Isolation and Purification of *C*-phycocyanin from *Nostoc muscorum* (Cyanophyceae and Cyanobacteria) Exhibits Antimalarial Activity *In vitro*

Pranay P. Pankaj[1], Ratanesh K. Seth[2], Nirupama Mallick[1] and Sukla Biswas[2*]

[1]*Agricultural & Food Engineering Department, IIT, Kharagpur, India.*
[2*]*National Institute of Malaria Research (ICMR), Dwarka, New Delhi, India.*

Abstract: The phycobilin pigments are intensively fluorescent and water soluble. They are categorized into three types, such as pigments containing high, intermediate and low energies are phycoerythrins (phycoerythrocyanins), phycocyanins and allophycocyanins, respectively. Besides light harvesting, the phycobiliproteins have shown industrial and biomedical importance. Among them, *C*-phycocyanin (*C*-PC) has been considered to be the most preferred one. The present study was undertaken to evaluate the antimalarial activity of *C*-PC isolated from a nitrogen-fixing cyanobacterium and *Nostoc muscorum*. *C*-PC was extracted and purified by acetone extraction and ammonium sulfate precipitation and dialysis followed by amicon filtration. It was isolated as a~124 kDa water soluble protein molecule. It showed antimalarial activity *in vitro* against chloroquine sensitive and resistant *Plasmodium falciparum* strains. Inhibitory concentrations at 50%, 90% and 95% were determined as 10.27±2.79, 53.53±6.26 and 73.78±6.92 µg/ml against the chloroquine-sensitive strains; 10.37±1.43, 56.99±11.07 and 72.79±8.59 µg/ml against chloroquine resistant of *Plasmodium falciparum* strains. *C*-PC was found to have antimalarial activity even at a concentration of 3.0µg/ml. The possible mechanism might be relied on the destruction of polymerization of haemozoin by binding of *C*-PC with ferriprotoporphyrin-IX at the water surface of the plasma membrane.

Keywords: *Plasmodium falciparum*, Antimalarial activity, *Nostoc muscorum*, *C*-phycocyanin and *In vitro* culture.

1. Introduction

Malaria remains a major public health problem resulting into an unacceptable toll on the health and economic welfare of the world's poor communities. Over 200–500 million cases and 0.7–2.7 million deaths occur each year due to malaria and making it one of the top three killers among communicable diseases [1]. Among Southeast Asian countries, India alone contributes more than 80% malaria cases and *Plasmodium falciparum* accounts for 35-40% cases [2]. Despite intensive efforts to control malaria, the disease continues to be one of the greatest health problems in 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 [3]. For 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. The development of safe and effective antimalarial agents has been realized as a challenge in recent years because of the rapid spread of drug resistant *P. falciparum* strains. Over the years chloroquine has been used as a safe, effective and readily available anti-malarial drug. The mechanism of chloroquine resistance in *Plasmodium* was first reported about two decades back and presently it is found by many users that chloroquine is no longer effective in most of the endemic areas as because of *P. falciparum* has developed resistance to it [4, 5].

Historically, the majority of antimalarial drugs has been derived from medicinal plants or from structures modelled on plant lead compounds. These include the quinoline-based antimalarials as well as artemisinin and

*Corresponding author:
E-mail: suklabiswas@yahoo.com; Tel.: +91-11-2530 7203; Fax: +91-11-25307177.

its derivatives [6]. Indian subcontinent boasts remarkably for its biodiversity and rich cultural traditions of plant use. Scientific understanding of medicinal plants is, however, largely unexplored and pharmacological investigation of the Indian flora only gained momentum recently [7].

Cyanobacteria are valuable organisms that contribute significantly to the total carbon biomass and primary productivity [8]. In cyanobacteria, the light-harvesting pigments are chlorophyll-a, carotenoids and phycobiliproteins. Phycobiliproteins are a family of hydrophilic, brilliantly coloured and stable fluorescent pigment proteins (Fig. 1) covalently linked with a linear tetrapyrrole prosthetic group (bilins) that, in their functional state, are covalently linked to specific cysteine residues of the proteins [9]. These intensely coloured proteins are occurring in Cyanophyceae, Rhodophyceae and Cryptophyceae. They are subdivided into three main groups according to their structural property, as phycocyanin (blue), allophycocyanin (blue) and phycoerythrin (red). Where phycocyanin and allophycocyanin are always present in Cyanophyceae and Rhodophyceae but phycoerythrin may be absent in the former [10]. The energy transfer occurs from phycocyanin to allophycocyanin to Chlorophyll-a (in the case of Cyanobacteria). The absorption maximum for *C*-phycocyanin is 620nm. Because of unique colour, proteinous nature, fluorescent and antioxidant properties, a wide range of promising applications of phycobiliproteins in diagnostics, biomedical research and therapeutics are possible [11].
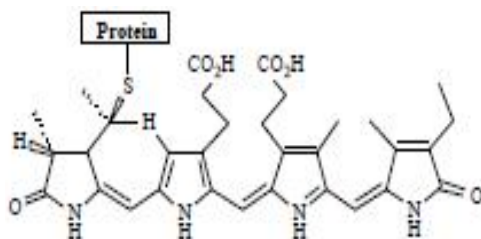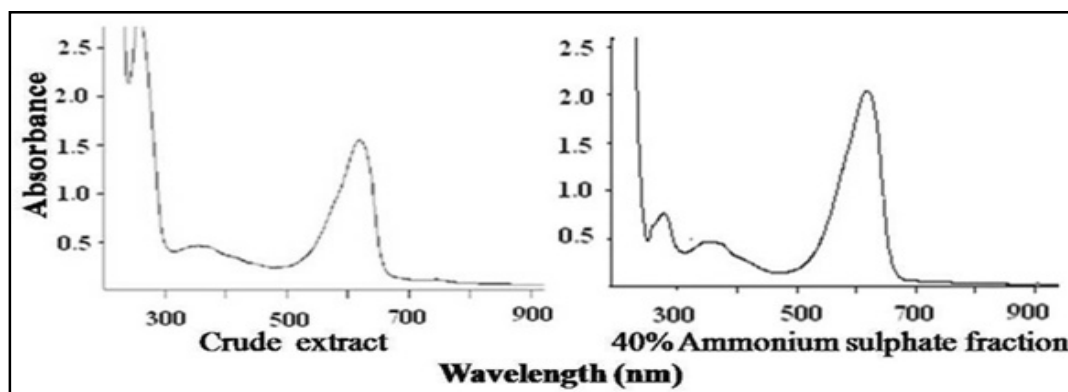

Fig. 1. Chemical structure of *C*-phycocyanin.

In the present study, we have isolated *C*-phycocyanin from nitrogen fixing cyanobacterium, *Nostoc muscorum*. It was purified and characterized. We have made an attempt to study the antimalarial activity of *C*-phycocyanin and its inhibitory effect on *P. falciparum* culture was described. *C*-phycocyanin has not been reported so far for its antiplasmodial activity.

2. Materials and Methods

2.1 Algal material, culture, extraction and purification of *C*-phycocyanin

Nostoc muscorum was cultured in BG-11 medium without any combined nitrogen sources at pH 8.0 [12]. The culture was done in conical flasks and maintained at temperature $25\pm2°C$ with light intensity of $75\mu M\ m^{-2}\ s^{-1}$ PAR at the light: dark cycles of 14:10 h and dry cell weight (DCW) was determined gravimetrically.

The *C*-phycocyanin (*C*-PC) was obtained by acetone extraction. Briefly, cell suspension of *Nostoc muscorum* was centrifuged at 5000 rpm for 10 min. The cell pellet was mixed well with 20ml of 80% acetone and incubated overnight at 4°C. After centrifugation, the pellet was mixed with 20 ml of sterile distilled water and kept at 50°C for 30 min. The suspension was centrifuged and the supernatant was stored as a crude extract of phycobiliproteins.

The extract of crude phycobiliproteins was subjected to ammonium sulphate precipitation at 35%, 40%, 45%, 50%, 60% and 70% saturation. Each precipitated fraction was dissolved in 1ml of 50mM Tris-buffer (pH 7.5) and dialysed. The optical density was measured at 280nm, 620nm and 650nm. The fraction with highest yield as per absorbance 620/280, i.e. 40% ammonium sulphate precipitate was taken for further work after concentrating by Amicon filtration with MW cut-off~30 kDa.

The UV-Visual absorbance spectra (250-820nm) were recorded on UV-Vis Diode-Array spectrophotometer (Shimadzu, Columbia, MD, USA) as shown in Fig. 2. The amount and purity ratio of *C*-PC were calculated using the published equations as described elsewhere [13].

Fig. 2. Absorption spectrum of *C*-phycocyanin crude and after 40% ammonium sulphate fractionation.

2.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified *C*-PC was put on polyacrylamide gel electrophoresis for molecular weight analysis on reducing and non-reducing gel. After electrophoresis, each lane of the gel was put separately for Coomassie brilliant blue and silver nitrate staining. The sizes of the resolved bands were determined by comparing with known molecular weight markers.

2.3 Biological evaluation: Antimalarial activity *in vitro*

Antimalarial activity was checked in six well-adapted *P. falciparum* culture lines, 3 each for chloroquine sensitive (CQ-S) and chloroquine resistant (CQ-R) following the published method [14]. The parasite isolates were collected from different places of India from patients reported with symptomatic malaria. They were adapted and maintained in vitro by candle-jar technique. Parasites were cultured in human O^+ RBCs in RPMI 1640 media enriched with 10% (v/v) AB^+ serum and supplemented with 25mM HEPES buffer and 25mM sodium bicarbonate.

The assay was done in synchronous culture in the ring form at 5% hematocrit containing 1% parasitaemia in 96-well flat bottom tissue culture plate. *C*-PC was dosed at two-fold dilutions in wells in duplicate at concentrations ranging from 192–1.5µg/ml.

The volume of culture in each well was kept 200µl including media, test sample, and parasite inoculum. Parasite culture only in enriched media was taken as controls. Chloroquine was used as reference antimalarial for comparison. To determine the activity of the compound, the assay was done in two sets. The first set was to determine the effect of compounds in schizont maturation after 24 h and the second set of assay was to determine the effect in total parasite growth after 48 h. Growth of the parasite from each well was monitored microscopically in Giemsa stained smears by counting number of schizonts per 200 asexual parasites and total number of parasites per 5000 RBCs. Percent schizont maturation inhibition and parasite growth inhibition were calculated by the formula;

$$(1 - Nt / Nc) \times 100$$

Where, Nt and Nc represent the number of schizont or number of parasites in the test and control wells, respectively. Inhibitory concentrations at 50% (IC_{50}), 90% (IC_{90}) and 95% (IC_{95}) were calculated.

3. Results and Discussion

3.1 Extraction and purification of *C*-phycocyanin

We have isolated crude phycobiliprotein from *Nostoc muscorum* and it was purified and further characterized. The concentration and purity of *C*-PC was increased on fractionation with ammonium sulphate. It was found to be maximum (3.25) in 40% ammonium sulphate fraction (Table 1). This simple step of purification yielded nearly 66% of *C*-PC is giving a final yield of 0.64mg/ml and 106.3mg/dcwg. Two sharp peaks were observed at 280nm and 620nm (Fig. 2). The purity and molecular weight of the sample obtained after 40% ammonium sulphate precipitation was confirmed using non-reducing and reducing PAGE (Fig. 3). Under non-reducing conditions, *C*-PC moved as a tight band of ~124 kDa suggesting it to be monomeric single population. Lane MW showed a molecular marker, while lanes NS and NC showed Silver nitrate and Coomassie brilliant blue staining of *C*-PC on non-reducing conditions. On reducing gel, *C*-PC was resolved as seven polypeptides of molecular masses of ~17, ~19, ~33, ~42, ~47, ~50 and ~72 kDa, respectively (Lane R).
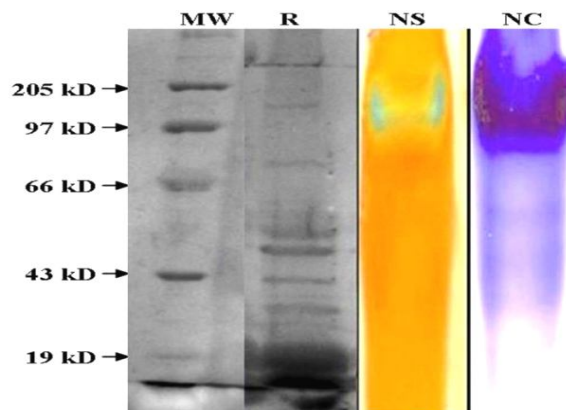

Fig. 3. Polyacrylamide Gel Electrophoresis of *C*-phycocyanin.

Lane MW - molecular weight marker; **Lane R** - reducing gel; **Lane NS** - Native PAGE stained with Silver nitrate, the band of *C*-PC showed greenish colour after staining; **Lane NC** - Native PAGE stained with Coomassie brilliant blue, the band of *C*-PC showed fluorescent bluish-violet colour after staining.

Table 1. Extraction and partial purification of *C*-phycocyanin obtained by acetone extraction and by ammonium sulphate fractionation (Values are mean of three independent observations).

Ammonium sulphate (%)	A_{650}	A_{620}	A_{280}	A_{620}/A_{280}	*C*-PC (mg/ml)	*C*-PC (mg/dcwg)	Recovery (%)
0	0.28	0.91	0.92	0.99	0.97	166.7	-
35	0.14	0.49	0.18	2.65	0.53	89.2	55.15
40	0.15	0.57	0.17	3.25	0.64	106.3	65.75
45	0.12	0.48	0.15	3.07	0.54	91.0	56.29
50	0.14	0.51	0.18	2.85	0.56	93.8	58.04
60	0.17	0.44	0.23	1.96	0.44	73.9	45.70
70	0.11	0.38	0.15	1.48	0.41	69.3	42.88

Several methods of extraction and purification of *C*-PC have been described in published literature. Briefly, *C*-PC was extracted and purified from *Aphanothece halophytica* by using DNase, RNase and proteinase followed by ion exchange and gel permeation chromatography [15]. From crude extracts of *Nostoc* sp. (PCC 9202) *C*-PC was purified by ultrafiltration and ammonium sulphate precipitation followed by gel filtration and ion-exchange chromatography [16]. Another method for the optimum extraction and isolation of *C*-PC was described from the cyanobacterium *Synechococcus* sp. IO9201 (isolated from Caribbean waters) by freezing and thawing of the extract followed by and purification, hydrophobic interaction and ion exchange chromatography [17]. Purification of *C*-PC from *Spirulina* (*Arthrospira fusiformis*) was achieved by a multi-step treatment of the crude extract with rivanol followed by ammonium sulphate precipitation [18]. Extraction and purification of *C*-PC from *Aphanizomenon flos-aquae* was done by ultracentrifugation and hydrophobic chromatography using a hydroxyapatite column [19]. Most recently, extraction and purification of *C*-PC from *Phormidium ceylanicum* has been described by ultrafiltration [20].

Compared to all these procedures, the present method described in this study was easy and gave maximum recovery without using expensive chemicals and equipment. Thus it could be recommended as a simple and cost-effective extraction and purification method for *C*-PC from *Nostoc muscorum*.

3.2 Effect of *C*-PC on *Plasmodium falciparum* growth

The purified *C*-PC was tested in *P. falciparum* culture. Results obtained from the total parasite growth inhibition of both CQ-S and CQ-R strains are summarized in Table 2 and Fig. 4. Parasite count was done microscopically per 5000 RBCs after incubation of 24 h and 48 h with *C*-PC. Parasite count, multiplication rate, and parasite growth inhibition were calculated as described earlier [21]. Parasite growth was inhibited completely above the concentration of 48μg/ml of *C*-PC. Figures 5-7 represent microscopical observation on the effect of *C*-PC in culture of *P. falciparum* at different time interval. Inhibitory concentrations of 50%, 90% and 95% were calculated in both CQ-S and CQ-R parasites by noting the parasite growth inhibition after 48 h (Table 3).
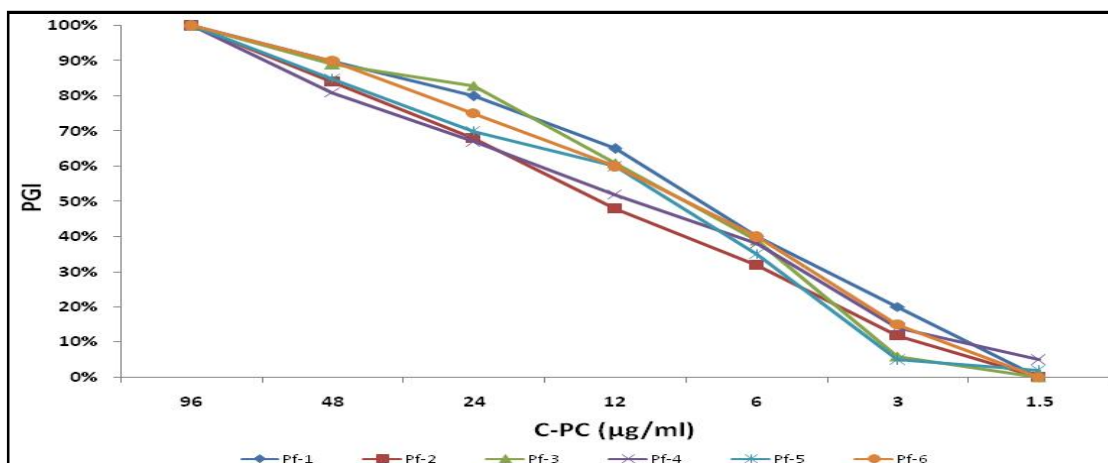

Fig. 4. Growth inhibition of six *P. falciparum* isolates in the presence of *C*-phycocyanin after 48 hours. *C*-PC was dosed at two-fold dilutions in wells in duplicate at concentrations ranging from 192 – 1.5μg per well. Total parasite growth inhibition (PGI) of both chloroquine sensitive (Pf-1,2,3) and resistant (Pf-4,5,6) strains is shown. Parasite count was done microscopically and parasitaemia was calculated as the number of parasites per 5000 RBCs after incubation of 48 h with *C*-PC. Parasite growth inhibition (%) was calculated.

Table 2. Effect of *C*-Phycocyanin in both chloroquine sensitive and chloroquine resistant *Plasmodium falciparum* culture lines. Parasite multiplication rate after 48 hours with or without *C*-phycocyanin (Values are mean of three independent observations).

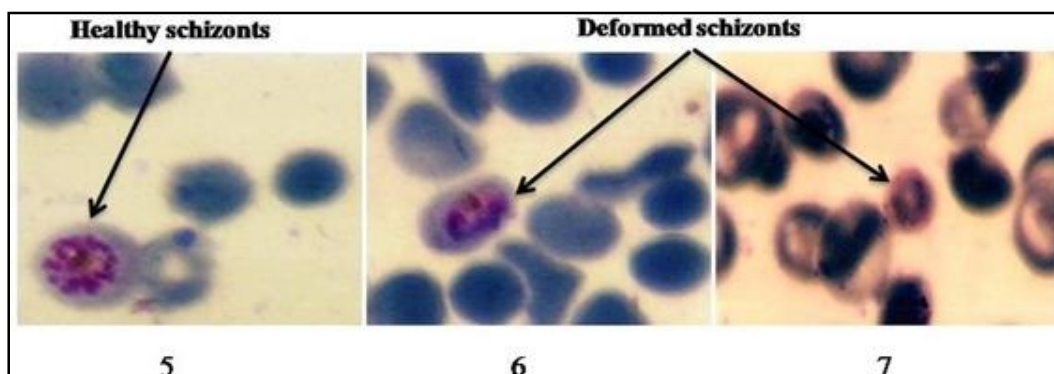|							
Parasite Count (PC) per 5000 RBCs							
(Multiplication Rate = No. of parasites at 48 h / No. of parasites at 0 h)							
Rows	*C*-PC (μg/ml)	Pf-1	Pf-2	Pf-3	Pf-4	Pf-5	Pf-6
A	96	0	0	0	0	0	0
B	48	0.25	0.5	0.4	0.57	0.5	0.4
C	24	0.5	1	0.6	1	1	1
D	12	0.87	1.63	1.4	1.43	1.33	1.6
E	6	1.5	2.13	2.2	1.86	2.17	2.4
F	3	2	2.75	3.4	2.57	3.17	3.4
G	1.5	2.63	3.2	3.68	2.86	3.27	4
H (Control)	Without *C*-PC	2.5	3.12	3.6	3	3.33	4
No. of parasites at '0' hr.	-	40	40	25	35	30	25

Fig. 5. Microscopic observations of *C*-PC addition to the *in vitro* culture of *Plasmodium falciparum*. 5. Culture after 24 h without *C*-PC is showing healthy schizont; Fig. 6. Culture after 24 h with *C*-PC is showing deformed schizont; Fig. 7. Culture after 48 h with *C*-PC, no further growth has observed.

Table 3. Inhibitory concentrations 50%, 90% and 95% in *P. falciparum* growth (Values are mean of three independent observations).

CQ sensitivity & parasite ID		*C*-PC in µg/ml		
		IC_{50}	IC_{90}	IC_{95}
CQ-S	Pf-1	8.4	47.9	66.1
	Pf-2	13.5	60.3	79.4
	Pf-3	8.9	52.5	75.9
	Pf-4	12.0	66.1	79.4
CQ-R	Pf-5	9.6	60.3	75.9
	Pf-6	9.6	44.7	63.1

The Inhibitory Concentration (IC) is commonly used as a measure of antagonist drug potency in pharmacological research. The IC_{50}, IC_{90}, and IC_{95} of *C*-PC were determined constructing a dose-response curve. The IC_{50} is a measure of the effectiveness of *C*-PC in inhibiting malaria parasite growth by half.

The problems of drug resistance in *P. falciparum* are escalating. New drugs of herbal origin discovered through ethnopharmacological studies have shown interesting results. The present screening of *C*-PC for *in vitro* antiplasmodial activity against *P. falciparum* was performed with an aim to investigate the activity of the compound in order to discover new lead structures or improve the traditional medicine and to understand the biomedical importance of algal pigments. 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 kDa, water soluble protein molecule.

We may postulate that the possible mechanism might be the destruction of polymerization of haemozoin by binding with ferriprotoporphyrin-IX (FP-IX). As *C*-PC is a large protein molecule, so it is unable to diffuse directly through the lipid bilayer 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 [22, 23].

Due to its nutritive and medicinal properties including hepatoprotective property and negligible toxicity, *C*-PC could act as an oral supplement. Thus, it may be concluded that *C*-PC could be used as a safe, potent and as an oral supplement along with known recommended antimalarial drugs for both CQ-S and CQ-R parasites and seem to be crucial in realizing the importance and use of algal pigment in the field of biomedical sciences.

Acknowledgments

References

[1]. World Health Organization (2003). WHO informal consultation on recent advances in diagnostic techniques and vaccines for malaria. Bull. WHO, 74: 47–54.

[2]. Sharma, V.P. (2000). Status of drug resistance in malaria in India. In Multi drug resistance in emerging and re-emerging diseases. Ed. R.C. Mahajan, p. 191-202. Indian National Science Academy, New Delhi, India.

[3]. Ridley, R.G. (2002). Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature*, 415: 686–693.

[4]. Krogstad, D.J., Gluzman, I.Y., Kyle, D.E., Oduola, A.M., Martin, S.K., Milhous, W.K. & Schlesinger, P.H. (1987). Efflux of Chloroquine from *Plasmodium falciparum*: mechanism of Chloroquine resistance. *Science*, 238: 1283-1285.

[5]. White, N.J., Nontprasert, A., Nosten, B., Pukrittayakame, S. & Vanijanonta, B. (1998).

Assessment of the neurotoxicity of Parenteral Artemisinin derivatives in mice. *Am. J. Trop. Med. Hyg.*, 59: 519-522.

[6]. Chen, M.C., Mwasumbi, L.B. & Heinrich M. (1994). Screening of Tanzanian medicinal plants for antimalarial activity. *J. Parasitic Disease*, 56: 65-77.

[7]. Simonsen, H.T., Nordskjold, J.B., Smitt, U.W., Nyman, U., Palpu, P., Joshi, P. & Varughese, G. (2001). *In vitro* screening of Indian medicinal plants for antiplasmodial activity. *J. Ethnopharmacol.*, 74: 195–204.

[8]. Viskari, P.J. & Colyer, C.L. (2003). Rapid extraction of phycobiliproteins from cultured cyanobacteria samples. *Anal. Biochem.*, 319: 263–271.

[9]. Santiago-Santos, M., Ponce-Noyola, T., Olvera-Ramý´rez, R., Ortega-Lo´pez, J. & Can~izares-Villanueva R.O. (2004). Extraction and purification of phycocyanin from *Calothrix* sp. *Process Biochemistry*, 39: 2047–2052.

[10]. Moreno, J., Rodriguez, H., Vargas M.A., Rivas, J. & Guerrero, M.G. (1995). Nitrogen-fixing cyanobacteria as source of phycobiliprotein pigments. Composition and growth performance of ten filamentous heterocystous strains. *J. Appl. Phycol.*, 7: 17-23.

[11]. Bermejo, R., Acie´n, F.G., Iba´n~ez, M.J., Ferna´ndez, J.M., Molina, E. & Alvarez-Pez, J.M. (2003). Preparative purification of B-phycoerythrin from the microalga *Porphyridium cruentum* by expanded-bed adsorption chromatography. *J. Chromatogr.*, 790: 317–325.

[12]. Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. & Stanier, R.Y. (1971). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.*, 85: 39-43.

[13]. Ravindra, A., Mishra, S., Pawar, R. & Ghosh, P. (2005). Purification and characterization of *C*-phycocyanin from cyanobacterial species of marine and freshwater habitat. *Protein Expr. Purif.*, 40: 248-255.

[14]. Biswas, S. (2003). 8-Hydroxyquinoline inhibits the multiplication of *Plasmodium falciparum in vitro*. *Annals Trop. Med. Parasitol.*, 97: 527-530.

[15]. Hilditch, C.M., Balding, P., Kakins, R., Smith, A.J. & Rogers, L.J. (1991). *C*-phycocyanin from the cyanobacterium *Aphanothece halophytica*. *J. App. Phycol.*, 3:345-349.

[16]. Reis, A., Mendes, A., Lobo-Fernandes, H., Empis, J.A. & Novais, J.M. (1998). Production, extraction and purification of phycobiliproteins from *Nostoc* sp. *Bioresource Technol.*, 66: 181–187.

[17]. Abalde, J., Betancourt, L., Torres, E. & Cid, A. (1998). Purification and characterization of phycocyanin from the marine cyanobacterium *Synechococcus* Sp. IO9201. *Plant Sci.*, 136: 109-120.

[18]. Minkova, K.M., Tchernov, A.A., Tchorbadjieva M.I., Fournadjieva, S.T., Antova, R.E. & Busheva, M.C. (2003). Purification of *C*-phycocyanin from a *Spirulina (Arthrospira) fusiformis*. *J. Biotechnol.*, 102: 55-59.

[19]. Benedetti, S., Benvenutia, F., Pagliarania, S., Francoglia, S., Scogliob, S. & Canestraria, F. (2006). Antioxidant properties of a novel phycocyanin extract from the blue-green alga *Aphanizomenon flos-aquae*. *Life Sci.*, 75: 2353-2362.

[20]. Singh, N.K., Parmar, V. & Madamwar, D. (2009). Optimization of medium components for increased production of *C*-phycocyanin from *Phormidium ceylanicum* and its purification by single step process. *Bioresource Technol.*, 100: 1663-1669.

[21]. Biswas, S. (2001). *In-vitro* antimalarial activity of azithromycin against chloroquine sensitive and chloroquine resistant *Plasmodium falciparum*. *J. Postgrad. Med.*, 47: 240-243.

[22]. Raynes, K. (1999). Bisquinoline antimalarials: their role in malaria therapy. *International J. Parasitol.*, 29: 367-379.

[23]. Egan, C.P. (2003). *In vitro* antiplasmodial activity of abietane and totarane diterpenes isolated from *Harpagophytum procumbens* (Devil's claw). *Planta Medica*, 8: 720-724.