

Anti-HIV / HCV Activity of Cyanobacterial Phycobiliproteins by a New Standardized Method Using Bacteriophage Surrogates

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The control of the viral infections requires efficient, rapid and safe testing procedures. We tested the efficiency of a quick and reliable alternative assay to the standard plaque assay (PA) in the detection of viral inhibition. Using MS-2 and ΦX-174 phages, the developed assay was used to evaluate the potential of cyanobacterial phycobiliproteins (PBS) as natural antiviral bioactive molecules against HIV and HCV. The antiviral activity was examined by mixing the phage with crude aqueous extracts in a 1:1 (v/v) ratio for 1hr at 4°C. The viral titer of MS-2 and ΦX-174 was reduced by 0.92 LR and 1.19 LR, respectively. In the case of the crude PBS extract of Spirulina platensis, similar result trends were obtained by virion titration which indicated that a strong correlation between the two assays was verified (≥90 %). The morphological characteristics of both phages were severely affected when treated with the extract, whereas the characteristic features of their hosts seem unaffected. The PBS was found to affect the adsorption of the phages on their bacterial hosts and consequently viral reproduction. In conclusion, MS-2 and ΦX-174 can be used in drug evaluation by the well standardized method developed in this study.

Keywords: Antiviral assay, surrogates, MS-2, ΦX-174, phycobiliproteins, cyanobacteria, virion titration

Bacteriophages are ubiquitous entities [1], nonpathogenic to human beings [2], easy to be cultured and detectable with unsophisticated laboratory methods [3]. Phages have been used as a workhorse in various biotechnological fields [4] including studies of DNA replication, gene expression and regulation, therapy and prevention, viral morphogenesis, and epidemiology [4]. The most interesting recent applications focused on using suitable phages as surrogates for highly contagious viruses in many studies [4]. This simulation is based on an architecture where both the mammalian viruses, and phages must be similar to each other, primarily in genome composition, size and replication modes [6]. The bacteriophage MS-2 is a small (25 nm), tail-less, naked icosahedral (20-sided structure), single stranded RNA bacteriophage that belongs to the *Leviviridae* family [2]. It is specific to male *E. coli* (F-pilus) [7]. Its RNA genome composed of 3569 bases [8]. MS-2 has also been used as a viral surrogate to human viruses, including poliovirus, enterovirus and hepatitis A virus [9]. The phage ΦX-174 belongs to the *Microviridae* family and is a positive sense, circular/linear, single stranded (ss) DNA [10]. Its genome is encapsulated in a 25 nm icosahedral protein capsid and has 5386 nucleotides [10]. ΦX-174 primarily infects its host, *Escherichia coli* C strain (*E. coli* C), and can also be used as a model for HBV, HCV and HIV [11].

The use of natural antiviral bioactive molecules may combat the rapidly evolving viral drug resistance. For instance, acyclovir (ACV), as an antiviral drug, has drastically lost its specificity and efficacy for many patients, due to the development of human simplex virus (HSV) resistant strain [12]. Similarly, ribavirin (RBV) has been under use to treat HSV [13] as well as HCV, but recent studies show that chronic HCV patients exhibit resistance to RBV [14,15]. Nearly all antiviral mechanisms of RBV require its import into the cell, and consequently,

cytotoxicity and damage to the host chromosomes is a highly probable result [12].

Cyanobacteria have gained much attention as rich resource of a variety of natural bioactive compounds that exhibit a broad spectrum of antiviral activity [16-19]. Cyanobacterial bio-active molecules include, to name a few, calcium spirulan (Ca-SP) from *Spirulina platensis*, cyanovirin from *Nostoc ellipsoforum* and a cyclic polypeptide produced by *Lyngbya majuscula* [16,17,19,20]. Phycobiliproteins (PBS) are accessory photosynthetic pigments found in cyanobacteria, red algae and cryptophytes [21]. They are located in the thylakoid membranes beside the reaction center of the photosystem II (PSII). PBS consist of allophycocyanin, phycocyanin and phycoerythrin [22]. They perform an important function by acting as light-harvesting complexes that transfer the energy of sunlight into chlorophyll in the thylakoid membranes. It is water-soluble and can easily be isolated and separated as pigment-protein complexes from other lipid soluble pigments [23]. PBS have been widely used in biomedical research as fluorescent markers [24,25], and for the preparation of the antimicrobial and anti-cancer therapeutics [26,27].

For decades, the laboratory characterization of bacteriophage growth has been conducted by the plaque assay (PA) as the most reliable technique [28]. Few studies have paid attention to the virion titration methods for the detection of viral concentrations [29-31]. In this context, the present investigation focuses on exploring the antiviral potential of cyanobacterial PBS by utilizing a simple spectrophotometric technique to compare its precision with classical plaque assay methods, in detection of viral inhibition of crude aqueous extracts of six different cyanobacterial species against MS-2 and ΦX-174 phages, as reliable surrogates of specific virulent human viruses.

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Materials and methods

Tested cyanobacteria

The cyanobacterial species were isolated from water samples collected along the eastern branch of the Nile river, at the delta region close to Mansoura University (31° 2' 45.7620"N, 31° 2' 18.6444"E) (EPA, 1985). The algal isolates were obtained from the culture collection of the Biotech International Research and Development (BIRD) Centre, Mansoura, Egypt (<https://www.petrotechffn.com>) and grown on *Navicula* nutrient medium [32]. The cultures were incubated for three weeks at 25 ± 1°C under 1.2 ± 0.2 Klux light intensity with continuous illumination provided by white, cool fluorescent tubes [33]. The growth was monitored every three days, to calculate the growth rate and generation time.

Biomass harvesting and spectrophotometric measurements of phycobiliproteins

The cyanobacterial biomass was harvested by centrifugation at 4000 rpm for 10 min [34] and washed twice with double distilled water. One gram of fresh biomass was suspended in 100 mL of 0.1 M sodium phosphate (pH 7.0), that contained 1 mM sodium azide, and subjected to 4 h freezing and thawing cycles [35]. The clear supernatant was collected after centrifugation (4000 rpm for 10 min at 4°C), for spectrophotometric analysis, and then lyophilized and stored until needed.

Using a Unico UV-2000, UV-Vis spectrophotometer, the absorbance of phycocyanin (C-PC), allophycocyanin (APC) and phycoerythrin (PE) in the supernatant were measured, at wavelengths 620, 652, and 562 nm respectively. The concentration of each pigment was calculated using the following the equations [36]:

$$\text{C-PC (mg mL}^{-1}\text{)} = [A_{620} - 0.474 A_{652}] / 5.34,$$

$$\text{APC (mg mL}^{-1}\text{)} = [A_{652} - 0.208 A_{620}] / 5.09,$$

$$\text{PE (mg mL}^{-1}\text{)} = [A_{562} - 2.41 \text{PC} - 0.849 \text{APC}] / 9.62$$

The purity of phycocyanin extracts were evaluated by the ratios A_{620}/A_{280} [35,36].

IR spectra of aqueous cyanobacterial phycobiliproteins

The IR spectra of the freeze-dried extracts were estimated by a Nicolet iS10IR spectrophotometer. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis was performed using a polyacrylamide slab gel with 1.5 mm thickness. A 300 µL of sample loading buffer was added to the samples, and pre-incubated for about 5-10 min at 95°C, followed by cooling on ice. The crude PBS extract, standard C-PC (Sigma Phycocyanin), and the protein ladder (MWP04, Nippon Genetics Europe GmbH, Germany) were loaded into separate wells that were filled carefully with electrode buffer of pH 8.3 using a micropipette. Vertical gel electrophoresis was carried out at room temperature for 2 h, at 115 Volt, and then the gel was washed and overlaid with gel fix solution in a plastic container, to prevent any possible out washing of the protein during gel staining. The gel was visualized by staining with Coomassie brilliant blue R-250. A de-staining solution was used for de-staining of the gel. Staining and de-staining of the gel were carried out on a rocking platform, for 2 h. The molecular weights of protein subunits were determined by using a protein marker [37,38].

Antiviral assays of the extracted PBS

Bacteriophages MS-2, ΦX-174 and their hosts *Escherichia coli* (*E. coli*) ATCC-15597 and ATCC-13706 respectively, were kindly provided by Prof. John Dennehy, Associate professor, Biology department, Queens College, City University of New York (CUNY) (<https://www.atcc.org>)

The strains of *E. coli* ATCC-15597 and ATCC-13706 were cultured on ATCC media, incubated overnight at 37°C, and then sub-cultured for 4 h at 37°C [39]. The 4 h aged bacterial cultures of ATCC-15597 and ATCC-13706 were inoculated with MS-2 and ΦX-174 respectively and incubated overnight at 37°C. The cell lysate was then harvested by centrifugation at 4000 rpm for 30 min at 4°C, and the supernatant filtered through a 0.45 µm syringe filter and used immediately or stored at -20°C as a phage stock [9].

Phage quantification (antiviral activity assay)

Plaque reduction assay was carried out by using the overlay technique (double agar layers) to monitor the antiviral activity of the PBS extracts. Briefly, the phage was mixed in a ratio of 1:1 (v/v) with different crude aqueous extracts, of final concentrations of 0.08, 0.32, 1.28, 5.12, 24.8 and 81.92 mg mL⁻¹, and incubated for 1.0 h, at 4°C [40]. The mixture was added to soft agar containing the respective host bacteria at its exponential growth phase, and then vortexed and overlaid on a solidified plate. The top agar was then allowed to solidify, and incubated overnight at 37°C. The plaques were counted and the log₁₀ reductions in the active phages were determined Standard C-PC with final concentration of 0.0125, 0.025, 0.125, 0.25 and 0.5 mg mL⁻¹, ribavirin and acyclovir (0.008, 0.032, 0.128, 0.512, 2.048 and 8.192 mg mL⁻¹) were used as positive controls [34,41,42]. Each treatment was run in triplicates, and a mean value was calculated.

Virion titrations

Briefly, the phage was added to different concentrations of the crude PBS extracts in a ratio of (1:1) as described before, and incubated for 1hr at 4°C. The mixture was then added to the bacterial host, vortexed, and incubated overnight at 37°C [27]. Phage concentration (virions titer) was determined. The measurement of concentration depends on the absorbance at 269 and 320 nm, which was measured using a Unico UV-2000, UV-Vis spectrophotometer [29]:

$$\text{Virions/ml} = \frac{(A_{269} - A_{320}) \times 6 \times 10^{16}}{\text{no. of bases/virion} \times \text{volume used (ml)}} \times 100 \times \text{dilution}$$

where: no. of bases/phage for MS-2=3569 [8] and that of ΦX-174=5386. Finally, the reduction in activity was determined as a percentage of number of phages treated with different test solutions, compared to that of the control (untreated phage). As mentioned, positive controls were used, and each treatment was run in triplicates.

Electron microscopy

Samples of treated and untreated phages were examined by a transmission electron microscope (TEM). A drop of purified phage was applied to a copper grid, and stained with 2 % uranyl-acetate for 5 min, and then examined in TEM (JEOL JEM-2100, USA) at the Electron Microscopy Unit, Faculty of Agriculture, Mansoura University, Egypt (<http://emunit.mans.edu.eg/>).

Statistical analysis

All values of antiviral activities (reduction in PFU and virion concentration) are reported as the mean of three replicates ± standard error. The data was subject to one-way ANOVA, and the mean values were separated based on a Duncan test at 0.05 probability level, using the COSTAT 6.3 package. Correlation between the two techniques (plaque assay and clarity method) was determined as a Pearson correlation, using the XL-Stat (2014) package, and the obtained results were expressed as determinants of R² (determination coefficient).

Table 1
THE AVERAGE VALUES OF SPECIFIC GROWTH RATE (μ), GROWTH DOUBLING PER DAY (Dd^{-1}) AND GENERATION TIME (G) OF DIFFERENT TEST CYANOBACTERIAL SPECIES

| Algal species | Specific growth rate (μ) (\pm SD) | Doubling per day (Dd^{-1}) (\pm SD) | Generation time (G) (\pm SD) |
|--------------------------------|--|--|---------------------------------|
| <i>Anabeanaanomala</i> | 0.163 (\pm 0.0001) | 0.236 (\pm 0.0002) | 4.24 (\pm 0.003) |
| <i>Aphanizomenonflos-aquae</i> | 0.156 (\pm 0.0003) | 0.226 (\pm 0.0004) | 4.44 (\pm 0.008) |
| <i>Chroococcusminimus</i> | 0.186 (\pm 0.001) | 0.268 (\pm 0.001) | 3.73 (\pm 0.019) |
| <i>Nostoc</i> sp. | 0.201 (\pm 0.001) | 0.290 (\pm 0.001) | 3.45 (\pm 0.014) |
| <i>Spirulinaplatensis</i> | 0.178 (\pm 0.001) | 0.257 (\pm 0.006) | 3.89 (\pm 0.095) |
| <i>Synechococcuscedrorum</i> | 0.228 (\pm 0.001) | 0.328 (\pm 0.006) | 3.04 (\pm 0.003) |

N.B. SD: Standard deviation

Results and discussions

The specific growth rate (μ) of different cyanobacterial species, divisions per day (Dd^{-1}), and the generation time (G) are listed in Table 1. The cyanobacterium *Synechococcus cedrorum* maintained the highest specific growth rate ($\mu = 0.228 \pm 0.001$), while *Aphanizomenon flos-aquae* maintained the lowest one (0.156 ± 0.0003). Growth doubling fluctuated within a narrow range of $0.226 Dd^{-1}$ (*Aphanizomenon flos-aquae*) and $0.328 Dd^{-1}$ (*Synechococcus cedrorum*).

Phycobiliprotein pigments include C-phycoyanin (C-PC), allophycoyanin (APC), and phycoerythrin (PE). Absorbance of the PBS pigments at different wavelengths, concentration of each pigment, and the purity of C-PC are listed in Table 2. The total PBS fluctuated within a wide range of $0.195 mg \cdot mL^{-1}$ (*Synechococcus cedrorum*), and $0.008 mg \cdot mL^{-1}$ (*Nostoc* sp.). The highest concentration of C-PC was recorded for *Synechococcus cedrorum* followed by *Spirulina platensis*, while the highest PE concentration was recorded for *Aphanizomenon flos-aquae*. The purity ratio (A_{620}/A_{280}) of C-PC ranged between 0.206 (*Anabeana anomala*) and 1.588 (*S. platensis*).

The IR spectra of *S. platensis* PBS were more or less identically similar to those of the pure C-PC. Data presented in Table 3, indicated consistent similarity between bands of amide C=O, N-H and C-N bonds of both crude PBS of *S. platensis*, and the pure C-PC.

Figure 1 illustrates two characteristic bands of the crude extract of *Synechococcus cedrorum* (Lane 1)

Table 3
IR SIGNIFICANT BANDS OF THE LYOPHILIZED CRUDE PHYCOBILIPROTEINS OF *S. PLATENSIS* AND PURE C-PHYCOCYANIN

| Sample | Significant bands (cm^{-1}) | | |
|----------------------------|---------------------------------|--------------|---------------|
| | N-H | C=O of amide | C-N stretchin |
| PBS of <i>S. platensis</i> | 3460 | 1687 | 1125 |
| Control: Pure C-PC | 3451 | 1660 | 1085 |

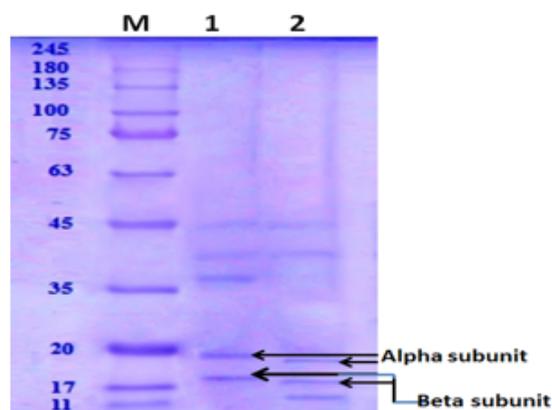


Fig. 1. Coomassie Brilliant blue-stained 14% SDS-PAGE showing the protein electrophoresis pattern of the lyophilized crude extract of *Synechococcus cedrorum* using freezing and thawing extraction method, and the pure C-PC (Lane 1 and Lane 2, respectively).

M: BlueStar Plus pre-stained protein ladder (MWP04, NIPPON GENETICS); (11, 17, 20, 25, 35, 45, 63, 75, 100, 135, 180, and 245 kDa)

Table 2
SPECTROPHOTOMETRIC ABSORBANCE FOR QUANTITATIVE DETERMINATION OF PBS CONCENTRATIONS AND PURITY RATIO OF PHYCOCYANIN POST FREEZING AND THAWING EXTRACTION METHOD FROM THE SIX TESTED CYANOBACTERIAL SPECIES

| Algal species | | <i>Anabeana anomala</i> | <i>Aphanizomenon flos-aquae</i> | <i>Chroococcus minimus</i> | <i>Nostoc</i> sp. | <i>S. platensis</i> | <i>Synechococcus cedrorum</i> |
|------------------------------|-----------|-------------------------|---------------------------------|----------------------------|-------------------|---------------------|-------------------------------|
| Purity (A_{620}/A_{280}) | | 0.206 | 0.470 | 0.089 | 0.312 | 1.588 | 0.790 |
| Abs | A_{280} | 0.160 | 0.071 | 0.045 | 0.125 | 0.365 | 1.304 |
| | A_{562} | 0.168 | 0.086 | 0.060 | 0.005 | 0.594 | 0.551 |
| | A_{652} | 0.159 | 0.297 | 0.047 | 0.002 | 0.349 | 0.280 |
| | A_{620} | 0.816 | 0.183 | 0.676 | 0.039 | 0.374 | 1.033 |
| Conc. (mg/ml) | C-PC | 0.017 | 0.010 | 0.007 | 0.007 | 0.079 | 0.169 |
| | APC | 0.025 | 0.010 | 0.006 | 0 | 0.047 | 0.013 |
| | PE | 0.010 | 0.028 | 0.003 | 0.001 | 0.013 | 0.014 |
| | Tot. PBS | 0.052 | 0.048 | 0.016 | 0.008 | 0.139 | 0.196 |

N.B. C-PC: C-phycoyanin, APC: Allophycoyanin, PE: Phycoerythrin and PBS: Phycobiliprotein pigments.

corresponding to 18 and 19.5 KDa for the α and β sub-units, respectively. Also, two sub-units of the pure C-PC, the α (16 KDa) and the β subunit (17.5 KDa) are shown. The selection of *Synechococcus* extract for SDS-PAGE analysis is based on the fact that its PBS was the richest in C-PC, Table 2, compared to other cyanobacteria, in addition to its high antiviral potential.

The antiviral activity of the cyanobacterial PBS crude aqueous extracts was detected by plaque reduction assay (PA) (Figure 2) and virion titration assay (Figure 3). The antiviral inhibition activities exhibit obvious ($p \leq 0.05$) variations depending on assay technique, phage surrogate, viral drugs and PBS extracted from different test cyanobacteria. It is clear from Figure 2 and 3 that the antiviral activity of pure C-PC on MS-2 was significantly higher than the commercial viral drugs ACV and RBV. Compared to these drugs, marked Φ X-174 activity inhibition was evident, at 0.5 mg mL⁻¹ of C-PC. According to the results of the PA (Figure 2), the aqueous crude PBS extracted from the cyanobacterium *Synechococcus cedrorum* (Fig. 2I) followed by *Anabeana anomala* (Fig. 2D), *Spirulina platensis* (Fig. 2H) and *Aphanizomenon flos-aquae* (Fig. 2E) exhibited significant antiviral activity against Φ X-174. Compared to pure C-PC, antiviral drugs, and extracted PBS, the crude PBS extracted from *Spirulina platensis* maintained significant inhibition against MS-2 (Fig. 2H).

Similar result trends are obtained by virion titration (spectrophotometric method) (Fig. 3). Compared to the

negative control (phage and bacterial host), ACV inhibited MS-2 activity between 3.75 and 40.08 % corresponding to the doses 0.008 and 8.192 mg mL⁻¹ respectively (Fig. 3B). However, RBV inhibited the MS-2 bacteriophage by 30.01 and 36.75 % at doses 0.008 and 8.192 mg mL⁻¹ respectively (Fig. 3C). Both drugs maintained relatively low antiviral activity against Φ X-174 with % of inhibition ranging between 0.0 and 15.74 %. Compared to the negative control, the inhibition (%) of different cyanobacterial PBS extracts on the MS-2 phage ranged between 32.23 % (*Chroococcus minimus*, Fig. 3F) and 87.89 % (*S. platensis*, Fig. 3H), and fluctuated from 17.97 % (*Nostoc* sp., Fig. 3G) to 98.23 % (*Synechococcus cedrorum*, Fig. 3I). The highest % of inhibition (87.89 %) of the ssRNA MS phage was recorded for phycobiliproteins extracted from the cyanobacterium *S. platensis* (Fig. 3H) followed by *Synechococcus cedrorum* (50.95 %, Fig. 3I), *Aphanizomenon flos-aquae* (45.19 %, Fig. 3E), *Anabeana anomala* (49.97 %, Fig. 3D), and *Nostoc* sp. (41.23 %, Fig. 3G). In case of the ssDNA Φ X-174, the highest % of inhibition (98.23 %) was maintained by PBS extract of the cyanobacterium *Synechococcus cedrorum*, Fig. 3I, followed by *Anabeana anomala* (95.29 %, Fig. 3D), *S. platensis* (93.62 %, Fig. 3H), *Aphanizomenon flos-aquae* (88.85 %, Fig. 3E), *Chroococcus minimus* (39.38 %, Fig. 3F) and *Nostoc* sp. (33.65 %, Fig. 3G).

Figure 4 presents a clear comparison between the antiviral activities of different cyanobacterial PBS extracts

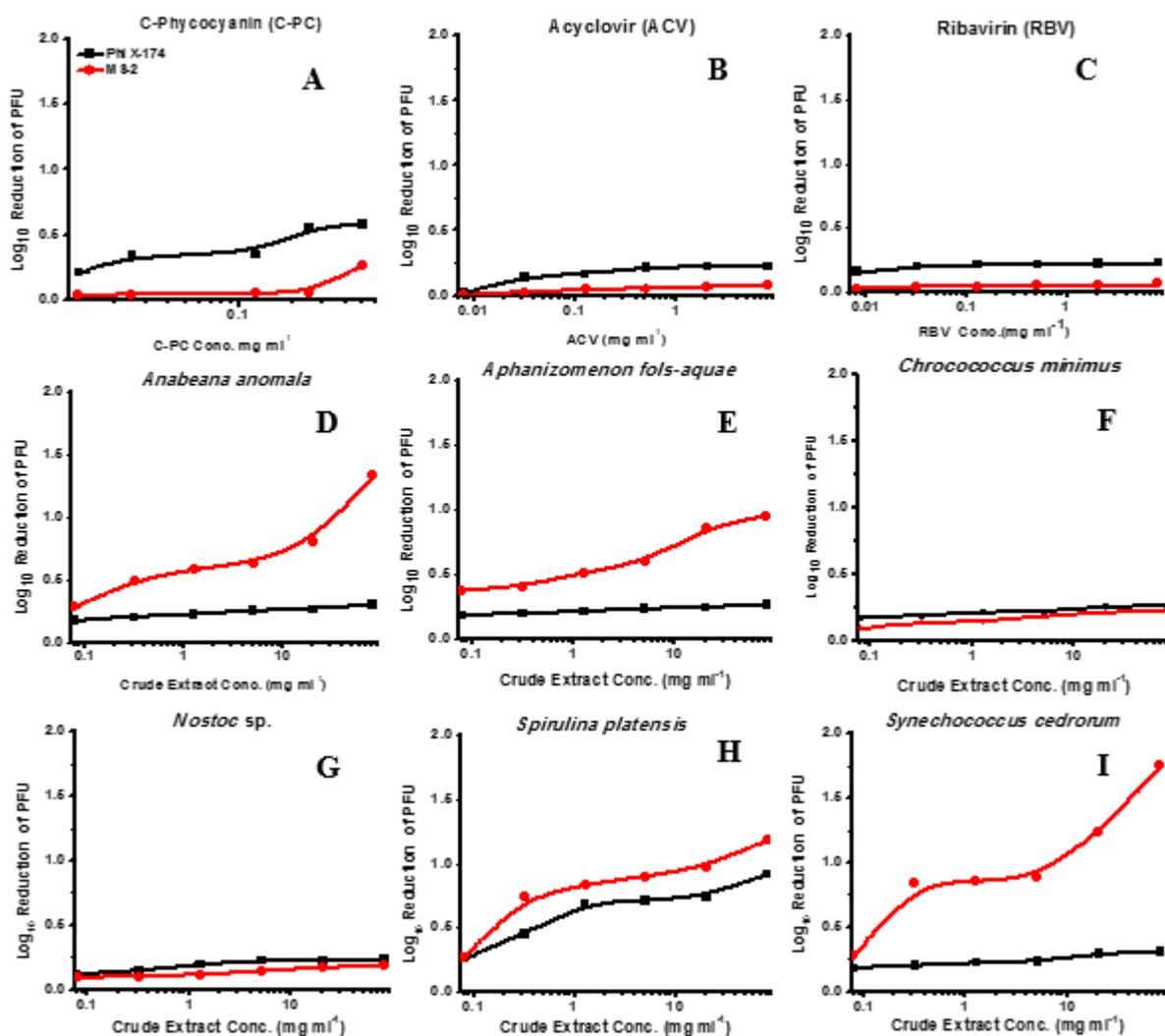


Fig. 2. The antiviral activity of the cyanobacterial phycobiliproteins compared with pure C-phycocyanin, acyclovir and ribavirin (positive controls) on the bacteriophage surrogates MS-2 and Φ X-174 using plaque reduction assay (log₁₀ reduction factor, LR)

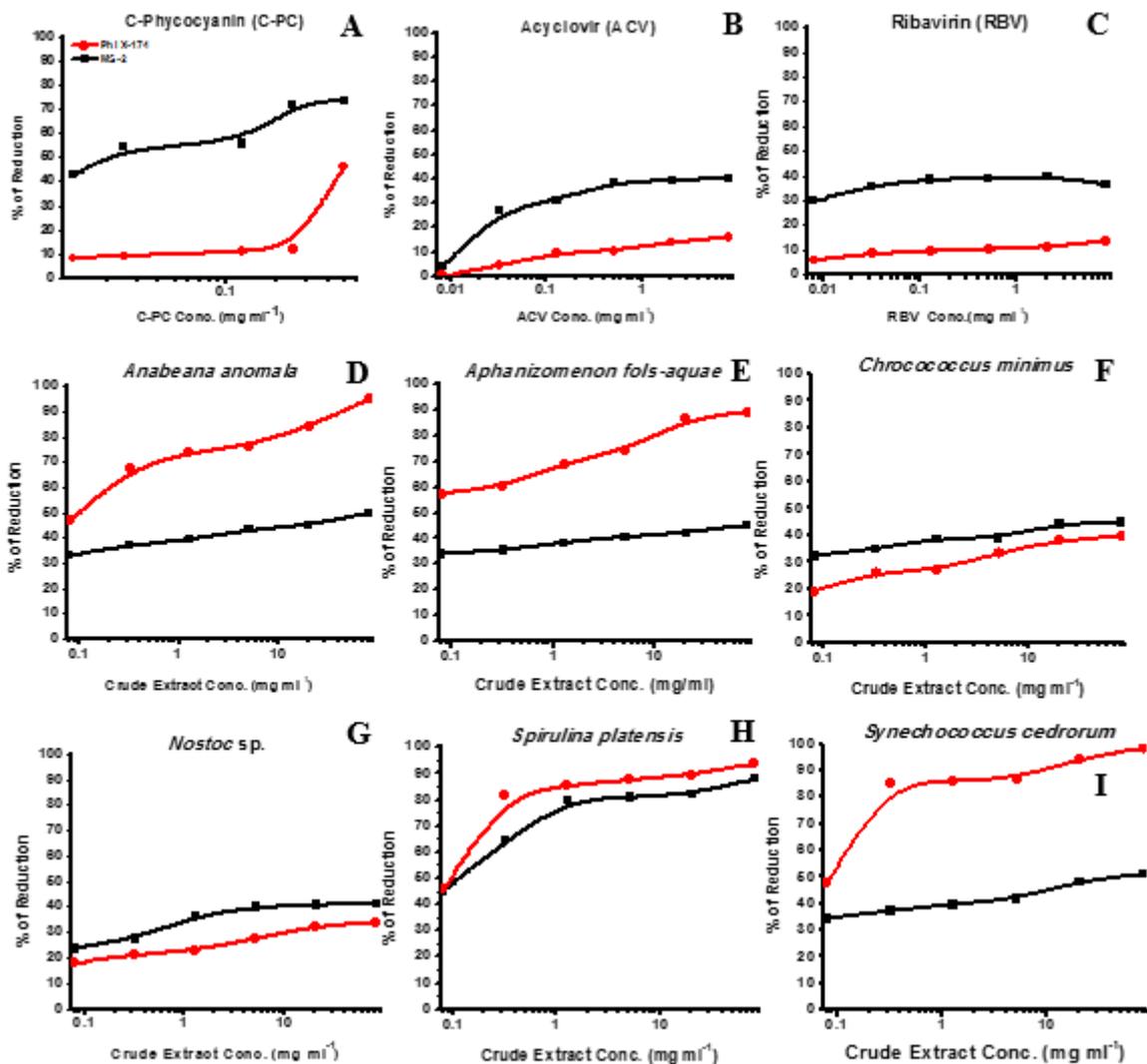


Fig. 3. Antiviral activity of the cyanobacterial phycobiliproteins compared with pure C-phycocyanin, acyclovir and ribavirin (positive controls) on the bacteriophage surrogates MS-2 and Φ X-174 using % of the reduction in virion concentration

on MS-2 phage using PA (Fig. 4A), and the spectrophotometric virion titration technique (Fig. 4B). The antiviral effects on the virulence of Φ X-174 using PA and the virion titration method are illustrated in Figure 4C and 4D,

respectively. The figure clearly displays great similarity between LR and % of inhibition as two different assays to monitor the effects of different cyanobacterial PBS on the activity virulence of MS-2, (Fig. 4A and B) and Φ X-174 (Fig.

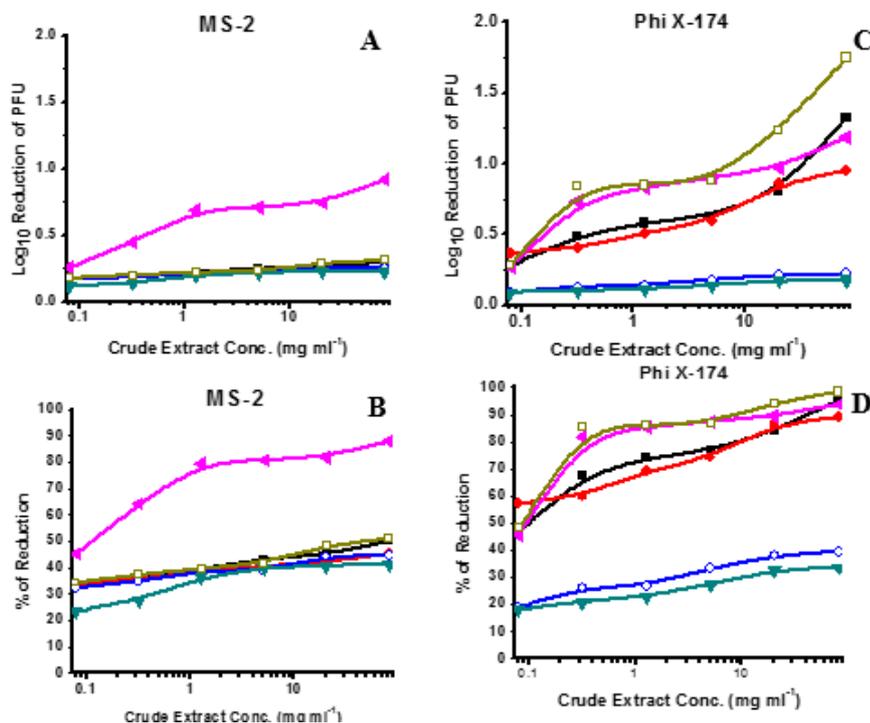


Fig. 4. Antiviral effects of different concentrations of six cyanobacterial crude extracts against MS-2 (A, B) and Φ X-174 (C, D) using Log₁₀ reduction factor (LR) and percent of reduction in virions

4C and D). The obvious antiviral similarities displayed in Figure 4 was confirmed with significant ($p \leq 0.05$) and very strong correlation between the two assays, monitoring the antiviral activities of different cyanobacterial PBS extracts against MS-2 ($R^2=0.94$) and Φ X-174 ($R^2=0.90$).

Based on the above results it is evident that the crude aqueous PBS extract of *S. platensis* maintained strong antiviral activities against both phages, MS-2 and Φ X-174 (Figure 4H). In addition, the extract maintained the highest PBS purity, (Table 2). Accordingly, studies were done to follow up on the impact on morphological changes in the two phages, after treatment with PBS extract at the highest dose (81.92 mg.mL^{-1}) giving the highest viral inhibition. Examination with transmission electron microscopy (TEM) was carried out to study the morphological changes in MS-2, Φ X-174 and their hosts after treatment with the PBS crude extract of *S. platensis* (81.92 mg.mL^{-1}). The icosahedral structure of MS-2 (Figure 5A), and Φ X-174 (Figure 5C), bacteriophages was highly affected after 1.0 hour of incubation with the extract at 4°C , which is clear in Figure 5B and 5D. It was also found that the adsorption of phages on their host cells one hour after the treatment was affected, compared to the untreated phages (Figure 6), where there was a decrease in the number of phage particles in both models than the control. The lysis of *E. coli* C (ATCC-15597) by Φ X-174 phage activity is illustrated in Figure 7.

Studying the epidemiology and management of viral infection, especially virulent viruses as HIV and HCV, requires some restrictions imposed by their antiviral assays.

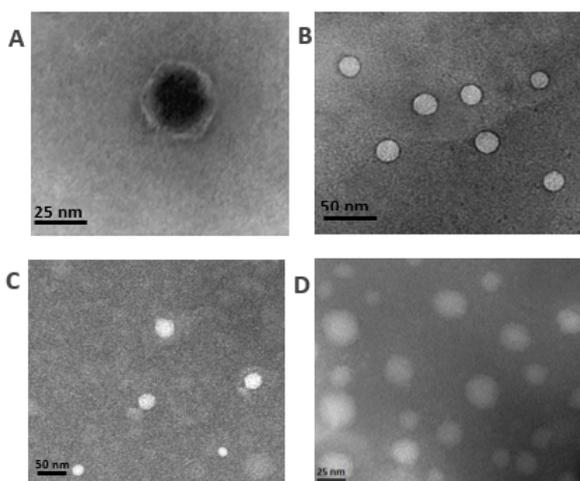


Fig. 5. Electron micrographs of MS-2 and Φ X-174 bacteriophage stained with 2% uranyl acetate showing the morphological characters of the virion before and after treatment with the crude phycobiliproteins extract of *Spirulina platensis*. A: The MS-2 phage was naked icosahedral and tailless with average diameter 24 nm. Bar corresponds to 25 nm; B: MS-2 bacteriophage coated with the extract after 1 h of incubation at 4°C . Bar corresponds to 50 nm; C: The Φ X-174 phage was naked icosahedral capsid with diameter 27 nm without envelope or tail. Bar corresponds to 50 nm; D: Irregular architecture of the Φ X-174 icosahedron after 1 h incubation with the PBS extract at 4°C . Bar corresponds to 25 nm.

The ever-evolving viral drug resistance, and their serious immune reactions, necessitate an urgent, serious need for drug alternatives. Peculiar structural characteristics, replication strategies, non-pathogenicity and ease of detection along with the economic feasibility of MS-2 (surrogate of enterovirus and HAV) and Φ X-174 (surrogate of HBV, HCV and HIV) bacteriophages qualify them as ideal models of virulent mammalian viruses in antiviral assays. The present study screens the antiviral potentialities of

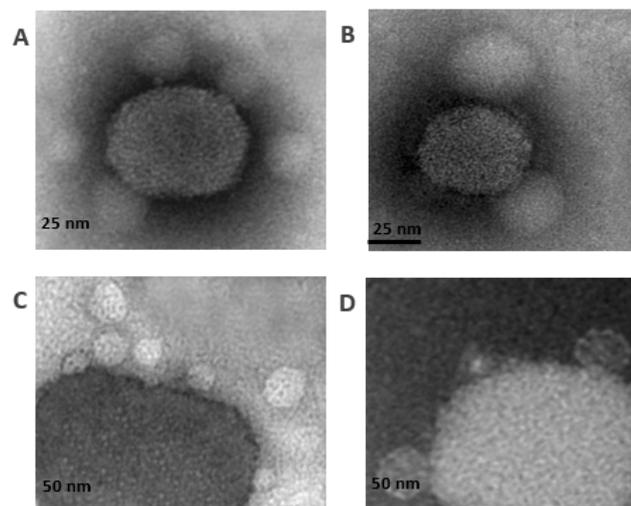


Fig. 6. Electron micrographs of the two phages with their bacterial host before and after treatment with *Spirulina platensis* phycobiliproteins crude extract. A: MS-2 phages attaching to F-pilus of *E. coli* C3000 (ATCC-15597). Bar corresponds to 25 nm; B: Adsorption of MS-2 phages to its bacterial host after 1 h of extract treatment; C: Φ X-174 phages binding to outer membranes of *E. coli* C (ATCC-13706). Bar corresponds to 50 nm; D: Adsorption of Φ X-174 phages to its bacterial host after 1 h of extract treatment

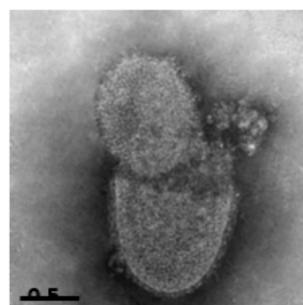


Fig. 7. Electron micrograph of *E. coli* C (ATCC-15597) negatively stained with 2% uranyl acetate showing the lysis phenomenon due to Φ X-174 phage activity. Bar corresponds to 500 nm

some PBS against MS-2 and Φ X-174 using the spectroscopic technique (virion titration) in the detection of antiviral activity as a promising technique. The choice of cyanobacterial PBS based on previous research [27] demonstrates the importance of cyanobacteria as a vital and rich renewable biosource, of a variety of antiviral drugs.

The physico-chemical studies indicate that the main active component was the C- phycocyanin where the IR spectra of lyophilized crude PSB of the tested cyanobacteria showed the primary significant bands of PC with the occurrence of bands at range of ($3500\text{-}3000 \text{ cm}^{-1}$), ($1640\text{-}1680 \text{ cm}^{-1}$) and ($1020\text{-}1200 \text{ cm}^{-1}$), confirming the presence of N-H bond, the specific protein amide I group ($\text{C}=\text{O}$) and C-N bond, respectively. These findings are similar to the spectra of the control C-PC and those reported earlier [44], thus indicating the presence of the major component of the crude extracts as C-PC. The sharp band indicates the presence of an α -helix as the main element of its protein secondary structure. SDS-PAGE analysis of the crude C-PC showed two bands corresponding to α and β subunits of the pure C-PC which confirms its purity and homogeneity. The molecular weight of α and β subunits was 19500 and 18000, respectively, which is in the range of the findings of other research [45].

Allophycocyanin extract was found to inhibit enterovirus 71-induced cytopathic effects, viral plaque formation, and viral-induced apoptosis [27]. The crude C-PC extract of *S. platensis* was also reported to inhibit activities against certain viruses (New castle virus, Avian adenovirus, HAV and Polio virus) [44]. Both studies relied on testing the activity of the extracts in cell culture, which impose human

significant risks associated with occupational exposure to viruses and increasing the likelihood of viral infection [2]. Therefore, viral assays with bacteriophage surrogates may be a powerful tool for screening new antiviral agents as they are safe to deal with, easily cultured, detectable with laboratory methods, rapid (18-24 h), and inexpensive [46-48].

It is clear from the results obtained (Fig. 3 and 4) that different cyanobacterial PBS exhibited significantly different antiviral activities. This may be attributed to different compositions of cyanobacterial PBS and in particular to their relative mass ratios and purity. Supporting results reported by Harden et al. [37] found significant variation of virucidal activities of polysaccharide extracts of four different algal species against herpes simplex virus. In this context, Kok *et al.* [49] have also reported widely different inhibitory effects of microalgal extracts against the Epstein-Barr virus. The most peculiar and interesting findings was the significantly higher anti-activity of cyanobacterial PBS on both MS-2 and Φ X-174 compared with the two tested drugs ribavirin and acyclovir. These findings highlight the test cyanobacteria as promising bio-resources for exploring novel antiviral compounds. Also, the relatively higher antiviral efficiencies of different PBS extracts compared to the pure C-PC can support the hypothesis that the purification and fractionation of C-PC can induce loss of the synergetic effect of the compounds mixture found in the crude extract [34,49].

A very interesting observation was the marked strong inhibitory effects of PBS of *Spirulina platensis* against both MS-2 (87.89 %, Fig. 4H) and Φ X-174 (93.62 %, Fig. 4H). Based on this significant outcome, *Spirulina platensis* may hold a promising future for the discovery of new effective antiviral drugs. In this respect, it was reported [34] a potential antiviral activity of C-PC extracted from *Spirulina platensis*. Moreover, the fact that *Spirulina platensis* is a super-human food lends itself to the superiority of this cyanobacterium as a potential, renewable, bio-source of a variety of safe health drugs against fetal human viruses.

The results indicated that virion titration technique exhibit narrow variations among different cyanobacterial extracts (Fig. 5D) while these variations were high significance in case of plaque reduction assay (PA) (Fig. 5C). This finding may indicate a higher sensitivity of virion titration compared to PA. This can also be supported by the fact that the PA is dependent on visually counting the plaques and therefore, may involve great personal error. However, the virion titration technique is an instrumental spectrophotometric method that exhibits higher sensitivity and low deviation errors. Accordingly, this technique can preferably substitute the classical PA in viral assays. In this respect, Adams indicated [20] that even though the traditional plaque assay is the current standard for quantitating phage titers, the obtained results may be significantly influenced by various factors.

The transmission electron microscope (TEM) micrographs elucidate the characteristic morphology of the purified MS-2 (Fig. 6A) and Φ X-174 (Fig. 6C) bacteriophages. The icosahedral capsid of MS-2 had a size of approx. 24 nm that agrees with previous studies [49], while the icosahedron of Φ X-174 was about 27 nm [10]. It is clear that the highest concentration of the *S. platensis* crude extract (81.92 mg mL⁻¹) induced marked morphological changes on both MS-2 and Φ X-174 compared to the control; where the characteristic icosahedral structures of their capsid became slightly spherical in shape. The *Spirulina platensis* extract exhibited a great effect on the phage adsorptions onto their hosts as

indicated by marked reduction in the number of adsorbed phages of either MS-2 (Fig. 7B) and Φ X-174 (Fig. 7D) in contrast to the untreated phages. This finding can correspond to the high reduction in phages titer of MS-2 (87.89 %) and Φ X-174 (93.62 %). The results clearly revealed that, the mode of action of *Spirulina platensis* PBS is assumed to interfere with the phage adsorption or penetration processes, *i.e.* the viral inhibition occurred at the early stage of the viral infection. The investigated cyanobacterial PBS seem to exhibit an antiviral mode of action disturbing the early stage of the viral life cycle with higher antiviral potential compared to the commercial medications (ACV and RBV).

Conclusions

The present study can provide a pioneering direction in the efforts for screening the antiviral activities of cyanobacterial phycobiliproteins against MS-2 and Φ X-174 bacteriophage surrogates simulating virulent, fatal human viruses.

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