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RESEARCH ARTICLE

Phycocyanin Extracted from *Oscillatoria minima* Shows Antimicrobial, Algicidal, and Antiradical Activities: *In silico* and *In vitro* Analysis

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Abstract: ***Background*:** Phycocyanin is an algae-derived protein, which binds to pigment for harvesting light. It has been reported in various different species, including that of red algae, dinoflagellates, and cryptophyta. Importantly, phycocyanin has enormous applications, including cosmetic colorant, food additive, biotechnology, diagnostics, fluorescence detection probe, an anticancer agent, anti-inflammatory, immune enhancer, *etc*. In addition, several different algae were utilized for the isolation of cyano-phycocyanin (C-PC), but most of the purification methods consist of several steps of crude extraction.

***Aim*:** To isolate C-PC from a new source of microalgae with better purity level and to evaluate its antimicrobial, algicidal, and antiradical activities.

***Methods*:** Biological activity, permeability, pharmacokinetics, and toxicity profile of C-PC were predicted by *in silico* studies. C-PC was purified and isolated by using ammonium sulphate precipitation, ion-exchange chromatography and gel-filtration chromatography. C-PC was characterized by SDS-PAGE and elution profile (purity ratio) analysis. Antimicrobial and algicial activities of C-PC were evaluated by the microtitre plate based assays. Antiradical activity of C-PC was evaluated by DPPH- and $ABTS^{*+}$ radical scavenging assays.

***Conclusion*:** C-PC was extracted from *Oscillatoria minima* for the first time, followed by its quantitative as well qualitative evaluation, indicating a new alternative source of this important protein. Furthermore, the antimicrobial, algicidal, and antiradical activities of the isolated C-PC extract have been demonstrated by both *in silico* as well as *in vitro* methods.

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1. INTRODUCTION

Phycocyanin is an algae-derived protein, which binds to pigment for harvesting light. The two major classes of this protein encompass cyanophycocyanin (C-PC) and recombinant phycocyanin, which are derived from blue-green algae and red algae, respectively [1, 2]. Phycocyanin is also characterized by its spectral property of λ_{max} at 620 nm. These are comprised of two polypeptides, namely α unit (MW: 12-19 kD) and β unit (MW: 14-21 kD) in equimolar quantity [3]. Its isoelectric point lies in the range of 4.1-6.4, subject to the methodology and extraction source utilized for purification [4, 5]. It should be purified at around 4°C [6], owing to its sensitiveness towards heat [7, 8]. However, C-PC is found in various different species, including that of red algae [9], dinoflagellates [10], and Cryptophyta [11]. Importantly, C-PC has enormous applications, including cosmetic colorant, food additive, fluorescence detection probe, anticancer agent, anti-inflammatory, immune enhancer, *etc*. [1]. In the context of cancer, C-PC has been shown to induce apoptosis, which led to the enhancement of topotecan effect on prostate cancer cells [12]. It is noteworthy, prostate cancer claims to be the second most cause for cancer-related mortality in men [13]. Its photodynamic effect has been shown again breast cancer cells *in vitro* [14]. It has also been used as a sensitive and selective biosensor for Hg^{2+} in the aquatic environment [15]. Recently, it has also been used in nanoformulation for effective delivery of paclitaxel in human glioma cells [16]. C-PC, being structurally similar to biliverdin, mammalian cells convert it into phycocyanorubin by enzyme biliverdin reductase. Phycocyanorubin has high similarity to one of the inhibitor (bilirubin) of crucial enzyme NAPDH oxidase, which generates ROS [17]. In addition to this, C-PC also prevents the accumulation of hypochlorous acid (HOCl) generated by myeloperoxidases. HOCl plays a critical role in inflammatory pathways [18]. Therefore, C-PC has also been used extensively in the neuroscience research, for example, it has been shown to activate oxidized astroglia cells to safeguard and repair the ischemic brain owing to its collective antioxidative, neurotrophic, and anti-inflammatory activities [19]. Nonetheless, mounting evidence also indicate the importance of Cyano compounds as antifungal and antibacterial activity might be due to membrane accumulation mechanism and lesser degradation potential by microbes [20, 21].

Until now, several different species of algae have been utilized to isolate cynophycocyanin as listed in Table **1** [22]. Most of the purification methods comprise several steps of crude extraction [23, 24]. In addition, the purity as well as recovery is comparatively small, which demands a more efficient alternative for the isolation of C-PC from a new source of microalgae with better purity level.

Table 1. Resolution of phycocyanin isolated from various sources.

Source	Resolution
Pseudanabaena sp. Lw0831	2.04
Thermosynechococcus elongatus	1.75, 2.5
Leptolyngbya sp. N62DM	2.61
Thermosynechococcus vulcanus	1.35-2.7
Synechocystis sp. PCC 6803	2.61
Synechococcus elongatus	1.45-2.2
Gracilaria chilensis	2.01
Arthrospira platensis	2.2
Cyanidium caldarium	1.65
Microchaete diplosiphon	1.66

In the present work, first, the computer-aided prediction models were utilized to study the biological activities, pharmacokinetics, toxicity, gastrointestinal permeability, and brain permeability of C-PC. Furthermore, the C-PC was isolated from the *Oscillatoria minima* for the first time, by using ion exchange chromatography and gel filtration chromatography, followed by their quantitative estimation. It was also evaluated qualitatively by running on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the antimicrobial activity of the isolated C-PC was examined against various bacterial species *viz*. *Pseudomonas fragi, Entherobacter erogens, Escthercia coli, Pseudomonas vulgarius, Bacillus subtilis, Staphlococcus aereus, Klebsilla oxytoca,* and *Streptococcus pyogen*e by using microtiter wells method. Besides, the algicidal activity of the isolated C-PC was evaluated against various species

of microalgae *viz*. *Nostoc*, *Gleocapsia*, *Spirulina*, and *Synechocystis*. Eventually, the anti-oxidant activity of the isolated C-PC was also evaluated by using diphenylpicrylhydrazyl (DPPH) radical scavenging assay and 2, 2'-azino -bis93-ethyl benzothiazoline-6-Sulfonic acid diammonium salt (ABTS) radical scavenging assay. The present study would present *Oscillatoria minima* as a new source of C-PC, with its demonstrated antimicrobial, algicidal, and antiradical properties.

2. MATERIALS AND METHODS

2.1. Simplified Molecular-Input Line-Entry System Notation of C-PC

The simplified molecular-input line-entry system (SMILES) for C-PC is CCCC1=C(NC(\C=C2\NC(=O)C(C)\C\2=C/C)=C1C)\C=C1\N=C(\C=C2\NC(=O)C(CC)=C2C)C(C)=C1CCC.

2.2. PASS Analysis

To obtain the predicted biological activity profile for C-PC, its structural formula was utilized for prediction of activity spectra for substances (PASS). Pa (probability "to be active") and Pi (probability "to be inactive") parameters were used to predict the most potent set of biological activity based on computer-aided algorithm [25].

2.3. Prediction of Gastrointestinal Absorption and Brain Penetration of C-PC by a Boiled-Egg Analysis

The gastrointestinal (GI) absorption and brain access at various stages of the drug discovery processes was predicted for C-PC by using the Brain Or IntestinaL EstimateD permeation method (BOILED-Egg) as described previously [26].

2.4. Prediction of Pharmacokinetics-, Toxicity-, Druglikeness-, and Medicinal Chemistry Profiles of C-PC by SwisADME

The pharmacokinetics and toxicity profiles of C-PC were predicted by using SwissADME as described previously [26].

3. EXPERIMENTAL

3.1. Organism and Culture Condition

The culture of *Oscillatoria minima* was maintained in standard laboratory conditions and was sub-cultured and grown in BG-11 media (Table **S1** and **S2**), which was used for culture of fresh water, soil, thermal, and marine cyanobacteria. They were incubated at 20 ± 2°C, providing 12/12 light and dark conditions with uninterrupted agitation and aeration (Fig. **S1**).

3.2. Extraction of Phycobiliproteins

C-PC was extracted from *Oscillatoria minima* as per previously published method with a slight modification [23]. In brief, fresh cyanobacterial cells were harvested. Then, it is centrifuged at 10000×g for 30 min at 4°C. It was then washed twice with distilled water and dried in vacuum. The mass was resuspended in 0.1(M) sodium phosphate buffer (pH 7.0) and subjected to the sonication for 60 s to disrupt the cell mass. Then, it was subjected to repeated freezing at -20°C and thawing at room temperature. Cell mass was centrifuged at 10,000×g for 30min at 4°C resulting into a clear supernatant.

3.3. Estimation of Phycobiliproteins

The absorbance of phycobiliproteins containing supernatant was measured on UV-visible spectrophotometer (Shimadzu-1800) at wavelengths of 620, 652, and 562 nm for calculating the concentrations of C-PC, allophycocyanin (AlloPC), and phycoerythrin (PE), respectively by using the following Eqs. (1-3) [27].

$$C\text{-}PC = - [A620-0.474(A652)]/ 5.34 \quad \text{(Eq. 1)}$$

$$AlloPC = -[A652-0.208(A620)]/5.09 \quad \text{(Eq. 2)}$$

$$PE = [A562-2.41(C\text{-}PC)-0.849(alloPC)]/9.62 \quad \text{(Eq. 3)}$$

3.4. Purification of C-PC by Ammonium Sulfate Precipitation

The extractions of C-PC were carried out in dark at 10-15°C as per previously published paper (Patel *et al.* 2005) [23]. The crude extracts were treated with a series of saturation steps with varying amount of solid ammonium sulfate to purify C-PC. Briefly, initial saturation was carried out by 25% ammonium sulfate and a precipitate was discarded. Following 50% ammonium sulfate saturation, the solution was allowed to stand for 4h at 4°C and then spun at 10,000×g for 30 mins at 4°C. The precipitated mixture containing mainly C-PC

was resuspended in 0.1M acetate buffer (pH-4.5) and spun again at 10,000g for 30mins at 4°C. The supernatant from this step was discarded and saturated again with 50% ammonium sulfate and allowed to stand for 4h at 4°C prior to spin at 10,000×g for 30 mins at 4°C. The resultant C-PC precipitate was finally dissolved in 5 ml of 5mM Na-phosphate buffer (pH-7). The partial purification of C-PC was done by dialyzing the obtained C-PC overnight at 4°C against the Na-phosphate buffer with same concentration in which it was dissolved in an earlier step.

3.5. Ion Exchange Chromatography

The complete purification of C-PC was carried out on DEAE-Cellulose anion exchange column [28]. Anion exchange column (1.5X10cms) was prepared and pre-equilibrated with 5mM Na-phosphate buffer (pH-7). Dialyzed filtered C-PC was employed on the pre-equilibrated column and developed with 5mM Na-phosphate buffer (pH-7) with flow rate 1ml/min. The absorption spectrum was also determined by scanning the eluted fractions through A280 to A800nm range to check the purity of each fraction. The elution profile of each C-PC fractions collected from the DEAE-Cellulose column was presented and the fractions, which displayed the purity ratio (A620/A280) >4 were contemplated as high purity of C-PC. All such fractions were pooled together and brought to 50% saturation with ammonium sulfate and precipitated. The final C-PC precipitate was further dissolved in a small volume of 5mM Na-phosphate buffer (pH-7) and dialyzed against water at 4°C.

3.6. Gel Filtration Chromatography

Gel filtration column Sephadex G-100 (25X 1cm) was utilized to find the molecular weight of native phycocyanin protein as described previously [23]. The column was pre-equilibrated with phosphate buffer (7.0). The column was calibrated using Blue- Dextran (2,000,000 Da), hemoglobin (64500Da) and with vitamin B12 (376Da). The flow rate was maintained at 300 µl/minute.

3.7. SDS-PAGE

The isolated protein was separated based on their size by using SDS-PAGE. It was carried out by using 10% polyacrylamide gel slab gel containing 0.1% SDS with a stacking gel of 5% acrylamide and 0.1% bisacrylamide as mentioned in Table **S3**. Samples were preincubated with gel loading buffer for about 5-10 min at 95°C. Gels were run at room temperature and stained with a solution of Coomassie brilliant blue R250, for visualization and imaging of bands.

3.8. Antimicrobial Assay

The antimicrobial activity of purified C-PC was examined by using microtiter wells method (Nunc-Immuno ^{TM} 96 Microwell ^{TM} Plate covered with a Nunc^{TM} standard lid) as per previously published method [29]. First, 1.06 mg/ml of sample was taken and it was serially diluted six times with deionized water to get a final concentration of 16.0 µg/ml. Aliquots of each of the solution were serially diluted by half with deionized water to obtain six 1ml aliquots with decreasing concentration. Then 20µl of purified phycocyanin sample was tested against the same volume of test bacterial organisms. The 20µl of nutrient broth and 20µl of test organisms was taken as positive controls. These plates were incubated at 37°C for 24 hours. Source of bacterial cultures was collected from Microbial Type Culture Collection (MTCC) as shown in Table **S4**.

3.9. Algicidal Assay

The algicidal activity of C-PC was examined by using microtiter well (Nunc-Immuno ^{TM} 96 Micro well ^{TM} Plate covered with a Nunc^{TM} standard lid) as described previously [27]. In brief, 1.06mg/ml of sample was taken and it was serially diluted 6 times with deionized water to obtain a final concentration of 16.0 µg/ml. Aliquots of each of the solution were serially diluted by half with deionized water to obtain six 1ml aliquots with decreasing concentration. 30µl of purified C-PC has been tested against the 100µl of test algal organisms. 30µl of deionised water and 100µl of test organisms were taken as positive controls. This plate was incubated at 21-22°C. Inhibition in the form of bleaching or decolourization of blue-green microalgae was observed. The code number of various microalgae is shown in Table **S5**.

3.10. Antiradical Activity

The antiradical activity was determined by using DPPH radical scavenging assay and ABTS

radical scavenging assay, as described previously [30].

3.11. DPPH$^{\cdot}$ Radical Scavenging Assay

This assay is widely used as it is fast and simple in terms of measuring the antioxidant activity which relies on the reduction of DPPH radical to yellow colored diphenyl picrylhydrazine in the presence of a hydrogen donor [31]. The DPPH radical scavenging activity was measured by spectrophotometric method. 100 µl of 10mg /ml sample and 700 µl of DPPH solution (200 µM) were incubated for 20 min. at room temperature. 100µl of methanol and 700 µl of DPPH were taken as control. Spectrophotometric absorbance was taken at 517nm. The percentage of radical scavenging activity was measured by the following Eq. (4).

% Radical Scavenging Activity =

[(Abs. of control - Abs. of test)/ Abs. of control]×100 (Eq. 4)

3.12. ABTS^{*+} Radical Scavenging Assay

ABTS^{*+} assay is an excellent tool to determine the antioxidant activity of hydrogen donating antioxidants. ABTS was dissolved in water to a 7mM concentration. ABTS^{*+} was produced by reacting ABTS stock solution with 2.45mM potassium persulfate and allowing the mixture to stand in the dark room temperature for 12-16 hours before use. Prior to the assay, the solution was diluted in ethanol (about 1:89 v/v) and equilibrated to 30°C to get an absorbance at 734 nm of 0.70 ± 0.02 in a 1cm cuvette [32]. The ABTS radical scavenging activity was measured by spectrophotometric method. 100µl of water and 700µl of ABTs were taken as control. 100µl of sample and 700µl of ABTs were taken as test samples. Both samples were incubated for 20 minutes at room temperature. Then absorbance was taken at 734 nm. The percentage of radical scavenging activity was measured by the following Eq. (5).

% Radical Scavenging Activity =

[(Abs. of control - Abs. of test)/ Abs. of control]×100 (Eq. 5)

3.13. Statistical Analysis

All the experiments were conducted in triplicates. Data are presented as mean ± standard deviation. *P<0.5, **P<0.01 were considered statistically significant.

4. RESULTS

4.1. *In silico* Studies of C-PC Revealed its Potential Biological Activities, Permeability, Pharmacokinetics, Toxicity, Druglikeness, Medicinal Chemistry

4.1.1. PASS Analysis Predicted Potential Pharmacological Activities of C-PC

In order to predict the potential biological activities, including some pharmacotherapeutic effects, biochemical mechanisms, metabolism, gene regulation expression, transporter-related activities of C-PC isolated from *Oscillatoria minima*, a computer-mediated tool, and PASS analysis was utilized [25]. It was found that the PASS analysis of C-PC based on its SMILES showed a total of 124 activities. Furthermore, the actives were categorized based on the Pa and Pi parameters. Importantly, the potential antimicrobial, algicidal, and antiradical activities were predicted among all the various biological activities (Table **2**).

4.1.2. Prediction of GI-and Brain Permeability of C-PC

Intestinal absorption and brain permeation establish key parameters for any drugs for its pharmacokinetics and bioavailability at their target site of action. Therefore, in order to predict the GI absorption and brain access for C-PC, the BOILED-Egg analysis was employed as described previously [26]. The white region is the physicochemical space of molecules with the highest probability of being absorbed by the gastrointestinal tract, and the yellow region (yolk) is the physicochemical space of molecules with the highest probability to permeate to the brain. Yolk and white areas are not mutually exclusive. Based on the analysis, it was found that C-PC could be among well-absorbed molecules (Fig. **1A**).

4.1.3. Prediction of Pharmacokinetics, Toxicity, Druglikeness, and Medicinal Chemistry of C-PC by SwissADME

The various properties of C-PC, including physicochemical properties, lipophilicity, water solubility, pharmacokinetics, druglikeness, and medicinal chemistry were studied by SwissADME tool, as

Table 2. Predicted pharmacological activities of C-PC by PASS analysis.

Pa	Pi	Activity
0.602	0.085	Antieczematic
0.295	0.104	Antiprotozoal (Leishmania)
0.137	0.050	Beta lactamase inhibitor
0.161	0.089	Anti-oxidant
0.177	0.125	RNA directed DNA polymerase inhibitor
0.177	0.134	Antiprotozoal
0.180	0.137	Antibacterial
0.023	0.006	Antibiotic beta Lactam-like
0.089	0.074	Antibiotic Glycopeptide-like

Pa: probability "to be active".

Pi: probability "to be inactive".

Table 3. Pharmacokinetics-, toxicity-, druglikeness-, and medicinal chemistry profile of C-PC.

Physicochemical Properties		**Pharmacokinetics**	
Formula	**$C_{33}H_{42}N_4O_2$**	**GI Absorption**	**High**
MW	526.71	BBB permeant	No
#Heavy atoms	39	Pgp substrate	Yes
#Aromatic heavy atoms	5	CYP1A2 inhibitor	No
Fraction Csp3	0.42	CYP2C19 inhibitor	Yes
#Rotatable bonds	8	CYP2C9 inhibitor	Yes
#H-bond acceptors	3	CYP2D6 inhibitor	No
#H-bond donors	3	CYP3A4 inhibitor	No
MR	174.07	log Kp (cm/s)	-5.93
TPSA	86.35		
Lipophilicity		**Druglikeness**	
iLOGP	4.24	Lipinski #violations	1
XLOGP3	5.04	Ghose #violations	4
WLOGP	5.62	Veber #violations	0
MLOGP	3.12	Egan #violations	0
Silicos-IT Log P	9.41	Muegge #violations	1
Consensus Log P	5.49	Bioavailability Score	0.55
Water solubility		**Medicinal Chemistry**	
ESOL Log S	-5.85	PAINS #alerts	0
ESOL Solubility (mg/ml)	7.48E-04	Brenk #alerts	0

(Table 3) contd…

Physicochemical Properties		Pharmacokinetics	
Formula	**$C_{33}H_{42}N_4O_2$**	**GI Absorption**	**High**
ESOL Solubility (mol/l)	1.42E-06	Leadlikeness #violations	3
ESOL Class	Moderately soluble	Synthetic Accessibility	6.07
Ali Log S	-6.59	-	-
Ali Solubility (mg/ml)	1.34E-04	-	-
Ali Solubility (mol/l)	2.54E-07	-	-
Ali Class	Poorly soluble	-	-
Silicos-IT LogSw	-9.59	-	-
Silicos-IT Solubility (mg/ml)	1.35E-07	-	-
Silicos-IT Solubility (mol/l)	2.56E-10	-	-
Silicos-IT class	Poorly soluble	-	-

Table 4. Concentration of various phycobiliproteins isolated from *Oscillatoria minima.*

Phycobiliprotein	% (w/w)
C-PC	60.345
Allophycocyanin	24.580
Phycoerythrin	25.665

shown in Table **3**. The SwissADME profile of C-PC is shown in Fig. (**1B**). The chemical structure of C-PC (Fig. **1C**), allophycocyanin, and phycoerythrin (Fig. **S2A**, **B**) depicted the structural differences. The source of C-PC was confirmed by microscopic image and mass culture as shown in Fig. (**1D**, **E**).

4.2. Quantitative Evaluation of Phycocyanin Isolated from *Oscillatoria minima*

It has been reported that phycobiliprotein possesses various pharmaceutical as well as biological properties, but their application is hindered by some keys factors, such as impedance due to the upstream and downstream processes, including the selection of strains of microalgae, bioreactor design, and condition of culture. In addition, the yield of purified phycobiliproteins from microalgae is small [33]. Therefore, it is quite important to purify the phycobiliproteins in possible enough amount. The isolated phycobiliproteins (C-PC, allophycocyanin, phycoerythrin) from *Oscillatoria minima* were first quantified. It was found that *Oscillatoria minima* contain a maximum amount of C-PC as compared to other phycobiliproteins, namely allophycocyanin, and phycoerythrin (Table **4**).

The crude extract is treated with ammonium sulphate by fractional precipitation and ion-exchange chromatography by using a DEAE cellulose column, which showed a better purity ratio (A620/A280). The saturation up to 25% involved in precipitating other proteins and C-PC remains soluble inthe fractional precipitation process with ammonium sulphate. The C-PC fractions are dissolved in acetate buffer to precipitate basic linker proteins. This shows the purity ratio and separation factor of a considerable level (Table **5**). Purification of C-PC was further carried out by ion-exchange chromatography using DEAE cellulose column. The elution profile further confirmed the C-PC fractions of *Oscillatoria minima* as shown in Table **5**. The samples of fractions collected through DEAE cellulose was evaluated for its purity through the purity ratio (A620/A280). The purity of C-PC fractions is considered high if the ratio > 4. In this case, the purity ratio of C-PC purified from *O. minima* was 8.91 (Table **5**).

4.3. Qualitative Evaluation of C-PC Isolated from *Oscillatoria minima*

As an additional confirmation, the cynophycocyanin was further purified using ion exchange chromatography by diethylaminoethyl cellulose (DEAE Cellulose). The elution profile of C-PC

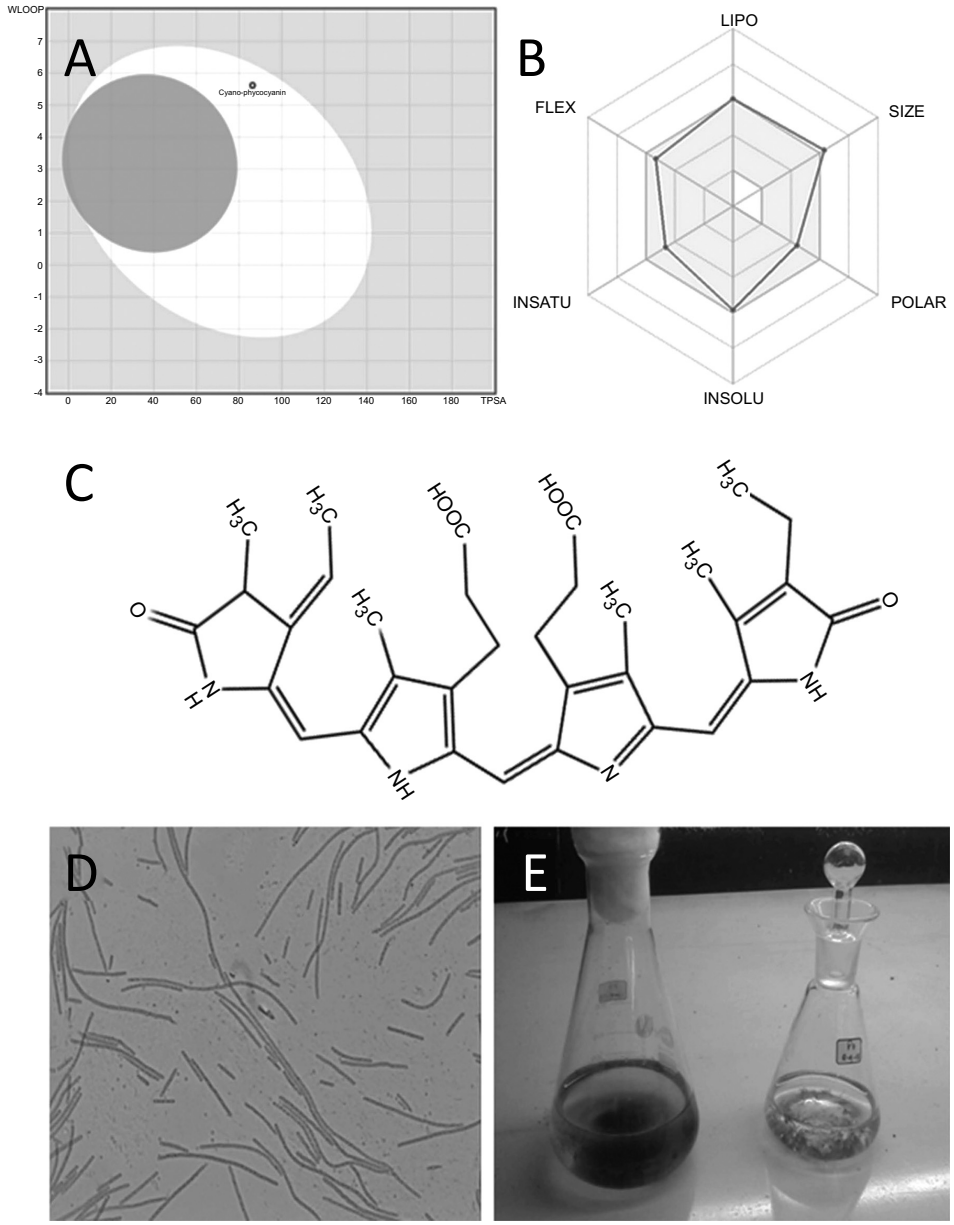

Fig. (1). (**A**) SMILES notation were subject to lipophilicity (WLOGP) and polarity (tPSA) computation. (**B**) SwissADME profile of C-PC. (**C**) Chemical structure of phycocyanin drawn by ChemBio Draw Ultra 14.0 software. (**D**) The morphology of *Oscillatoria minima* as observed under 40X phase contrast microscope. (**E**) Mass culture of *Oscillatoria minima.*

Table 5. Characterization of different parts at various steps of isolation of C-PC from *Oscillatoria minima*.

Purification Steps	*Zero scillatoria minima*	
	Purity Ratio (A620/A280)	**Separation Factor (A620/A652)**
Crude extract	0.536749	1.048533
Fractional precipitation with $(NH_4)_2S0_4$ [25% saturation]	0.97861	1.49921
DEAE cellulose	8.9166	4.458

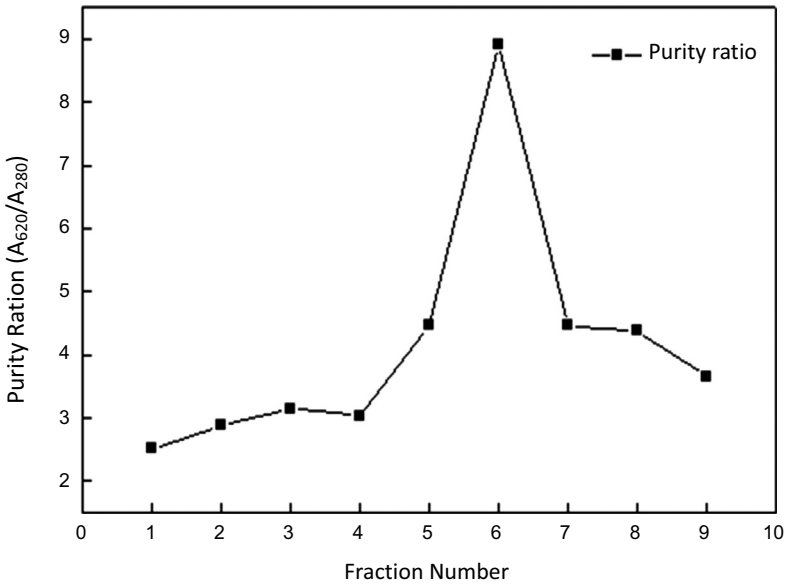

Fig. (2). The plot between purity ratios (A620/280) *vs*. fraction number depicting the elution profile of C-PC shows the maximum purity ratio around fraction number 6.

fractions after DEAE- cellulose column is as shown in Fig. (**2**).

In order to further characterize the isolated C-PC, several other parameters were established, including molecular weight. Therefore, by using gel filtration chromatography on Sephadex G-100 column, the molecular weight of native purified C-PC was found to be 64.5 kDa. Furthermore, the SDS-PAGE analysis of C-PC yielded two bands corresponding to α- and β- subunits (Fig. **3**). The predicted molecular weight of α- subunits was found to be around 14.0 kDa, whereas that of β- subunit was found to be ranging between 29-43 kDa, as detected by Gel doc (BIO-RAD) system. Lane A represents the bands corresponding to the molecular weight of protein marker, and lane B represents the loaded protein of C-PC isolated from *Oscillatoria minima* (Fig. **3**).

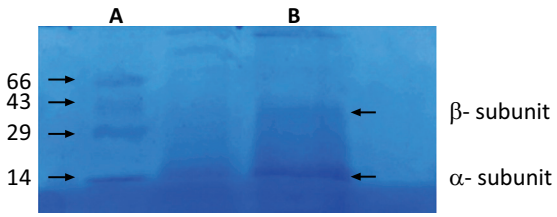

Fig. (3). Coomassie staining of C-PC protein separated on SDS-PAGE. Lane A represents the protein marker, were as lane B represents the loaded C-PC protein isolated from *Oscillatoria minima.*

4.4. C-PC Extracted from *Oscillatoria minima* Showed Antimicrobial Activity

The antimicrobial activity of C-PC extracted from *Oscillatoria minima* were examined for the first time against bacterial species *viz*. *Pseudomonas fragi*, *Entherobacter erogens*, *Escthercia coli*, *Pseudomonas vulgarius*, *Bacillus subtilis*, *Staphlococcus aereus*, *Klebsilla oxytoca*, and *Streptococcus pyogene* by using microtiter wells method. The final concentration of 16 µg/ml C-PC used to evaluate the inhibitory effect against each bacterial culture. Importantly, it was found that amongst the aforementioned bacterial species, only six bacterial species *viz*. *Pseudomonas fragi*, *Escthercia coli*, *Pseudomonas vulgarius*, *Bacillus subtilis*, *Klebsilla oxytoca*, and *Streptococcus pyogene* were inhibited by C-PC extracted from *Oscillatoria minima* (Table **6**).

4.5. C-PC Extracted from *Oscillatoria minima* Showed Algicidal Activity

In order to evaluate the algicidal activity of C-PC extracted from *Oscillatoria minima*, 16 µg/ml of C-PC was tested against variousmicroalgae *viz*. Nostoc, Synechocystics, Spirulina, and Gloeocapsa by using microtiter well (Nunc-Immuno ^{TM} 96 Microwell ^{TM} Plate). Importantly, it was found that amongst the aforementionedmicroalgae, all the microalgae were inhibited by C-PC except *Synechocystis* (Table **7**).

Table 6. Effect of C-PC against various bacterial species.

Bacterial Cultures	Inhibition by 16 µg/ml C-PC
Pseudomonas fragi	+
Entherobacter erogens	-
Escherichia coli	+
Pseudomonas vulgarius	+
Bacillus subtilis	+
Staphlococcus aereus	-
Klebsilla oxytoca	+
Streptococcus pyogene	+

(+) Antibacterial activity (-) No effect.

Table 7. Effect of C-PC against various species of microalgae.

Algal Culture	Inhibition by 16 µg/ml C-PC (6 Days)
Nostoc	+
Gleocapsia	+
Spirulina	+
Synechocystis	-

(+) Algicidal activity (-) No effect.

4.6. C-PC Extracted from *Oscillatoria minima* Showed Anti-Oxidant Activity

To further examine the anti-oxidant activity of C-PC extracted from *Oscillatoria minima,* DPPH free radical scavenging assay and $ABTS^{\cdot+}$ radical decolonization assay were performed. In the context of DPPH free radical scavenging assay, it works on the principle of reduction of DPPH to diphenyl picrylhydrazine (yellow in colour) in the availability of a hydrogen donor. The percentage of radical scavenging activity is directly related to the bleaching of the reaction mixture. Remarkably, the C-PC at the concentration of 1mg/ml, showed 44% of DPPH radical scavenging activity. Besides, the butylated hydroxyl anisole (BHA) showed around 90% of DPPH radical scavenging activity, indicating the effective performance of positive control.

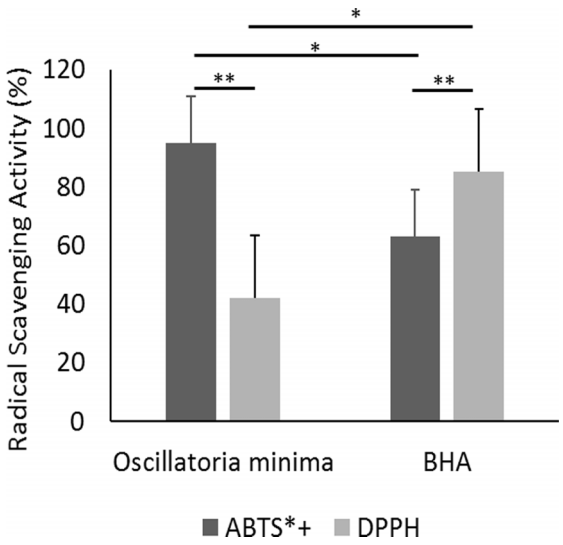

Fig. (4). The percentage $ABTS^{\cdot+}$ and DPPH radical scavenging activity of purified C-PC of *Oscillatoria minima* at the concentration of 1mg/ml and compared with standard BHA at the concentration of 1mg/ml.

In the context of $ABTS^{\cdot+}$ radical decolonization assay, it works on the principle of generation of $ABTS^{\cdot+}$ by the oxidation of ABTA with potassium persulfate and its reduction in the occurrence of hydrogen-donating anti-oxidants. This method takes into account both the concentration of anti-oxidant and duration of reaction on the inhibition of the radical cation absorption [34]. Interestingly, it was found that the purified C-PC at the concentration of 1mg/ml from *Oscillatoria minima* showed 95% of $ABTS^{\cdot+}$ radical scavenging activity. In addition, the C-PC showed better $ABTS^{\cdot+}$ radical scavenging activity as compared to the BHA, which was used as a positive control (Fig. **4**). The association may to a certain extent result from a similar mechanism and both anti-oxidants are soluble in aqueous/ethanol systems. Consequently, the purified C-PC has anti-oxidant property, which can suppress the formation of polar radicals and protect against oxidative damages.

5. DISCUSSION

One of the most important factors for the use of natural peptides in therapy is the method by which they are extracted. The separation and purification of C-PC from *Oscillatoria minima* are achieved through a convenient and single step chromatographic method. The results of the purification process (Table **2**) show the better efficiency of the

methods employed. The percentage purity puts any product which is more of a micrograph, that decides how a product might behave when it enters intothe host cell. The purity ratio of C-PC till the end of the process was significantly augmented. The difference in their biochemical properties was observed by the differences in subunits as detected by the SDS-PAGE analysis of the C-PC. The quantitative evaluation of phycobiliproteins (Tables **2** and **3**) in the *Oscillatoria* species showed C-PC as a major component along with other phycobiliprotein *i.e*. allophycocyanin and phycoerythrin, which gives an added advantage to the purification process. Since phycoerythrin is present in very low amount, it could not interfere in the purification process. This may be due to the species specificity in the phycobiliprotein content and the culture conditions of *Oscillatoria* species. During the extraction process, cyanobacterial cells were frozen at -20°C and thawed at room temperature, allowing allo-phycocyanin and phycoerythrin, along with other proteins and nucleic acids to be extracted besides phycocyanin. The presence of phycocyanin, allo-phycocyanin, other proteins, and nucleic acids correspondsto their maximum absorption at 620 nm, 652 nm, and 280 nm wavelengths, respectively.

The crude extract purification involves fractional precipitation with ammonium sulfate and ion-exchange chromatographic step. The first step of fractional precipitation is carried out with ammonium sulfate showing 25% saturation, which salted out other proteins with scanty improvement in purity ratio (A620/A280) and separation factor (A620/A652). Further, C-PC was salted out with 50% saturation with ammonium sulfate dissolving in acetate buffer (4.5, 0.1M), eliminated other basic proteins to a remarkable level with simultaneous improvement in purity ratio (A620/A280) as well as separation factor (A620/A652). The final purification of C-PC was achieved by ion-exchange chromatography method for purifying proteins, including phycobiliproteins. In the present study, purification was performed on a DEAE cellulose column as it is widely used for the separation and purification of C-PC and allophycocyanin from *Oscillatoria minima*. We have employed this column for the separation and purification of C-PC from *Oscillatoria minima* with few optimization steps in the purification steps as detailed, in the studies showing the better efficiency of the whole process. The C-PC was eluted by using phosphate buffer and after the initial two, three fractions; C-PC was eluted with high purity ratio. C-PC is considered as pure (for biochemical studies and especially for application as a fluorescent label) when its purity ratio (A620/A280) is greater than 4.0. The molecular weight of native C-PC is determined by gel filtration chromatography yielded two bands corresponding to α- and β- subunit of phycocyanin on SDS-PAGE (Fig. **3**) which further confirm its purity and homogeneity. The monomer molecular weight of *Oscillatoria minima* species was found to be 32.1(18.1+14.0) kD. The major role of dialysis in biological research is to remove the small molecular weight constituents from the sample. In the present case, the dialysis membrane with a pore size of 10-14 kD was used. Additionally, phycocyanin of *Oscillatoria minima* has been tested for an antibacterial and anti-oxidant property. Intriguingly, C-PC has shown specificity in inhibition potential against a wide range of bacterial and fungal species. Moreover, C-PC also revealed better free radical scavenging activity in DPPH assay indicating its potent anti-oxidant nature. Therefore, it has a wide scope in the pharmaceutical and biotechnological application for treating immuno-compromised patients, prevention against oxidative stress damage and bacterial infections, such as enteric fever, impetigo, and erysipelas and skin diseases. However, our future perspective will include the further characterization of this C-PC in order to determine the quality and purity in detail by using Infrared and Circular Dichroism.

CONCLUSION

ROS generating pathways and inflammatory pathways playa horrendous role in many inflammatory and neurodegenerative disorders as they area double-edged sword, which can go haywire if not controlled. Till now, drugs available to control it have equal chances of putting normal cells at risk in terms of non-specificity, toxicity level, and metabolic potential of cells. However, the use of natural peptides like C-PC could be pivotal as it could be easily metabolized and are more target specific. Moreover, remarkable structural similari-

ty to inhibitors of NAPH oxidase and clearing ability of HOCl provides C-PC an advantage compared to other drugs used for anti-inflammation, including neuroprotection. By the present study, we report here a simple and rapid isolation method of the C-PC from *Oscillatoria minima* with a better purity by using ion exchange chromatography and gel filtration chromatography. The quality check of isolated C-PC by SDS-PAGE and quantitative estimation validates our data and confirms the presence of α- and β- subunits at an expected size of 14.0 kDa and 29-43 kDa, respectively. This indicates that C-PC is a grouping of dimers in its native state. Therefore, by optimization, a cost-effective method of purifying C-PC from *Oscillatoria minima* was established. Importantly, apart from anti-oxidant activity, the isolated C-PC also exhibited antimicrobial and algicidal properties. This provides a scope for its further investigation to fetch useful role for pharmaceutical as well as in many biotechnological applications.

AUTHORS' CONTRIBUTIONS

VCV, AT, and LKC conceived the experimental design. VCV performed the experimental part. AT and PR performed the *in silico* study. LKC supervised the experimental part. CM, KS, and AR collected the information. VCV and AT wrote the manuscript. All the authors approved the content of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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