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Antioxidant properties of a novel phycocyanin extract from the blue-green alga *Aphanizomenon flos-aquae*

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Abstract

Aphanizomenon flos-aquae (AFA) is a fresh water unicellular blue-green alga (cyanophyta) rich in phycocyanin (PC), a photosynthetic pigment with antioxidant and anti-inflammatory properties.

The purpose of this study was to evaluate the ability of a novel natural extract from AFA enriched with PC to protect normal human erythrocytes and plasma samples against oxidative damage in vitro. In red blood cells, oxidative hemolysis and lipid peroxidation induced by the aqueous peroxyl radical generator [2,2′-Azobis (2-amidinopropane) dihydrochloride, AAPH] were significantly lowered by the AFA extract in a time- and dose-dependent manner; at the same time, the depletion of cytosolic glutathione was delayed. In plasma samples, the natural extract inhibited the extent of lipid oxidation induced by the pro-oxidant agent cupric chloride ($CuCl_2$); a concomitant increase of plasma resistance to oxidation was observed as evaluated by conjugated diene formation.

The involvement of PC in the antioxidant protection of the AFA extract against the oxidative damage was demonstrated by investigating the spectral changes of PC induced by AAPH or $CuCl_2$. The incubation of the extract with the oxidizing agents led to a significant decrease in the absorption of PC at 620 nm accompanied with disappearance of its blue color, thus indicating a rapid oxidation of the protein. In the light of these in vitro results, the potential clinical applications of this natural compound are under investigation.
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Keywords: Blue-green algae; *Aphanizomenon flos-aquae*; Phycocyanin; Antioxidant; Lipid oxidation

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Introduction

Oxidative stress is an important factor in the genesis of many pathologies, from cancer to cardiovascular and degenerative diseases (Galli et al., 1999; Parthasarathy et al., 2001; Cooke et al., 2003). In order to protect the body against the consequences of oxidative stress, an efficacious approach consists in improving the antioxidant nutrition. In this regard, scientific studies have shown that the synergistic action of a wide spectrum of antioxidants is better than the activity of a single antioxidant, and that antioxidants from natural sources (primarily foods) have a higher bioavailability and therefore higher protective efficacy than synthetic antioxidants (Gey, 1998).

Focusing our attention on natural and bioavailable sources of antioxidants, we undertook to investigate the antioxidant properties of the cyanophyta *Aphanizomenon flos-aquae* (AFA), a fresh water unicellular blue-green alga that spontaneously grows in Upper Klamath Lake (Oregon, USA) and that is consumed as a nutrient-dense food source and for its health-enhancing properties (Jensen et al., 2000, 2001; Pugh and Pasco, 2001; Pugh et al., 2001). AFA is an important source of the blue photosynthetic pigment phycocyanin (PC), which has been described as a strong antioxidant (Bhat and Madyastha, 2000, 2001; Romay and Gonzalez, 2000) and anti-inflammatory (Romay et al., 1998; Reddy et al., 2000) natural compound, as evidenced by in vitro and in vivo studies on PC from the cyanophyta *Spirulina platensis*. PC is a water soluble phycobiliprotein composed of α and β subunit polypeptides which associate into (αβ)-monomers (Glazer, 1988). (αβ)-monomers, in turn, have a high affinity to assemble together to form $(\alpha\beta)_3$-trimers and finally $(\alpha\beta)_6$-examers. α and β subunits are constituted of a protein backbone to which linear tetrapyrrole chromophoric groups are covalently bound (Padyana et al., 2001). The chromophore, named phycocyanobilin, is similar in chemical structure to bilirubin, and like the latter acts as a powerful scavenger of reactive oxygen species (Stocker et al., 1990; Bhat and Madyastha, 2001).

Taking into account that AFA is used as dietary supplement in many countries and that PC represents around 15% of algal dry wet, in our opinion it was of prime importance to investigate the biochemical properties of the protein in vitro. In this report, we evaluated for the first time the antioxidant activity of a novel natural extract from AFA enriched with PC. The extract was used for the inhibition of aqueous peroxyl radicals [2,2′-Azobis (2-amidinopropane) dihydrochloride, AAPH]-induced oxidative hemolysis and lipid peroxidation of normal human erythrocytes. We also tested the protective role of the AFA extract in human plasma samples treated with the pro-oxidant agent cupric chloride ($CuCl_2$).

Methods

Chemicals

AAPH was purchased from Trimital srl (Milan, Italy); $CuCl_2$, trichloracetic acid (TCA), 2-thiobarbituric acid (TBA) and 5,5′-dithio-bis 2-nitrobenzoic acid (DTNB) were obtained from Sigma-Aldrich (Milan, Italy).

Preparation of the AFA extract

The AFA extract (PC content 30–40% of dry wet) was kindly provided by Nutratec (Urbino, Italy) and stored in the dark at 4°C. The dried extract was first dissolved in phosphate saline buffer (PBS) pH

7.4 and centrifuged at 2500 g at 4°C for 10 min to remove any insoluble material. The spectrophotometric analysis of the blue supernatant revealed the characteristic peak of PC at 620 nm (Fig. 1); the protein could not be considered pure, in fact the absorbance ratio 620 nm/280 nm was less than 4 (Bhat and Madyastha, 2000, 2001). The concentration of PC in the aqueous extract was evaluated using its molar extinction coefficient at 620 nm taking into account that, as revealed by native PAGE, the protein was in the form of $(\alpha\beta)_3$-trimer (M_r 121 000, ε = 770 000 M^{-1} cm^{-1}). The stock solution of the AFA extract used for all the experiments (PC concentration 10 μM) was stable in the dark at 4°C for at least one week.

Preparation of erythrocyte suspensions

Heparinized blood samples were obtained from healthy volunteers via venapuncture after obtaining informed consent. Red blood cells (RBC) were isolated by centrifugation at 1500 g for 10 min, washed three times with PBS and finally re-suspended using the same buffer to an hematocrit level of 5%. In the time-dependent experiments, the RBC suspension was pre-incubated with the AFA extract containing 100 nM PC for 15 min at 37°C; then samples were incubated with 50 mM AAPH for up to 6 hours at 37°C in order to induce the free radical chain oxidation in RBC. In the dose-dependent experiments, RBC were incubated with 50 mM AAPH for 4 hours at 37°C in the presence of the AFA extract containing PC at different concentrations (range 10–1000 nM). Erythrocyte suspension incubated with PBS served as control.

Hemolysis assay

At the indicated time, an aliquot of reaction mixture (1 ml) was removed and centrifuged at 3000 g for 2 min; the content of hemoglobin in supernatants was determined at 540 nm by the use of Drabkins

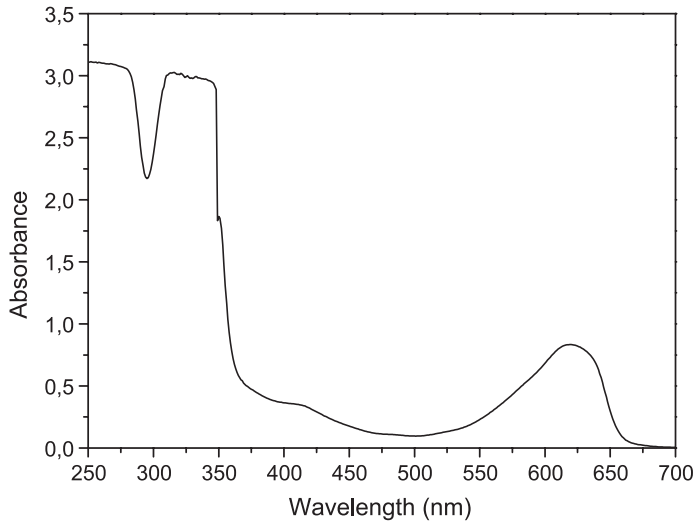

Fig. 1. Spectrophotometric analysis of the AFA extract in aqueous solution. The characteristic peak of PC can be recorded in the visible region at 620 nm.

solution (Hseu et al., 2002). References values were obtained using the same amount of RBC in distilled water (100% hemolysis).

Estimation of erythrocyte lipid peroxidation

TBARS, mainly malonyldialdehyde (MDA), as indicators of lipid peroxidation, were assayed as previously described (Hseu et al., 2002). Briefly, a 1 ml reaction mixture was incubated at 95°C for 1 h with 250 μl of TBA (0.67%) and 100 μl of H_3PO_4 (0.44 M); then 150 μl of TCA (20%) were added. After centrifugation, the peroxide content in the supernatant was determined using the molar extinction coefficient (OD_{535}) of MDA.

Determination of glutathione (GSH) content in erythrocytes

At the indicated time, the reaction mixture (2 ml) was centrifuged and 0.6 ml of distilled water was added to the RBC pellet in order to lyse the cells. The content of GSH in the lysate was determined at 412 nm by titration with DTNB (Hseu et al., 2002).

Preparation of plasma samples

Plasma samples were obtained after centrifugation of the heparinized blood from healthy volunteers at 1500 g for 10 min. Aliquots were stored at 4°C until used. Lipid oxidation was carried out at 37°C by treating plasma samples with 100 μM $CuCl_2$ as oxidant for the indicated time; to evaluate the protective role of the AFA extract, samples were pre-incubated with the extract containing PC at different concentrations (range 10–150 nM) for 15 min at 37°C.

Measurement of oxidizability of plasma

The copper-induced oxidation in plasma samples (a 50-fold dilution was requested) were monitored spectrophotometrically by recording diene formation at 245 nm for 3 h at 37°C as previolusly described (Schnitzer et al., 1995). The lag-phase preceding oxidation of plasma can be used as a standard measure of the "oxidation resistance" of plasma lipids.

Estimation of plasma lipid oxidation

The extent of lipid oxidation in plasma samples incubated for 2 h at 37°C with PBS (control) or with 100 μM $CuCl_2$ in presence of the AFA extract with increasing concentrations of PC was assayed by measuring TBA-reactive substances at 535 nm as described earlier (Bhat and Madyastha, 2000).

Interaction of PC with AAPH and $CuCl_2$

To evaluate the interaction of PC with AAPH or $CuCl_2$, the aqueous extract (containing 150 nM PC) was taken in a 1 ml quartz cuvette thermostated at 37°C. The reaction was initiated by adding freshly prepared AAPH or $CuCl_2$ solutions (final concentrations 50 mM and 100 μM, respectively). The spectral changes of PC were recorded between 500 and 700 nm every 5 min for 45 min (Bhat and Madyastha, 2000).

Statistics and data processing

Results are expressed as means ± standard deviation (SD). The statistical analysis was carried out using the Student's t-test for paired data. Probability values of <0.05 were accepted. Statistics and graphs were obtained using the software Microcal™ Origin 6.0 (Microcal Software, Inc., Northampton, Ma, USA).

Results

Protection of the AFA extract against erythrocyte oxidative damage

When human RBC were incubated as a 5% suspension in PBS, they were stable and little hemolysis was observed after 6 h of incubation at 37°C (4.2 ± 0.3%). When the water-soluble radical initiator AAPH (final concentration 50 mM) was added to the RBC suspension, it induced hemolysis in a time-dependent manner (the degree of hemolysis at 6 h approached 100%). The onset of AAPH-induced hemolysis was significantly delayed in the presence of the AFA extract containing 100 nM PC (Fig. 2, panel A).

As regard TBARS formation, AAPH caused lipid peroxidation in the RBC suspension in a time-dependent manner; in fact, the MDA content increased from 12.9 ± 0.6 to 48.8 ± 2.5 μmol/l after 5 h of incubation with AAPH 50 mM. However, the addition of the AFA extract containing 100 nM PC significantly inhibited the AAPH-induced MDA formation (Fig. 2, panel B).

GSH content in RBC incubated as a 5% suspension in PBS (control) remained almost unchanged during the 5 h of incubation at 37°C (mean value 1.92 ± 0.12 mmol/l). The presence of 50 mM AAPH caused a significant consumption of the cytosolic GSH in a time-dependent manner with a decrement from 1.96 ± 0.14 to 0.32 ± 0.02 mmol/l after 5 h of incubation. The addition of the AFA extract containing 100 nM PC significantly inhibited the AAPH-induced depletion of the cytosolic GSH (Fig. 2, panel C).

The addition of the AFA extract to the RBC suspension in absence of AAPH did not cause hemolysis or changes in MDA and GSH content after 6 h of incubation (data not shown).

The dose-dependent protection of the AFA extract against erythrocyte oxidative damage was evaluated in the RBC suspension after 4 h of incubation with AAPH 50 mM as shown in Table 1. Erythrocyte hemolysis as well as MDA and GSH content were comparable to control values when RBC were pre-incubated in the presence of the AFA extract containing 1000 nM PC.

Protection of the AFA extract against plasma oxidizability and lipid peroxidation

The kinetic of conjugated diene formation at 245 nm upon exposure of plasma samples to 100 μM $CuCl_2$ is shown in Fig. 3. Initially, the lipoprotein-associated antioxidants became oxidized and the absorbance at 245 nm increased only slightly (lag phase); when most of the antioxidants became oxidized, oxidation of lipoprotein began, leading to diene accumulation. The presence of the AFA extract with increasing concentrations of PC (15–150 nM) led to a progressive inhibition

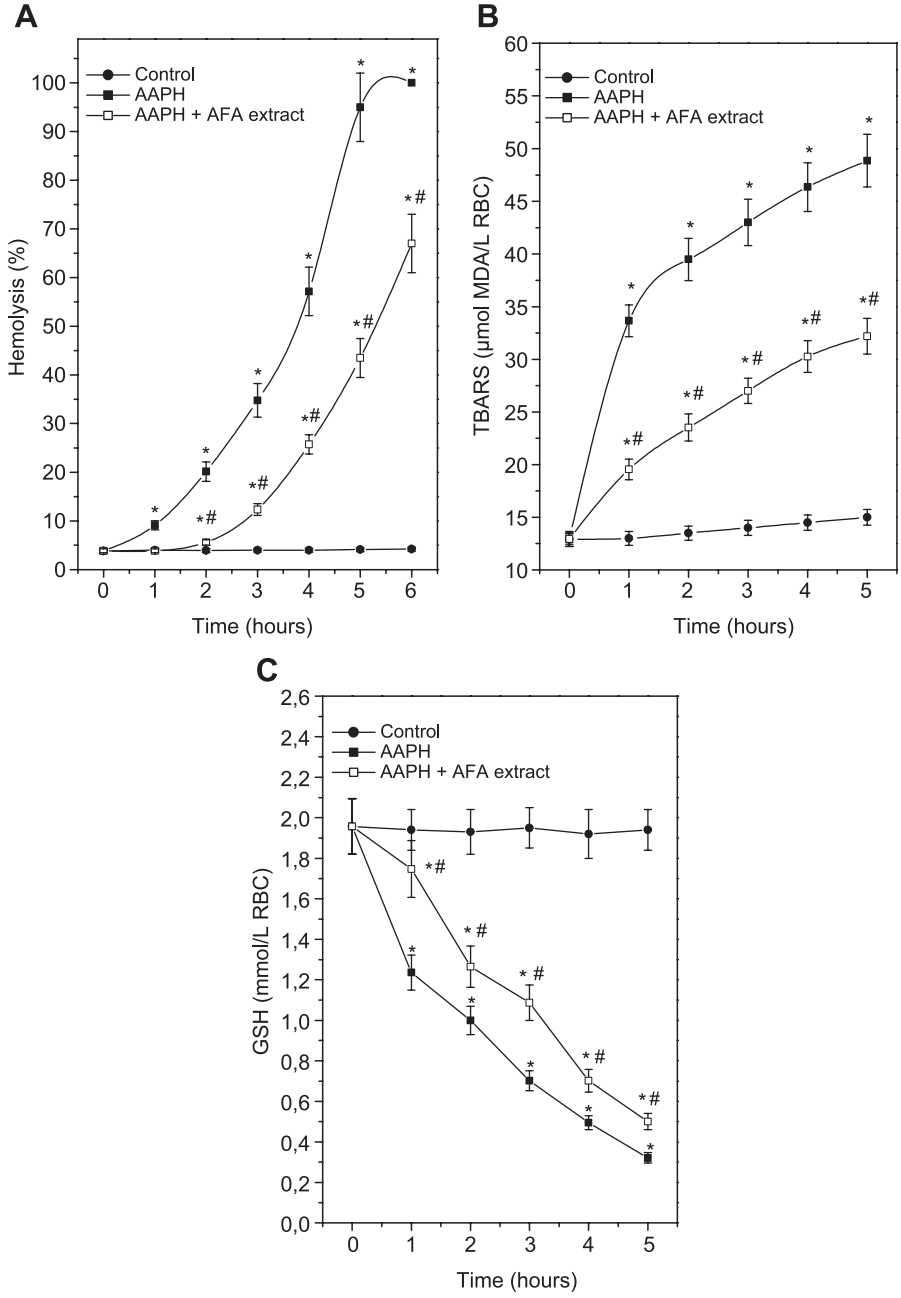

Fig. 2. Time-dependent protection of the AFA extract against erythrocyte oxidative damage. RBC suspension (5%) were incubated with PBS (control), or pre-incubated with the natural extract containing 100 nM PC for 15 min. Then RBC were incubated with or without 50 mM AAPH for 6 h at 37°C. A: Time course of hemolysis of human RBC induced by AAPH. B: Formation of TBARS induced by AAPH. C: Depletion of GSH induced by AAPH. *p<0.05 from control group; # p < 0.05 from AAPH group.

Table 1
Dose-dependent protection of the AFA extract against RBC oxidative damage after 4 h of incubation with AAPH 50 mM

PC concentration in the AFA extract (nM)	Hemolysis (%)	TBARS (μmol MDA/L RBC)	GSH (mmol/L RBC)
0	58.2 ± 4.6	46.3 ± 3.9	0.49 ± 0.04
10	49.8 ± 3.5	42.6 ± 3.3	0.55 ± 0.05
50	37.6 ± 3.4	35.2 ± 2.9	0.64 ± 0.05
100	25.7 ± 1.9	30.1 ± 2.7	0.72 ± 0.06
250	13.0 ± 1.1	23.8 ± 2.2	1.18 ± 0.08
500	5.9 ± 0.5	16.9 ± 1.4	1.66 ± 0.11
1000	4.3 ± 0.4	15.3 ± 1.4	1.87 ± 0.12

Control values (RBC suspension incubated with PBS): hemolysis 4.0 ± 0.3%; TBARS 14.5 ± 1.1 μmol MDA/L RBC; GSH 1.94 ± 0.14 mmol/L RBC.

of the copper-induced diene formation with a concomitant increase of plasma resistance to oxidation.

The exposure of plasma samples to 100 μM $CuCl_2$ for 2 h at 37°C led to a strong accumulation of MDA levels with respect to control (plasma incubated in presence of PBS) (Fig. 4). However, the addition of the AFA extract to the reaction mixture significantly inhibited the copper-induced MDA formation in a concentration-dependent manner (86% of inhibition when the extract contained 100 nM PC).

Effect of AAPH and $CuCl_2$ on PC spectroscopic properties

When the AFA extract (PC concentration 150 nM) was incubated with 50 mM AAPH or 100 μM $CuCl_2$ at 37°C for 45 min, there was a progressive decrease in the absorption of PC at 620

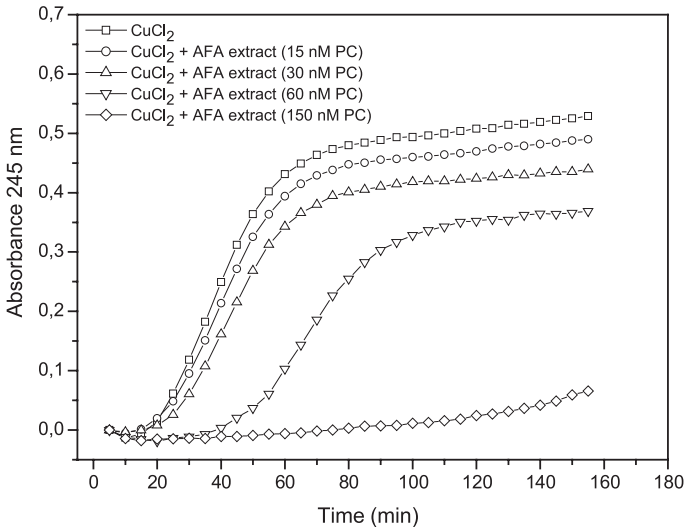

Fig. 3. Time-dependent diene accumulation during copper-induced oxidation of plasma for 3 h at 37°C. 50-fold diluted plasma samples were pre-incubated with the AFA extract containing 15–150 nM PC for 15 min; then 100 μM $CuCl_2$ was added.

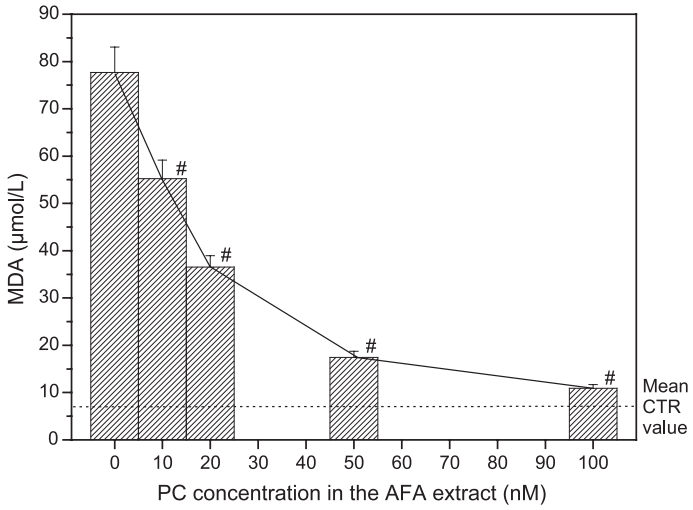

Fig. 4. Dose-dependent inhibitory effect of the AFA extract containing 10–100 nM PC on copper-induced lipid oxidation (TBARS formation) in human plasma samples treated for 2 h at 37°C with 100 μM $CuCl_2$. #$p < 0.05$ from AAPH group.

nm accompanied with disappearance of the characteristic blue color of the pigment (data not shown).

Discussion

Phycocyanin is a water-soluble, highly fluorescent protein derived from cyanobacteria (blue-green algae) used in food coloring (Yoshida et al., 1996), cosmetics (Cohen, 1986) and biomedical research (Glazer and Stryer, 1990). Recently, it has been demonstrated that PC from *Spirulina platensis* has significant antioxidant and radical scavenging properties both in vivo and in vitro models (Bhat and Madyastha, 2000, 2001; Romay and Gonzalez, 2000), becoming a potential therapeutic agent in oxidative stress-induced diseases.

In this contest, we focused our attention on the edible microalga *Aphanizomenon flos-aquae*, in which PC represents around 15% of algal dry wet. In particular, we tested the efficacy of a novel natural extract from AFA enriched with PC in protecting human erythrocytes and plasma samples against the oxidative damage induced by AAPH or $CuCl_2$.

In RBC treated with 50 mM AAPH, free radicals attack erythrocyte membrane components, such as proteins and lipids, and cause changes in the structure and function of membranes; as a result, a time-dependent RBC hemolysis was observed during cell incubation with AAPH. At the same time, a significant accumulation of TBARS, used as indicators of lipid peroxidation, was found during the incubation period with AAPH. Moreover, the treatment of RBC with AAPH resulted in up to 75% loss of intracellular GSH after 5 h at 37°C, indicating that GSH is very susceptible to AAPH mediated oxidative insult.

In RBC suspensions pre-incubated with the natural AFA extract, our findings clearly indicated that the extract significantly reduced in a time- and dose-dependent manner the extent of lipid peroxidation and hemolysis of RBC treated with AAPH, thus protecting the cell against the

oxidative damage. Contemporarily, the depletion of intracellular GSH induced by AAPH was delayed.

The AFA extract showed a similar antioxidant activity in human plasma samples treated with the pro-oxidant agent $CuCl_2$ (100 μM): by evaluating diene formation at 245 nm, we found that the extract increased the plasma resistance to oxidation in a dose-dependent manner; in the same way, MDA accumulation induced by $CuCl_2$ was significantly inhibited.

The involvement of the photosynthetic pigment PC in the antioxidant protection of the AFA extract against the oxidative damage was demonstrated by recording the spectral changes of PC induced by AAPH or $CuCl_2$. In fact, the incubation of the extract with the oxidizing agents led to a significant decrease in the absorption of PC at 620 nm accompanied with disappearance of its blue color, thus indicating a rapid oxidation of the protein. Similar spectral changes underline a direct involvement of the chromophore phycocyanobilin in the radical scavenging activity of PC; in fact, the spectroscopic properties and the brilliant blue color of the protein depend in large measure on the chemical nature of its chromophore (Glazer, 1988).

In the light of these in vitro results, and taking into account that PC also has anti-inflammatory properties, we think that the oral supplementation with the natural AFA extract could be a helpful co-factor in the treatment of clinical conditions related to oxidative stress and inflammation.

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