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Antioxidant potential of C-phycocyanin isolated from cyanobacterial species *Lyngbya, Phormidium* and *Spirulina* spp.

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The antioxidant activity of C-Phycocyanin (C-PC) isolated from three cyanobacterial species *Lyngbya* (marine), *Phormidium* (marine) and *Spirulina* (fresh water) was studied *in vitro*. The results demonstrate that C-PCs from *Lyngbya*, *Phormidium* and *Spirulina* spp. are able to scavenge peroxyl radicals (determined by crocin bleaching assay) with relative rate constant ratio of 3.13, 1.89 and 1.8, respectively. C-PCs also scavenge hydroxyl radicals (determined by deoxyribose degradation assay) with second order rate constant values of 7.87×10^{10}, 9.58×10^{10} and 6.42×10^{10}, respectively. Interestingly, *Lyngbya* C-PC is found to be an effective inhibitor of peroxyl radicals (IC_{50} 6.63 μ*M*), as compared to *Spirulina* (IC_{50} 12.15 μ*M*) and *Phormidium* C-PC (IC_{50} 12.74 μ*M*) and is close to uric acid (IC_{50} 2.15 μ*M*). Further, the studies suggest that the covalently-linked tetrapyrrole chromophore phycocyanobilin is involved in the radical scavenging activity of C-PC. The electron spin resonance (ESR) spectra of C-PCs indicate the presence of free radical active sites, which may play an important role in its radical scavenging property. This is the first report on the ESR activity of native C-PCs without perturbations that can cause radical formation.

Keywords: C-Phycocyanin, cyanobacteria, antioxidant, hydroxyl and peroxyl radical scavenger, electron spin resonance (ESR) spectra, *Lyngbya*, *Phormidium, Spirulina*

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Oxidative stress, induced by oxygen radicals is believed to be a primary factor in various diseases as well as in aging[1]. The reactive oxygen species (ROS), formed during normal metabolic processes can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. Free radical scavengers and antioxidants can reduce lipid peroxidation and the generation of ROS.

Phycobiliproteins, the brilliantly colored, water-soluble proteins, bearing covalently attached open chain tetrapyrroles, are the major photosynthetic accessory pigments in cyanobacteria. They, in association with the outer surface of the photosynthetic lamellae, are the constituents of the photosystem-II light-harvesting apparatus. C-Phycocyanin (C-PC), isolated from cyanobacteria, and is a major component of the phycobiliprotein family, is gaining increasing importance for its promising antioxidant property. It has a structure similar to bilirubin, a well-known effective scavenger for various reactive species. Recently, the antioxidant and anti-inflammatory properties of C-PC isolated from *Arthrospira platensis* have been reported[2-4]. Studies have also shown that C-PC can prevent and inhibit cancer in human and animals[5-9]. In view of the increasing applications of C-PC in the field of biomedicals, recently, we reported the denaturation kinetics of C-PC, purified from a cyanobacterium *Spirulina platensis*[10].

In the present study, the antioxidant potential of C-PC from three cyanobacterial species i.e., *Spirulina, Phormidium* and *Lyngbya* spp. has been evaluated against peroxyl and hydroxyl radicals and their efficiency is compared. In addition, the role of tetrapyrrole chromophores in the radical scavenging property and the possible mechanism of the antioxidant action are also studied. The presence of free radical species in C-PC was confirmed by electron spin resonance (ESR) spectroscopy.

Materials and Methods

Materials

2,2'-Azo-bis(2-amidinopropane) hydrochloride (AAPH), 2-deoxyribose and 2-thiobarbituric acid (TBA) were obtained from Sigma, St. Louis, MO,

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Abbreviations: C-PC, C-phycocyanin; TBA, thiobarbituric acid; AAPH, 2,2′-azo-bis (2-amidinopropane) hydrochloride; MDA, malonaldehyde; ROS, reactive oxygen species; TCA, thiobarbituric acid; ESR, electron spin resonance.

USA. Crocin was isolated from saffron by water/methanol (9:1) extraction and its concentration estimated using the molar extinction coefficient of 89,000 $M^{-1}cm^{-1}$ in aqueous solution[11]. All other reagents used were of A. R. grade available from commercial sources.

Methods

Purification of C-PC

The *Phormidium* and *Lyngbya* spp. were isolated from the rocky surface near the sea coast of Gujarat (Lat. 21°38' N and Long. 69°37'), the west coast of India. These organisms were grown in batch cultures in the standard artificial seawater medium ASN-III[12] at *p*H 7.5. The fresh water *Spirulina* sp. was grown in batch cultures in Zarrouk's medium[13] at *p*H 10. All the organisms were grown at temperature 20 ± 2°C, with optimum light intensity of 60 μ E m^{-2} s^{-1}, provided by the cool-white fluorescent tubes with a dark: light cycle of 12:12 hr.

Fresh biomass was harvested after 15 days of incubation by centrifugation at 10,000 × *g* for 30 min and cell mass was washed twice with distilled water and suspended in Na-phosphate buffer (0.1 *M*, *p*H 7.0 containing 1 m*M* sodium azide). The cells were disrupted by sonication for 60 sec. Repeated freezing at –20°C and thawing at room temperature in dark, followed by centrifugation at 10000 × *g* for 30 min at 4°C yielded a clear supernatant containing a mixture of C-PC, allophycocyanin (APC) and phycoerythrin (PE). C-PC from the three species was purified as described earlier[14].

Protein preparations were analyzed for purity by absorbance ratios (A_{620}/A_{280} and A_{620}/A_{652})[15] and SDS-PAGE. The molecular mass of native purified C-PC was determined by gel filtration on Sephadex G-100 column and native-PAGE.

C-PC interaction with peroxyl and hydroxyl radicals

AAPH and Fenton reagent were used for the generation of peroxyl and hydroxyl radicals, respectively. C-PC of the three species (10 μ*M*) dissolved in 1.0 ml of Na-phosphate buffer (10 m*M*, *p*H 7.4) was taken in a 1 ml quartz cuvette thermostated at 37°C. To study the interaction of C-PC with peroxyl radicals, the reaction was initiated by adding 20 μl of freshly prepared AAPH solution (0.5 *M*). The peroxyl radicals were generated by the thermolysis of AAPH.

The effect of hydroxyl radicals on C-PC was studied by generating hydroxyl radicals by the reaction of a mixture of $FeCl_3$ (20 μ*M*), EDTA (100 μ*M*), H_2O_2 (2.8 m*M*) and ascorbic acid (100 μ*M*) in a total volume of 1 ml containing 10 μ*M* C-PC in 10 m*M* Na-phosphate buffer (*p*H 7.4). $FeCl_3$ and EDTA were pre-mixed prior to addition to the reaction mixture. Ascorbic acid was added in order to start the reaction.

The final reaction mixture contained C-PC (10 μ*M*), Na-phosphate buffer (10 m*M*, *p*H 7.4) and AAPH (10 m*M*)/Fenton reagent in a total volume of 1 ml was incubated at 37°C for 1 hr. The changes in the UV-vis spectra of C-PC were recorded between 300-700 nm at 5 min intervals in Shimadzu UV-3101 PC thermostated spectrophotometer.

Measurement of peroxyl radical activity

Reactivity of C-PC was measured from the competition kinetics of crocin bleaching rate in the presence of peroxyl radicals generated by adding AAPH (10 m*M*). The reaction was carried out at 37°C in Na-phosphate buffer (10 m*M*, *p*H 7.4) containing crocin (10 μ*M*) and increasing concentration of C-PC (0-30 μ*M*) in a total volume of 1 ml. Uric acid (0-30 μ*M*) was used as a known peroxyl radical scavenger. The rate of crocin bleaching was monitored at 440 nm in a thermostated spectrophotometer. As bleaching rate was linear after 1.5 min, the rate from 2 to 6 min was used for calculation after the addition of AAPH. The equation for competition reaction is given as:

$$V_b/V_a = 1 + K_a/[K_c\{[\text{C-PC}]/[\text{crocin}]\}]$$

where V_b is basal crocin bleaching rate in the absence of C-PC, V_a is bleaching rate of crocin in the presence of C-PC. K_a and K_c are the rate constants for the reaction of peroxyl radicals with C-PC and crocin, respectively. A plot of V_b/V_a vs [C-PC]/[crocin] gives a straight line, intersecting the ordinate at unity. The slope $[K_a/K_c]$ of the straight line is the rate constant for the reaction of peroxyl radicals with C-PC, relative to the rate constant with crocin.

Estimation of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of C-PC was estimated by the inhibition of 2-deoxyribose degradation and measured as formation of the complex of thiobarbituric acid (TBA) and the degraded product of 2-deoxyribose. Reaction mixtures contained the following reagents at the final concentrations: C-PC variable concentration (0-0.082

m*M*), 2-deoxyribose (2.8 m*M*), KH_2PO_4/KOH buffer, *p*H 7.4 (15 m*M*), $FeCl_3$ (20 µ*M*), EDTA (100 µ*M*), H_2O_2 (2.8 m*M*) and ascorbic acid (100 µ*M*). The total volume of solution was 1 ml. Solutions of $FeCl_3$ and ascorbic acid were prepared immediately before use in deaerated water and were pre-mixed prior to addition into the reaction mixture. Reaction mixtures were incubated at 37°C for 1 hr and subsequently quenched by the addition of 1 ml of TBA 1% (w/v) (in 0.05 *M* NaOH) and 1 ml of trichloroacetic acid (TCA) 2.8% (w/v) (in water) and centrifuged at 10,000 × *g* for 15 min at 4°C. C-PC reacts with TBA at higher temperature yielding a pink chromogen with an absorption peak at 528 nm. To prevent this reaction, C-PC was removed by centrifugation at 10,000 × *g* for 10 min at 4°C after addition of stopper solution. The supernatant was transferred and heated for 20 min in boiling water bath. The pink chromogen that progressively developed was then measured at 532 nm after cooling against appropriate blanks.

$$\text{OH} + \text{2-Deoxyribose} \rightarrow \text{Fragments} \xrightarrow[\text{TBA + TCA}]{\text{Heated with}} \text{Malonaldehyde (MDA)}$$

$$2\ \text{TBA} + \text{MDA} \rightarrow \text{Chromogen}\ (\lambda_{max}, 532\ \text{nm})$$

The second order rate constant K_S were calculated using the equation:

$$1/A = 1/A_0\ [1+K_S\{S\}/K_{DR}\{DR\}]$$

where A and A_0 are the absorbances in the presence of C-PC at concentration [S] and in absence of C-PC, respectively. Hence, a plot of $1/A_{532\ nm}$ against [S] gives a straight line of slope $K_S/K_{DR}[DR]A_0$ with an intercept of $1/A_0$. The K_S for the reaction of C-PC with OH· was obtained from the slope of the line, using a K_{DR} value of $3.1 \times 10^9\ M^{-1}s^{-1}$ as described[16].

ESR spectrum

ESR measurements of freeze-dried C-PC samples were carried out on a Bruker EMX spectrometer operating at room temperature (X band, 100 kHz field modulation, 9.7 GHz).

Results

Purification of C-PC

The purification procedure resulted in a homogenous preparations, as evidenced by native and SDS-PAGE (Figs 1a and b). The position of bands on native PAGE (Fig. 1a) is not similar for all the three C-PCs, indicating some variation associated with charge or molecular mass. The molecular mass of native C-PC as determined by gel filtration chromatography is almost similar for *Spirulina* sp. (112 kDa) and *Phormidium* sp. (131 kDa), but lower for *Lyngbya* sp. (81 kDa). SDS-PAGE (Fig. 1b) gave two bands corresponding to the α- and β-subunits of C-PC, further confirming its purity and homogeneity. Moreover, the molecular mass of β-subunits of all the C-PCs is calculated to be 24.4 kDa, whereas that of α-subunits is found to be 17 kDa, 19.1 kDa and 15.2 kDa for *Spirulina, Phormidium* and *Lyngbya* C-PC, respectively. The monomer molecular mass of *Spirulina* and *Phormidium* C-PC is 41.4 kDa (17+24. 4 kDa) and 43.5 kDa (19.1+24.4 kDa), respectively and is consistent with a trimer structure of this molecule[14]. However, the monomer molecular mass of C-PC of *Lyngbya* sp. is 39.6 Da (15.2 + 24.4 kDa), while that of native C-PC is 81 kDa, suggesting a dimer structure.

C-PC interaction with peroxyl and hydroxyl radicals

Exposure of C-PC to the free radical source (AAPH) leads to a progressive decrease in the visible absorbance, suggesting the involvement of phycocyanobilin in the radical scavenging activity. A significant decrease in absorbance at 620 nm and a shift in absorption maxima towards lower wavelength (~ 598 nm) were observed (Fig. 2), when C-PC was incubated with AAPH (10 m*M*) at 37°C for 1 hr. Furthermore, the decrease in absorption was not

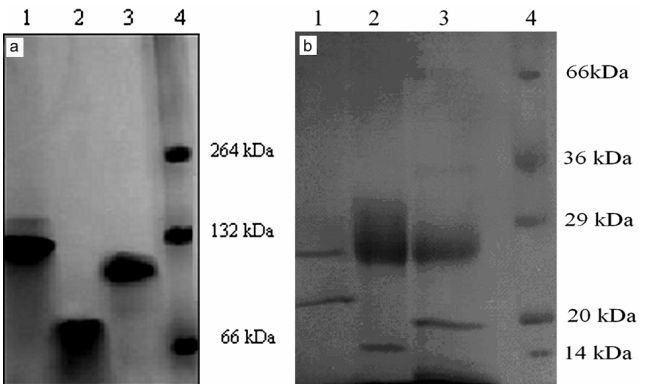

Fig. 1—(a): Native PAGE of C-PCs [C-PCs were subjected to Native PAGE (8%) followed by staining with Coomassie blue. Lane1, *Phormidium* sp.; lane 2, *Lyngbya* sp.; lane 3, *Spirulina* sp.; and lane 4, BSA standard; and (b): SDS PAGE of C-PCs [C-PCs were subjected to SDS-PAGE (15%), followed by staining with Coomassie blue. Lane1, *Phormidium* sp.; lane 2, *Lyngbya* sp.; lane 3, *Spirulina* sp.; and lane 4, markers]

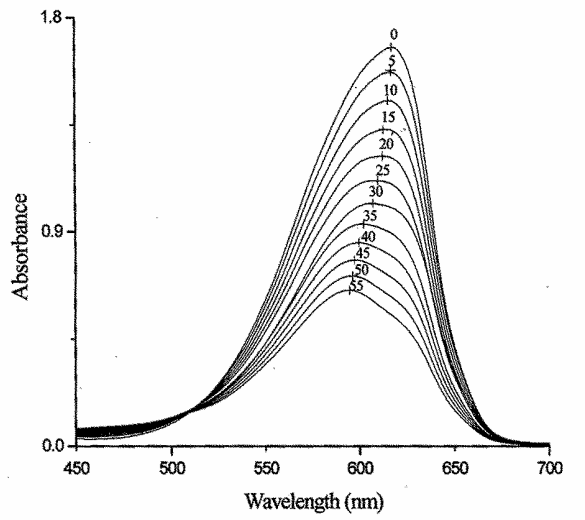

Fig. 2—Time-dependent spectral changes associated with the AAPH (0-55 min) induced oxidation of C-PC (10 μ*M*) in Na-phosphate buffer (10 m*M*, *p*H 7.4)

accompanied by the appearance of a new band in visible region of the spectra. All the three C-PCs exhibited almost similar decrease in the absorbance at 620 nm (~ 60% decrease) accompanied by ~ 22 nm blue shift in the absorption maxima (Fig. 2). However, only 15-20% decrease in the absorbance at 620 nm, without any significant shift in the position of absorption maxima was observed, when C-PC was incubated with hydroxyl radical generating system (Fenton reagent), indicating slow reactivity of hydroxyl radicals towards C-PC, in comparison to peroxyl radicals.

Bleaching rates of the three C-PCs in the presence of peroxyl and hydroxyl radicals are shown in Fig. 3. The results indicate that the bleaching rate of C-PC in the presence of peroxyl is much faster, compared to hydroxyl radicals. Moreover, the rate of interaction is almost similar for the three C-PC samples with peroxyl radicals, whereas slight differences are observed with hydroxyl radicals.

Peroxyl radical scavenging activity of C-PC

The ability of C-PC to scavenge peroxyl radicals was further analyzed by studying the competition kinetics of crocin bleaching (Fig. 4). The results demonstrate that C-PC is a potent radical scavenger. The relative rate constant ratio and IC_{50} values of three samples of C-PC and uric acid, a known peroxyl radical scavenger are given in Table 1. The data presented in Table 1 and Fig. 5 show that *Lyngbya* C-PC exhibited the maximum peroxyl radical

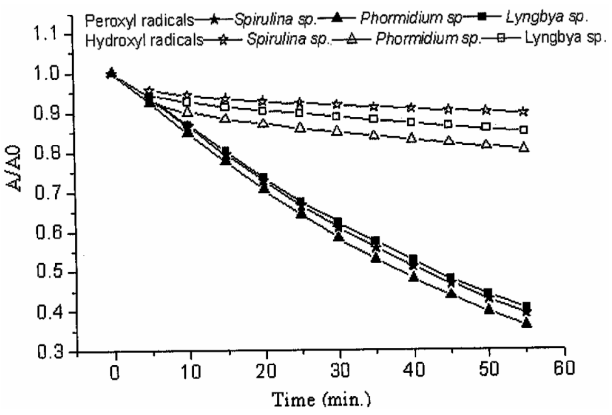

Fig. 3—Change in relative absorbance of C-PC incubated at 37°C in the presence of peroxyl and hydroxyl radicals

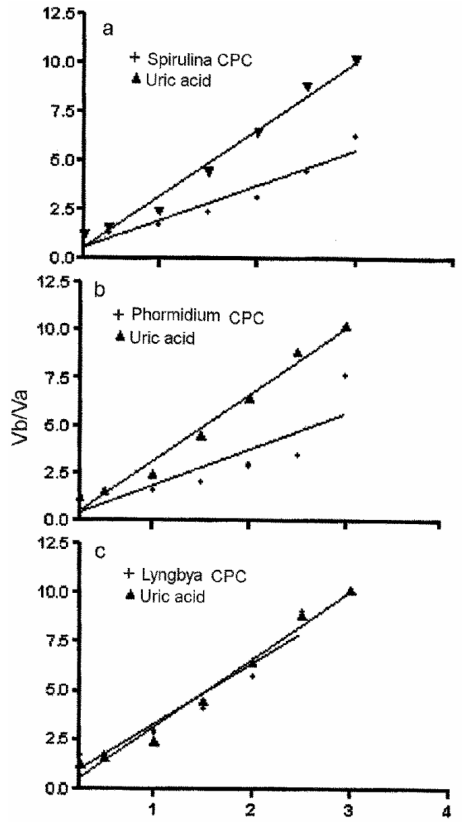

Fig. 4—Competition kinetics plots of C-PCs and uric acid towards crocin in AAPH-induced radical reaction

scavenging activity, followed by *Phormidium* and *Spirulina* C-PC.

Hydroxyl radical scavenging activity of C-PC

C-PC inhibited the deoxyribose degradation in a concentration-dependent manner. Fig. 6 shows a typical linear competition profile of C-PC concentration vs $1/A_{532\,nm}$. The slope of competition

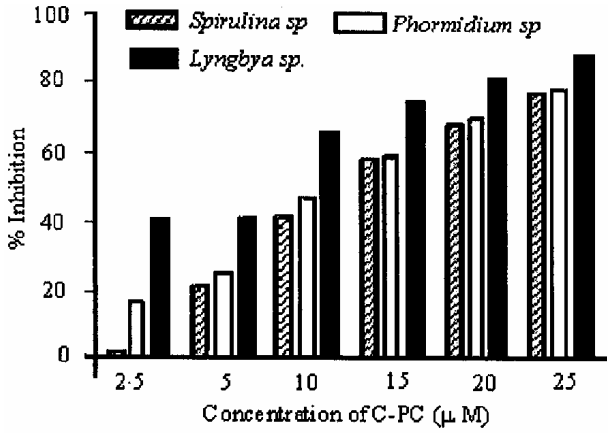

Fig. 5—Percentage inhibition of crocin bleaching as a function of C-PC concentration

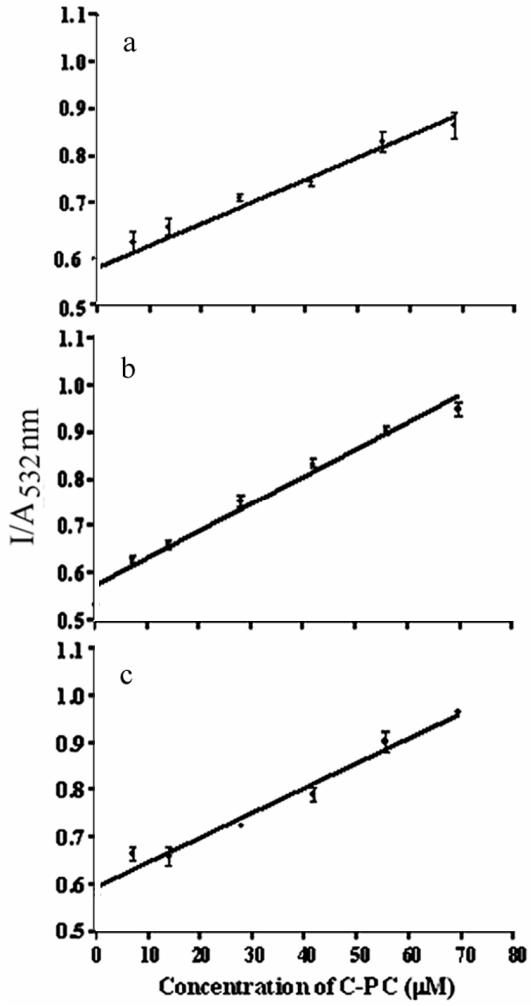

Fig. 6—Plots of $1/A_{532nm}$ vs concentration of C-PC using data obtained by 2-deoxyribose degradation [Values for each point are mean ± SD of three experiments. (a) *Spirulina* sp.; (b) *Phormidium* sp.; and (c) *Lyngbya* sp.]

Table 1—Inhibitory effect of C-PC on peroxyl radical-induced bleaching of crocin and second order rate constants (K_s) for reaction of C-PC with hydroxyl radicals

Test compound	Relative rate constant ratio (K_{rel})	IC_{50} (μ*M*)	Second order rate constants ($M^{-1} s^{-1}$) with hydroxyl radicals
C-PC (*Spirulina* sp.)	1.8	12.15	6.42×10^{10}
C-PC (*Phormidium* sp.)	1.89	12.74	9.58×10^{10}
C-PC (*Lyngbya* sp.)	3.13	6.63	7.87×10^{10}
Uric acid	3.47	2.15	—

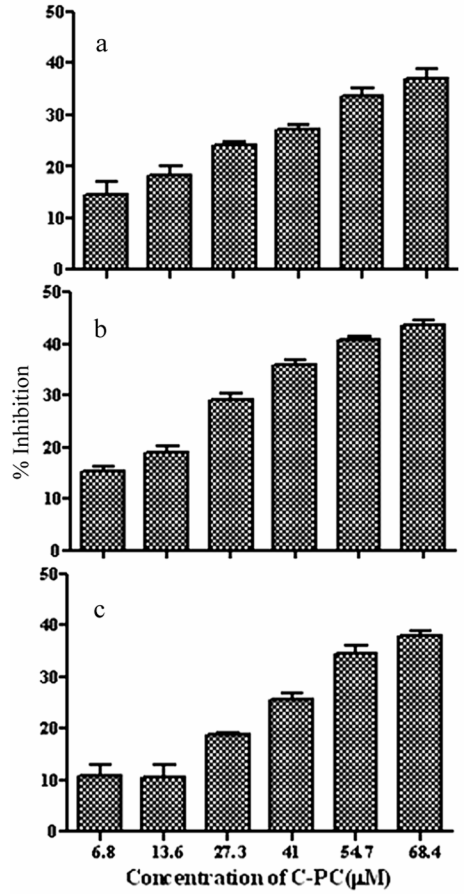

Fig. 7—Percentage inhibition of 2-deoxyribose degradation as function of C-PC concentration [Values represent mean ± SD of 3 determinations. (a) *Spirulina* sp.; (b) *Phormidium* sp.; and (c) *Lyngbya* sp.]

profile was used to calculate the second order rate constant (K_S= slope × K_{DR} × [DR] × A_0) for the reaction of C-PC with $OH^{\cdot}$. The values of second order rate constant of three C-PCs show only slight differences and are given in Table 1. The inhibitory effect of three C-PC samples on hydroxyl radicals is shown in Fig. 7. *Phormidium* C-PC is found to exhibit

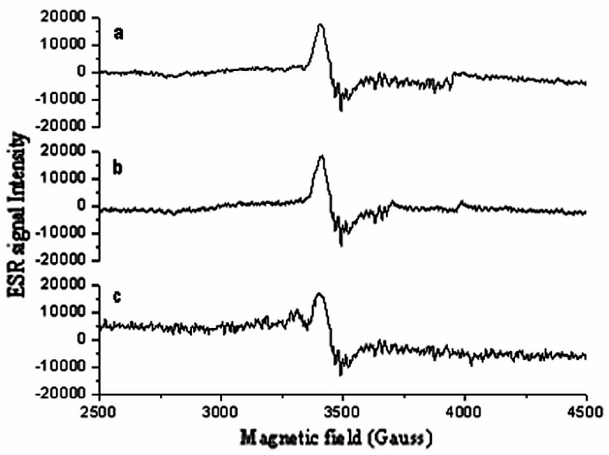

Fig. 8—ESR spectra of C-PC at 9.7 GHz. *Spirulina* sp.; (b) *Phormidium* sp.; and (c) *Lyngbya* sp.

better activity against hydroxyl radicals, as compared to *Lyngbya* and *Spirulina* C-PCs, at all the concentrations tested.

ESR spectra

The ESR spectra of freeze-dried C-PC are shown in Fig. 8. The spectra showed one broad singlet for all the three C-PC samples. The calculated *g* values, corresponding to the free radical active species were 2.002, 2.004 and 2.011 for C-PC from *Spirulina, Phormidium* and *Lyngbya* spp., respectively and were close to the typical *g* value of 2.004 for a carbon-centered organic free radical.

Discussion

In present study, the antioxidant potential of C-PC isolated from *Spirulina, Phormidium* and *Lyngbya* spp was evaluated *in vitro* against hydroxyl and peroxyl radicals. Interaction of C-PC with peroxyl radicals results in a rapid decrease of the absorbance at 620 nm, with concomitant blue shift of the maxima (Fig. 2). The high bleaching rate of the 620 nm band (Fig. 3) indicates that the main target of the peroxyl radicals is the phycocyanobilin (chromophore) moiety. The high reactivity of phycocyanobilin moiety may be due to high degree of conjugation of double bonds, which strongly stabilizes the radicals formed. Thus, the interaction of C-PC with peroxyl radicals can be explained under the following reaction scheme:

$ROO^{\cdot}$ +C-PC → C-PC* (+ ROOH or ROO-)

C-PC* + $ROO^{\cdot}$ → Non-radical products

2 C-PC* → Non-radical products

$ROO^{\cdot}$ + C-PC → Bleaching

On the other hand, the interaction of C-PC with hydroxyl radicals results in 15-20% decrease in absorbance at 618 nm, without any significant spectral shift. The slow bleaching rate of C-PC indicates that the reactivity of phycocyanobilin moiety against hydroxyl radicals is less, as compared to peroxyl radicals. However, a possible contribution of the apoprotein part in the total antioxidant activity of C-PC can't be ruled out, since it contains antioxidant amino acid residues[17].

The peroxyl radical scavenging ability of C-PC was further evidenced by competition kinetics of crocin bleaching assay (Fig. 4). *Lyngbya* C-PC is found to be a better peroxyl radical scavenger than *Spirulina* and *Phormidium* C-PC, with an IC_{50} value of 6.63 μ*M* and relative rate constant ratio (K_{rel}) of 3.13. Indeed, it appears to be as efficient as uric acid, a physiological antioxidant (IC_{50} 2.15 μ*M*; K_{rel} 3.47) in its peroxyl radical scavenging activity. *Spirulina* and *Phormidium* C-PCs have shown moderate peroxyl radical scavenger activity. The significant difference in peroxyl radical scavenging activity, exhibited by *Lyngbya* C-PC may possibly be due to the differences in the amino acid residues in the apoprotein part of C-PC. Some amino acid residues in a polypeptide chain are known to exhibit antioxidant activity. In an earlier study[18], it has been suggested that tryptophan, tyrosine and histidine residues may trap peroxyl radicals in a less polar environment.

The results of hydroxyl radical scavenger activity of C-PC as determined by deoxyribose degradation assay (Table 1) demonstrate that C-PC is a potent hydroxyl radical scavenger (Fig. 7). Thus, the values of second order rate constant of all the three C-PCs of are better than the mannitol[19] and are comparable to those reported for the non-steroidal anti-inflammatory drugs indomethocin and ibuprofen[20]. The observation made in the present study is consistent with the earlier report on hydroxyl radical scavenger activity of C-PC of *Arthrospira maxima*[2]. Although *Lyngbya* C-PC exhibits higher peroxyl radical scavenger activity than *Spirulina* and *Phormidium* C-PC, its antioxidant potential against hydroxyl radicals is found to be similar to that of *Spirulina* and *Phormidium* C-PC.

ESR spectra of C-PC reveal the presence of free radical active sites on C-PC, which might play an important role in the radical scavenging activity. In earlier studies[21,22], the generation of ROS was reported from the photo-sensitization of C-PC in the presence of oxygen. Here, the chromophore group can

act as an electron donor in the presence of a proper acceptor, thus it was inferred that photo-induced electron transfer from C-PC to oxygen might occur and lead to the formation of ROS, which can be explained under the following reaction scheme:

$$\text{C-PC} \xrightarrow{h\upsilon} \text{C- CPC*}$$

$$\text{C- PC*} + O_2 \longrightarrow \text{Reactive oxygen species } (O_2^{-}\cdot + OH\cdot)$$

The ROS can react with free radicals to give non-radical species, suggestive of another pathway of antioxidant activity of C-PC in the presence of oxygen. However, to our knowledge, this is the first report of the ESR activity of native C-PCs without perturbations that can cause radical formation.

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