



## IN VITRO ANTIMALARIAL ACTIVITY OF C-PHYCOCYANIN FROM NOSTOC MUSCORUM

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Antimalarial activity

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*Nostoc muscorum*

*Plasmodium falciparum*

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## ABSTRACT

The phycobilin pigments are intensively fluorescent and water-soluble. These are categorized into three types by bilin energy: those of high energy (phycoerythrins or phycoerythrocyanin), intermediate energy (phycocyanins) and low energy (allophycocyanins). Besides light harvesting, the phycobiliproteins possess several properties of industrial and biomedical importance. Among them *C*-phycocyanin (*C*-PC) is the most preferred one. The present study was performed to evaluate the antimalarial activity of *C*-phycocyanin from *Nostoc muscorum* by *in vitro* testing on resistant and sensitive strains of *Plasmodium falciparum*. *C*-PC was extracted and purified from *Nostoc muscorum* by using acetone extraction, ammonium sulphate precipitation and dialysis followed by amicon centrifugation. *C*-PC used in the present study was a ~ 124 KD, water soluble protein molecule. *C*-phycocyanin showed an antimalarial activity *in vitro* with minimum inhibitory concentration ranging from 8.41 mg/mL to 13.48 mg/mL against the chloroquine-sensitive strains and 0.95 mg/mL to 12.02 mg/mL against the chloroquine resistant of *Plasmodium falciparum* strains. The possible mechanism relies on the fact of destruction of polymerization of haemozoin by binding with FP-IX (which is responsible for the polymerization of haemozoin) at the water surface of plasma membrane. As *C*-PC is a large protein molecule of ~ 124 KD so it is unable to diffuse directly through the lipid bilayer of plasma membrane.

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## INTRODUCTION

Cyanobacteria contribute significantly to the total carbon biomass and primary productivity (Viskari and Colyer, 2003). Phycobilisomes are large supramolecular aggregates attached to the thylakoid membrane of cyanobacteria (Blue Green Algae) and rhodophyta (red algae) that function in light harvesting and energy migration (Marsac, 2003). In cyanobacteria, the light-harvesting pigments are chlorophyll-a, carotenoids and phycobiliproteins. Phycobiliproteins are a group of intensely colored proteins occurring in Cyanophyceae, Rhodophyceae and Cryptophyceae. It can be subdivided into three main groups according to their structure: phycocyanins (blue), allophycocyanins (blue) and phycoerythrins (red) (Brown *et al.*, 1997). Phycocyanin and allophycocyanin are always present in Cyanophyceae and Rhodophyceae but phycoerythrin may be absent in the former (Moreno *et al.*, 1995). Phycobiliproteins are organized in supramolecular aggregates “phycobilisomes” in order to maximize energy transfer to the chlorophyll-protein complexes located at the thylakoid membrane. No such aggregates have ever been detected in Cryptophyceae (Rowan, 1989). These phycobilisomes are composed of phycobiliproteins: a family of hydrophilic, brilliantly coloured and stable fluorescent pigment proteins covalently linked with linear tetrapyrrole prosthetic group (bilins) that are covalently linked to specific cysteine residues of the proteins (Santiago *et al.*, 2004). The absorption maxima for C-phycocyanin, is 620 nm (Bermejo *et al.*, 2003). The protein nature, unique colour, fluorescence, antioxidant properties with wide range of promising applications in diagnostics, biomedical research and therapeutics has made attention in scientific field (Rossano *et al.*, 2003). The main application of phycobiliproteins is as fluorescent markers of cells and macromolecules in biomedical research in highly sensitive fluorescent techniques (Bermejo *et al.*, 2003).

Malaria remains a major problem resulting into an unacceptable toll on the health and economic welfare of the world's poorest communities. Over 200–500 million cases and 0.7–2.7 million deaths occur each year due to malaria (Berman, 2001) and making it one of the top three killers among communicable diseases (WHO, 2003). Despite intensive efforts to control malaria, the disease continues to be one of the greatest health problems facing Indian sub continent. Although a number of advances have been made towards the understanding of the disease, relatively few antimalarial drugs have been developed in the last 30 years (Ridley, 2002). Since the treatment and control of malaria depends largely on a limited number of chemoprophylactic and chemotherapeutic agents, there is an urgent need to develop novel, affordable antimalarial treatments. Over the years chloroquine has been used as an anti-malarial drug due to its availability, effectiveness and low toxicity. Presently it is found by many users that chloroquine is no longer effective in most of the world because of the resistance to it that has developed (White *et al.*, 1998). The mechanism of chloroquine resistance in *Plasmodium* was first reported by Donald Krogstad (Krogstad *et al.*, 1987). The development of safe and effective anti-malarial agents has been realized as a challenge in recent years because of the rapid spread of drug resistant *P. falciparum* strains. Historically, the majority of antimalarial drugs have been derived from medicinal plants or from structures modeled on plant lead compounds. These include the quinoline-based antimalarials as well as artemisinin and its derivatives (Chen *et al.*, 1994). Indian sub continent boasts remarkable biodiversity and rich cultural traditions of plant use. Scientific understanding of medicinal plants is however, largely unexplored and pharmacological investigation of the Indian sub continent flora only gained momentum recently (Simonsen *et al.*, 2001).

But no work has been done on the Antimalarial activity of C-phycocyanin extracted and purified from a nitrogen fixing cyanobacterium, *Nostoc muscorum* in *Plasmodium falciparum*, hence the present work has been undertaken.

## MATERIALS AND METHODS

### Organism and growth conditions

*Nostoc muscorum* was cultured in BG-11 medium without any combined nitrogen sources at pH 8 (Rippka *et al.*, 1971). The culture flasks were maintained in culture racks at temperature  $25 \pm 2^\circ\text{C}$  and light intensity

of 75  $\mu\text{mol m}^{-2}\text{s}^{-1}$  PAR at light: dark cycles of 14:10 hr.

### Dry weight measurement

Dry cell weight (dcw) was determined gravimetrically according to Rai *et al.* (1991).

### Extraction of C-phycoyanin

#### Acetone Extraction

Cell suspension of *Nostoc muscorum* was taken in a centrifuge tube. The suspension was centrifuged at 5000 rpm for 10 min. The supernatant was discarded. 20 mL of 80% acetone was mixed with the pellet and incubated over night at 4°C. The suspension was again centrifuged at 5000 rpm for 10 min. The supernatant was then discarded. 20 mL of distilled water was added to the pellet and kept at 50°C for 30 min. The suspension was centrifuged at 5000 g for 10 min to get the supernatant containing phycobiliproteins.

#### Purification of C-phycoyanin

Crude phycobiliproteins obtained was then precipitated sequentially with 35%, 40%, 45%, 50%, 60% and 70% of  $(\text{NH}_4)_2\text{SO}_4$ . Pellet obtained after centrifugation was dissolved in minimal volume of 50 mM Tris-buffer (pH 7.5) and optical density was measured at 620 nm, 650 nm and 280 nm. The samples with higher purity ratio were collected and were subjected to dialysis with same buffer overnight followed by Amicon centrifugation for 5 min at 5000 rpm.

#### Native page

Native polyacrylamide gel electrophoresis was employed for electrophoretic separation of C-PC. C-PC was run in non-denaturing conditions. The purity of the samples obtained after 40%  $(\text{NH}_4)_2\text{SO}_4$  precipitation was confirmed using native PAGE in three different lanes. The molecular weight of C-PC was determined by comparing with molecular marker of lane 2. The molecule contains single protein band at ~124 KD as observed in electrophoretic separation on native gel.

#### Spectrophotometric estimation of phycobiliproteins

The UV-Vis absorbance spectra (250-820 nm) were recorded on UV-Vis Spectrophotometer. The amount of C-phycoyanin was calculated using the equation of Ravindra (2005) and the purity ratio (R) following Minkova *et al.* (2003).

#### Malarial parasites

Malarial parasites were cultured in complete RPMI-1640 medium using candle jar technique originally described by Trager and Jensen (1976) for the continuous cultivation of *Plasmodium falciparum* later on modified by Rai Chowdhuri *et al.*, 1979. Following strains were used for present work (Table 1).

#### Parasite Growth Inhibition Assay

The assay was done by the method described by Biswas, 2003. The measurement of Haemozoin in alkaline solution (sodium hydroxide) along with Triton-X-100 gives absorbance peak at 400 nm this is used for the detection and quantification of haemozoin (Rieckmann *et al.*, 1978). Percentage Growth Inhibition of parasitemia as well as Total parasite growth was calculated using the formulae (Biswas, 2001). Inhibitory concentration 50 and 95 were calculated in both CQ-S and CQ-R parasites by noting the C-Phycocyanin concentration at which 50% and 95% Schizont Maturation and total parasite growth were affected at 48 hrs.

**Table 1: Parasites strains used**

Type of <i>P. falciparum</i>	Code	Place of collection	Source of parasite isolate	year	chemotherapy
CQ-S	Pf1	Delhi	Delhi	1991	Chloroquine
CQ-S	Pf2	Delhi	Delhi	1992	Chloroquine
CQ-S	Pf3	Shajahanpur (UP)	Shajahanpur (UP)	1989	Chloroquine
CQ-R	Pf4	Shankargarh(UP)	Shankargarh(UP)	1992	Sufadoxine-pyrimethamine (SP)
CQ-R	Pf5	Delhi	Satna (MP)	2006	(SP)
CQ-R	Pf6	Delhi	Manipur	1995	(SP)

Percent Schizont Maturation inhibition =  
 $[1 - (\text{No. of Schizont/Parasite in the test}) / (\text{No of Schizont/Parasite in control})] \times 100$

## RESULTS

### Extraction of C-phycoyanin

The purity ratio ( $R = A_{620}/A_{280}$ ) was found to be 0.99 in case of acetone extraction. The purity ratio and C-phycoyanin concentration was increased on fractionation with ammonium sulphate. It was found to be maximum (3.25) at 40% ammonium sulphate fractionation (Table 2).

**Table 2: Summary of Extraction and Partial purification of C-phycoyanin obtained by acetone extraction**

Stages of Purification	A <sub>650</sub>	A <sub>620</sub>	A <sub>A280</sub>	A <sub>A620/A280</sub>	C-PC (mg/mL)	C-PC (mg/dcwg)	Recovery efficiency (%)
Crude extract	0.28	0.91	0.92	0.99	0.97	166.65	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (35%)	0.14	0.49	0.18	2.65	0.53	89.16	55.15
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (40%)	0.15	0.57	0.17	3.25	0.64	106.29	65.75
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (45%)	0.12	0.48	0.15	3.07	0.54	90.99	56.29
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (50%)	0.14	0.51	0.18	2.85	0.56	93.82	58.04
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (60%)	0.17	0.44	0.23	1.96	0.44	73.88	45.7
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (70%)	0.109	0.38	0.148	1.48	0.41	69.33	42.88

### Absorption spectrum of C-PC

Two sharp peaks were observed at 280 nm and 620 nm corresponding the protein and C-PC respectively (Fig. 1a, 1b). The protein peak got reduced upon fractionation with ammonium sulphate (Fig. 1b).

### Native PAGE

The purity of the sample obtained after 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation was confirmed using native PAGE (Fig. 1c). Lane 2 shows molecular marker while lane 1 and lane 3 shows silver staining and coomassie staining of C-PC respectively. The molecule contained a single protein band at ~124 KD.

### In vitro assay of C-PC as antimalarial compound

*In vitro* efficacy of C-PC for its antimalarial activity was monitored indirectly by quantification of haemozoin

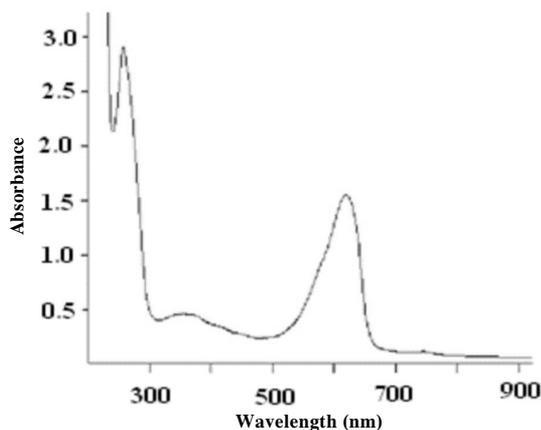


Figure 1a: Absorption spectrum of crude C-PC

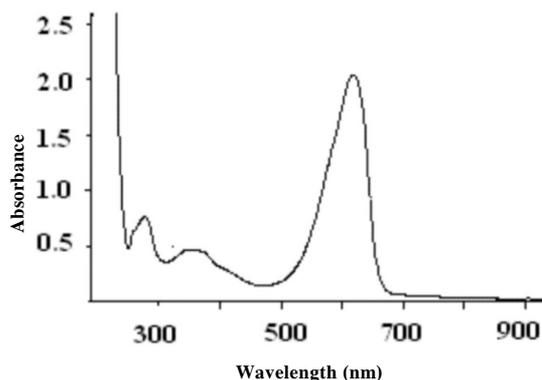


Figure 1b: Absorption spectrum of C-PC after 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation

(Table 3). The measurement of Haemozoin in alkaline solution (sodium hydroxide) along with Triton-X-100 gives absorbance peak at 400 nm, this is used for the detection and quantification of haemozoin (Rieckmann *et al.*, 1978). In the presence of ineffective concentrations of the drug, ring stages (which are devoid of pigment) are able to mature to schizonts and in the process, the digestion of the haemoglobin leads to the progressive accumulation of pigment.

### Microscopy

Parasite count, multiplication rate and parasite growth inhibition (Table 4, 5) were calculated microscopically according to Biswas and Valecha (1996). Parasite count was done per 5000 RBC after incubation of 48 hr with C-PC (Table 4). Above the concentration of 47.7  $\mu\text{g/mL}$  of C-PC, parasite growth was inhibited completely. Fig. 2 represents the microscopic observations of C-PC addition to the *in vitro* culture of *Plasmodium falciparum* at different time interval.

### Effect of C-PC on *Plasmodium falciparum*

The inhibitory concentration (IC) is commonly used as a measure of antagonist drug potency in pharmacological research. The  $\text{IC}_{50}$  is a measure of the effectiveness of C-PC in inhibiting malaria parasite growth by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC or  $\text{IC}_{50}$ ). The  $\text{IC}_{50}$  of C-PC was determined constructing a dose-response curve and examining the effect of different concentrations of antagonist on reversing agonist activity.  $\text{IC}_{50}$  values are dependent on conditions under which they are measured. In general, the higher the concentration of inhibitor, the more the agonist activity is lowered. Similar method was followed to determine  $\text{IC}_{90}$ ,  $\text{IC}_{95}$  (Table 6).

## DISCUSSION

It was observed while compiling the extraction and purification procedures of C-phycoerythrin that the existing methods are completed with several steps and expensive. For example, Hilditch *et al.* (1991) extracted and purified C-PC from *Aphanothece halophytica* by using DNase, RNase and proteinase followed by ion exchange and gel permeation chromatography. Reis *et al.*, (1998) purified C-PC from crude extracts of *Nostoc sp.* PCC 9202 by means of ultrafiltration and  $(\text{NH}_4)_2\text{SO}_4$  precipitation followed by gel filtration and ion-exchange chromatography. In the same year, Abalde *et al.*, (1998) described a suitable method for the optimum extraction and isolation of C-PC from the cyanobacterium *Synechococcus sp.* IO9201, isolated from Caribbean waters, which comprised extraction by freezing and thawing and purification, by hydrophobic interaction chromatography and ion exchange chromatography. C-PC was purified from *Spirulina (Arthrospira) fusiformis*

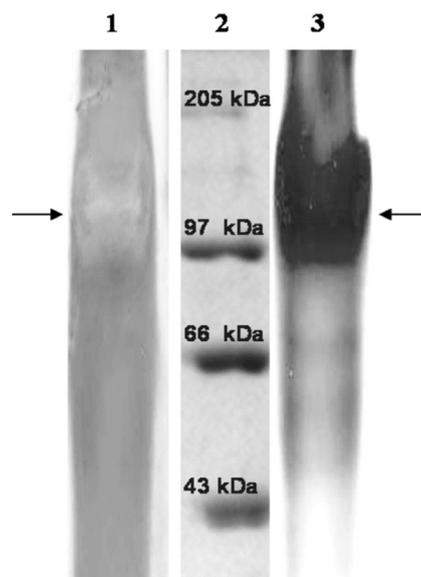


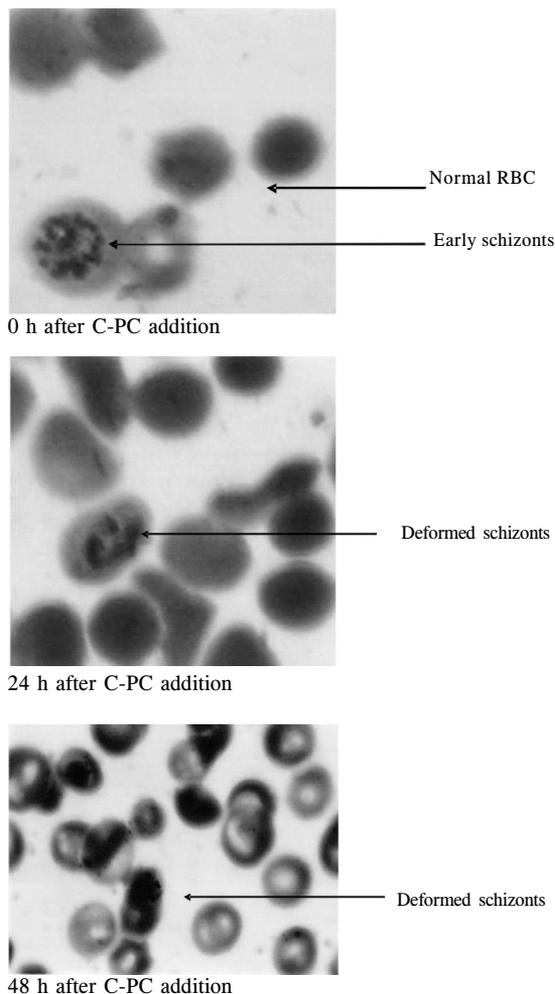
Figure 1c: Native PAGE of C-PC

Table 3: Micro Plate-ELISA reader assay of different malarial strains measured at 400 nm after incubation with C-PC for 48 hr

Rows	Malarial Strains					
	pf1	pf2	pf3	pf4	pf5	pf6
A	1.20±0.04	1.17±0.02	1.21±0.8	1.18±0.04	1.19±0.4	1.13±0.03
B	1.48±0.03	1.50±0.04	1.51±0.048	1.53±0.06	1.49±0.04	1.46±0.02
C	1.58±0.1	1.60±0.10	1.61±0.12	1.62±0.10	1.57±0.1	1.51±0.04
D	1.61±0.13	1.63±0.02	1.66±0.012	1.63±0.02	1.64±0.08	1.58±0.04
E	1.65±0.01	1.67±0.12	1.68±0.052	1.65±0.09	1.67±0.14	1.64±0.08
F	1.75±0.13	1.70±0.14	1.76±0.18	1.74±0.10	1.78±0.1	1.71±0.05
G	1.81±0.20	1.79±0.17	1.80±0.17	1.79±0.17	1.81±0.2	1.73±0.12
H	1.90±0.01	1.87±0.02	1.85±0.04	1.85±0.04	1.82±0.02	1.86±0.02

by a multi-step treatment of the crude extract with rivanol followed by 40% saturation with ammonium sulfate. After removal of rivanol by gel-filtration on Sephadex G-25, the pigment solution was saturated to 70% with ammonium sulfate (Minkova *et al.*, 2003). Most recently, Benedetti *et al.*, (2006) extracted and purified C-PC from *Aphanizomenon flos-aquae* by ultracentrifugation and hydrophobic chromatography using a hydroxyapatite column. As compared to these methods, the present method is easy and gave maximum recovery without using expensive chemicals and equipments, thus could be recommended as a simple and cost-effective extraction and purification method for C-PC from *Nostoc muscorum*.

The problems of resistant lines of *plasmodium* are escalating. Laboratory studies revealed the development of resistance to almost every antiplasmodial drugs known today, which means that there is an urgent need of new antimalarial drug (Farnsworth, 1994). New drugs of herbal origin discovered through ethnopharmacological studies have shown interesting results (Chen *et al.*, 1994), *e.g.* artesimnin from *Artemisia annua* L., a plant from traditional Chinese medicine. The present screening of C-PC for *in vitro* antiplasmodial activity against *Plasmodium falciparum* was performed as part of a project, the aim of which was to investigate their *in vitro* activity in order to discover new lead structures or improve the traditional medicine and to understand the biomedical importance of algal pigments.



**Figure 2: Microscopic observations of C-PC addition to the *in vitro* culture of *Plasmodium falciparum***

In this study, C-PC was found to be effective against both chloroquine-sensitive and chloroquine-resistant even at very low concentrations. C-PC used in the present study was a ~ 124 KD, water soluble protein molecule. We may postulate that the possible mechanism relies on the fact of destruction of polymerization of haemozoin by binding with ferriproto-porphyrin-IX (FP-IX), which is responsible for the polymerization of haemozoin, at the water surface of plasma membrane. As C-PC is a large protein molecule, so it is

**Table 4: Parasite count/5000 RBCs (Assay time 48 hr)**

C-PC ( $\mu\text{g/mL}$ )	Malarial strains					
	pf1	pf2	pf3	pf4	pf5	pf6
191	NG	NG	NG	NG	NG	NG
95.5	NG	NG	NG	NG	NG	NG
47.7	10	20	10	20	15	10
23.9	20	40	15	35	30	25
11.9	35	65	35	50	40	40
6.0	60	85	55	65	65	60
3.0	80	110	85	90	95	85
Control	100	125	90	105	100	100
0 hour	40	40	25	35	30	25

Values are means of three observations; NG: No growth

**Table 5: Parasite growth inhibition**

C-PC ( $\mu\text{g/mL}$ )	Malarial strains					
	pf1	pf2	pf3	pf4	pf5	pf6
191	100%	100%	100%	100%	100%	100%
95.5	100%	100%	100%	100%	100%	100%
47.7	90%	84%	88.9%	80.9%	85%	90%
23.9	80%	68%	83.3%	66.7%	70%	75%
11.9	65%	48%	61.1%	52.4%	60%	60%
6.0	40%	32%	38.9%	38.1%	35%	40%
3.0	20%	12%	5.6%	14.3%	5%	15%
Control	0%	0%	0%	0%	0%	0%

Values are means of three observations

**Table 6: Summary of the effect of C-PC on *Plasmodium falciparum* chloroquine-sensitive and resistant culture lines at 48 hr**

Type of <i>P. falciparum</i>	Malarial strains	IC <sub>50</sub> of C-PC in (mg/mL)	IC <sub>90</sub> of C-PC in (mg/mL)	IC <sub>95</sub> of C-PC in (mg/mL)
CQ-S	pf1	8.4	47.9	66.1
CQ-S	pf2	13.5	60.3	79.4
CQ-S	pf3	8.9	52.5	75.9
CQ-R	pf4	12.0	66.1	79.4
CQ-R	pf5	9.6	60.3	75.9
CQ-R	pf6	9.6	44.7	63.1

Values are means of three observations

unable to diffuse directly through the lipid bi-layer of plasma membrane. One mechanism could be the proteolytic cleavage of C-PC molecule and fragmented active functional moiety that may traverse through the plasma membrane or trans-membrane region linked with another carrier protein (Raynes, 1999 and Egan, 2003). Due to its nutritive and medicinal properties and negligible toxicity including hepatoprotective property, C-PC could act as oral supplement.

Thus, it may be concluded that C-PC could be used as a safest, potent oral supplement along with known recommended antimalarial drug for both CQS and CQR parasites and seem to be crucial to realizing the importance and use of algal pigment in the field of biomedical sciences.

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