J., 2001. Temperature induced decoupling of phycobilisomes from reaction centers. *Biochimica et Biophysica Acta*, 1504:229-234. doi:10.1016/S0005-2728(00)00250-4

Liu, Y., Xu, L., Cheng, N., Lin, L., Zhang, C., 2000. Inhibitory effect of phycocyanin from Spirulina platensis on the growth of human leukemia K562 cells. *Journal of Applied Phycology*, 12:125-130. doi:10.1023/A:1008132210772

Lorenz, R.T., 1998. Quantitative Analysis of C-Phycocyanin from Spirulina pacifica (Low Temperature Method). Cyanotech Corporation, *Spirulina Pacifica Technical Bulletin #004*.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin-Phenol reagents. *Journal of Biological Chemistry*, 193:265-275.

Madhyastha, H.K., Radha, K.S., Sugiki, M., Omura, S., Maruyama, M., 2006. Purification of c-phycocyanin from Spirulina fusiformis and its effect on the induction of urokinase-type plasminogen activator from calf pulmonary endothelial cells. *Phytomedicine*, 13:564-569. doi:10.1016/j.phymed.2005.09.006

Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E., McLaughlin, J.L., 1982. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica*, 45:31-34. doi:10.1055/s-2007-971236

Minkova, K.M., Tchernov, A.A., Tchorbadjieva, M.I., Fournadjieva, S.T., Antova, R.E., Busheva, M.Ch., 2003. Purification of C-phycocyanin from *Spirulina* (*Arthrospira*) *fusiformis*. *Journal of Biotechnology*, 102:55-59. doi:10.1016/S0168-1656(03)00004-X

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1-2):55-63. doi:10.1016/0022-1759(83)90303-4

Okadjima, T., Tanabe, T., Yasuda, T., 1993. Nonurea sodium dodecyl sulfate-polyacrylamide gel electrophoresis with high-molarity buffers for the separation of protein and peptides. *Analytical Biochemistry*, 211:293-300. doi:10.1006/abio.1993.1272

Pardhasaradhi, B.V.V., Ali, A.A.M., Kumari, A.L., Reddanna, P., Khar, R., 2003. Phycocyanin mediated apoptosis in AK-5 tumor cells involves down-regulation of Bcl-2 and generation of ROS. *Moleculer Cancer Therapeutic*, 2:1165-1170.

Qureshi, M., Ali, RA., 1996a. Phagocytic potential of Feline macrophages after exposure to a water soluble extract of Spirulina in vitro. *Immunopharmacol Immunotoxicolology*, 18(3):342-354.

Qureshi, M., Ali, RA., 1996b. Spirulina platensis exposure enhances macrophage phagocytic function in cats. *Immunopharmacol Immunotoxicolology*, 18(3):457-463. doi:10.3109/08923979609052747

Reddy, M.C., Subhashin, J., Mahipal, S.V.K., Bhat, V.B., Reddy, S.P., Kiranmai, G., Madyastha, K.M., Reddanna, P., 2003. C-Phycocyanin, a selective cyclooxygenase-2 inhibitor, induces apoptosis in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications*, 304:385-392. doi:10.1016/S0006-291X(03)00586-2

Reis, A., Mendes, A., Lobo-Fernandes, H., Empis, J.A., Novais, J.M., 1998. Production, extraction and purification of phycobiliproteins from *Nostoc* sp. *Bioresource Technology*, 66:181-187. doi:10.1016/S0960-8524(98)00064-9

Romay, C., Armesto, J., Remirez, D., Gonzalez, R., Ledon, N., Garcia, I., 1998. Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. *Inflammation Research*, 47:36–41. doi:10.1007/s000110050256

Romay, C., Gonzalez, R., 2000. Phycocyanin is an antioxidant protector of human erythrocytes against by peroxyl radicals. *Journal of Pharmacy and Pharmacology*, 52:8-367. doi:10.1211/0022357001774093

Romay C., González, R., Ledón, N., Ramirez, D., Rimbau, V., 2003. C-Phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. *Current Protein and Peptide Science*, 4:207-16. doi:10.2174/1389203033487216

Schreiber, E., 1927. Die Reinkultur von marinen phytoplankton und deren bedeutung für die erforschung der produktionsfähigkeit des meerwassers. *Wiss Meeresuntersuch N.F.*, 10:1-34.

Singh, R., Singh, S., Kumar, S., Arora, S., 2007. Free radical-scavenging activity of acetone extract/fractions of *Acacia auriculiformis* A. Cunn. *Food Chemistry*, 103:1403-1410. doi:10.1016/j.foodchem.2006.10.056

Sreemantula, S., Nammi, S., Kolanukonda, R., Koppula, S., Boini, K.M., 2005. Adaptogenic and nootropic activities of aqueous extract of *Vitis vinifera* (grape seed): an experimental study in rat model. *BMC Complementary and Alternative Medicine*, 5(1):1-19. doi:10.1186/1472-6882-5-1

Stocker, R., Mcdonagh, A.F., Glazer, A.N., Ames, B.N. 1990. Antioxidant activities of bile pigments: biliverdin and bilirubin. *Methods in Enzymology*, 186-301.

Subhashini, J., Mahipal, S.V., Reddy, M.C., Mallikarjuna, R.M., Rachamallu, A., Reddanna, P., 2004. Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562. *Biochemical Pharmacology*, 68:453-462. doi:10.1016/j.bcp.2004.02.025

Surakka, A., Sihvonen, L.M., Lehtimäki, J.M., Wahlsten, M., Vuorela, P., Sivonen, K., 2005. Benthic cyanobacteria from the Baltic Sea contain cytotoxic *Anabaena*, *Nodularia* and *Nostoc* strains and an apoptosis-inducing *Phormidium* strain. *Environmental Toxicology*, 20:285-292. doi:10.1002/tox.20119

Velioğlu, Y.S., Mazza, G., Gao, L., Oomah, B.D., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *Journal of Agricaltural and Food Chemistry*, 46:375-379. doi:10.1021/jf9801973

Xu, J., Chen, S., Hu, Q., 2005. Antioxidant activity of brown pigment and extracts from black sesame seed (*Sesamum indicum* L). *Food Chemistry*, 91(1):79-83. doi:10.1016/j.foodchem.2004.05.051

Yi-Ming, Z., Fheng, C., 1999. A simple method for efficient separation and purification of C-Phycocyanin and Allophycocyanin from *Spirulina platensis*. *Biotechnology Techniques*, 13:601-603. doi:10.1023/A:1008914405302

Yoshida, A., Takagaki, Y., Nishimune, T., 1996. Enzyme immunoassay for phycocyanin as the main component of Spirulina color in foods. *Bioscience Biotechnology and Biochemistry*, 60:57-60. doi:10.1271/bbb.60.57

Zarrouk, C., 1966. Contribution A L'etude du Cyanophyceae. Influence de Divers Facteurs Physiques et Chimiques Sur La Croissance et La Photosynthese de Spirulina Maxima (Setch Et Gardner) Geitl., Phd, Paris.