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Original Article

Anti-hepatitis Viruses of Phycobiliprotiens Aqueous Extract of the Cyanobacterium *Synechococcu scedrorum* Sauvageau Using Bacteriophages MS-2 / ΦX-174 as Model Systems

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Abstract

Cyanobacteria produced a variety of secondary metabolites includes potent antiviral bioactive molecules. The cyanobacterium *Synechococcus cedrorum* Sauvageau (Family: *Synechococcaceae*) was cultivated on Navicula nutrient medium and left to grow under the growth favorable conditions. For phycobiliprotiens extraction the biomass was harvested and suspended in 0.1 M sodium phosphate (pH 7.0), containing 1 mM sodium azide, and subjected to freezing and thawing cycles (6 cycles). The mixture was centrifuged at 4000 rpm and the clear supernatant was collected for spectrophotometeric analysis resulting 0.196 mg/ml phycobiliprotiens. MS-2 and ΦX-174 were used in this study as bacteriophage models. MS-2 replaced enteroviruses and hepatitis A virus (HAV) in antiviral assays and ΦX-174 models HBV, HCV and HIV as they are similar in shape. Phage suspension (negative control), phage-drugs suspensions (positive controls), phage pure C-Phycocyanin suspensions (positive control) and phage-phycobiliprotiens suspensions (treated) were incubated for 1h at 4^{o}C. Equal volumes of different phage suspensions were separately added to the respective bacterial host suspension of constant volumes. Antiviral activities were assayed by plaque reduction assay and clarity assay. The results indicates that the extracted phycobiliprotiens reduced ΦX174 and MS-2 titers by 5.16 and 9.197 Log_{10} PFU/ml, respectively. The clarity method also confirmed reduction in concentration of phage ?X174 and MS-2 by 83.07 and 41.94 %, respectively. So it can conclude that, phycobiliprotiens may hold great future as antiviral agents.

1. Introduction

It has become evident that viral infections pose great threat to the humankind (Grabow, 2007), in 1998 Enterovirus 71(EV71) reaped many victims through the outbreak in Taiwan (Shia *et al.,* 2002). Over 40 millions become infected by HIV (Piot, 1998) and about 170 mil-

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lions have HCV infection around the world (WHO, 1997), so it is perhaps relevant to give concise and precious attention about some of the most dangerous animal viruses infecting humans.

Enterovirus (EV71) is a member of the family *Picornaviridae* (Grabow, 2007) with a single-stranded RNA (ssRNA) (Bienz, 2005). Enteroviruses reach their target organs through the bloodstream and transmitted via food and water, their syndromes are HFMD (hand, foot, and mouthdisease) and acute hemorrhagic conjunctivitis (Bienz, 2005). Human immunodefficiency virus (HIV) is a lentivirus of family *Retroviridae* that causes the AIDS (Weiss, 1993 and Douek *et al.,* 2009). HIV has a spherical shape with approximately 120 nm diameter and a ssRNA (McGovern, 2002). As an exception of retroviruses, HIV's life cycle is of the lytic type (Bienz, 2005). HIV transmitted sexually and via body fluids, it can be also transmitted from mother to her child during the pregnancy and nursery (Bienz, 2005). HCV (Hepatitis C Virus) is a member of the family *Flaviviridae* (Wagner *et al.,* 2008), with a ssRNA and a size of 50nm (Bienz, 2005). Transmission occurs through direct exposure to infected blood or intravenous drugs and rarely via sexual exposure (George *et al.,* 2001 and Wagner *et al.,* 2008). In Egypt, HCV is a viral pandemic; where about 10-20% of population was infected (Kamel *et al.,* 1992; Darwish *et al.,* 1993; Waked *et al.,* 1993 and Abdel-Wahab *et al.,* 1994) and currently, no vaccine against hepatitis is available (Bienz, 2005).

The search for new antiviral drugs has become a vital research demand of natural origin. (Chatis and Crumpacker, 1991; Darville *et al.,* 1998 and Shia *et al.,* 2002). In general, algae have been widely reported as promising renewable bioresources of a variety of bioactive molecules with potent antiviral activities (Zhu *et al.,* 2003). In this respect, cyanobacteria attracted more research attention (Dahms *et al.,* 2006).

Cyanobacteria have vast applications in biotechnology such as mariculture, fuel, fertilizer, medicine and in combating pollution (Prabhakaran and Subramanian, 1995; Sundararaman *et al.,* 1996 and Subramaninan and Uma, 1996). Most of bioactive natural products produced by cyanobacteria maintain a broad spectrum of antiviral activity (Hayashi *et al.,* 1996 a,b; Lee *et al.,* 2001, 2004 and Jha and Zi-rong, 2004). There antiviral bioactive molecules include, for instant, calcium spirulan (Ca-SP), from *Spirulina platensis* (Hayashi *et al.,* 1996a), cyanovirin from *Nostoc ellipsosporum* (Boyd *et al.,* 1997) and a cyclic polypeptide produced by *Lyngbya majuscule* (Jha and Zi-rong, 2004).

Synechococcus cedrorum Sauvageau is a freshwater, unicellular cyanobacterium that possesses a therapeutic value according to Schaeffer and Krylov, 2000 and Noaman *et al.,* 2004. Kok *et al.,* found that the methanolic extract of *Synechococcus* sp. and its fractions exhibited antiviral activities against Epstein-Barr virus (EBV) (Kok *et al.,* 2011), also lipophilic and hydrophilic extracts of *Synechococcus elongates* had anti-HIV activity (Lau *et al.,* 1993 and Schaeffer and Krylov, 2000). Many studies have already focused on *Synechococcus* sp. as a source of phycobiliprotien production for various purposes whether in medicine or in industry (Abalde *et al.,* 1998; Schaeffer and Krylov, 2000; Sekar and Chandramohan, 2008 and Kok *et al.,* 2011).

Phycobiliproteins (PBS) are found in cyanobacteria, red algae and cryptophytes (Zilinskas, 1986). They are the major accessory photosynthetic pigments in the blue-green algae (Zhang *et al.,* 2010). PBS are found on the thylakoid membranes beside to the reaction center of the photosystem II (PSII) (MacColl, 1998), and consist of allophycocyanin, phycocyanin and phycoerthrin (Gantt, 1981and Bazire and Bryant, 1982). They act as light-harvesting complexes that transfer the energy of sunlight to chlorophyll a in the thylakoid membranes. Phycobiliproteins are water-soluble proteins, with a 3-dimensional structure (Wolfgang *et al.,* 1996).They are used in biomedical researches as fluorescent markers (Hardy, 1986 and Ganapathi and Raghavarao, 2007), and exhibit therapeutic characteristics (Bhat and Madyastha, 2001 and Farooq *et al.,* 2006). Shin *et al* reported that, allophycocyanin, a protein-bound pigment purified from *Spirullina platensis*, exhibit anti-enterovirus 71 activity. Allophycocyanin was found to inhibit enterovirus 71-induced cytopathic effects, viral plaque formation, and viral-induced apoptosis (Shin *et al.,* 2003).

Blood-borne viruses, like HCV are difficult to be cultured in *vitro* and mammalian cell culture is very expensive and time consuming process (Hu and Aunins, 1997 and Aranha-Creado and Brandwein, 1998), so scientists tends to replace cell culture protocol with bacteriophage model assay where phages are used as simulants for mammalian viruses in medical applications (Aranha-Creado and Brandwein, 1998 and Dennehy, 2009). ΦX-174 and MS-2 bacteriophages were used as models in many virological studies, such as filtration size, medical and environmental virology applications (Lytle *et al.,* 1991and Jain and Srivastava, 2009).

The objective of this study was to determine the impact of phycobiliprotiens aqueous extract isolated from an Egyptian cyanobacterium *Synechococcus cedrorum* Sauvageau on enteric viral models as an antiviral agent.

2. Materials and Methods

2.1. Culturing of microalga (cyanobacterium)

The algal isolate was obtained from the culture collection of the Biotech International Research and Development (BIRD) Centre, Mansoura, Egypt, and grown on *Navicula* nutrient medium (Starr, 1978).The culture incubated and monitored for two weeks at 25 ±1°C under 1.2 ± 0.2 Klux light intensity with continuous illumination (Pelizer *et al,* 2003 and Walter *et al.,* 2003).

2.2. Preparation of algal extracts

The cyanobacterium biomass was harvested, centrifugation at 4000 rpm for 10 min (Murugan and Radhamadhavan, 2011) and washed twice. One gram of fresh biomass suspended in 100 ml of 0.1 M sodium phosphate (pH 7.0), containing 1 mM sodium azide, and subjected every 4 hrs for freezing and thawing cycles (Abalde *et al.,* 1998; Patel *et al.,* 2005 and Singh *et al.,* 2010). The clear supernatant was collected after centrifugation (4000 rpm/ 10 min/ 4°C) for spectrophotometeric analysis (Abalde *et al.,* 1998), then lyophilized and stored at 4°C till needed.

2.3. Analysis of phycobiliprotiens

Using a Unico UV–2000, UV–Vis spectrophotometer, the absorbance of phycocyanin (C-PC), allophycocyanin (APC) and phycoerthrin (PE) in the supernatants was measured at wavelengths 620, 652, and 562 nm respectively. The concentration of each pigment was calculated using the following equations (Sigelman and Kycia, 1978):

C-PC (mg ml^{-1}) = [A_{620} – 0.474 A_{652}] / 5.34,

APC (mg ml^{-1}) = [A_{652} – 0.208 A_{620}] / 5.09,

PE (mg ml^{-1}) = [A_{562} – 2.41 PC – 0.849 APC]/ 9.62

The purity of phycocyanin extracts evaluated by the ratios A620/A280 (Patel *et al.,* 2005).

2.4. PCR detection of HCV virus in serum

Bacteriophages MS-2, ΦX-174 and their hosts *Escherichia coli* (E. coli) ATCC-15597 and ATCC-13706 were generously provided by Prof. John Dennehy, Associate professor at Biology department, Queen College, City University of New York (CUNY).

2.5. Preparation of bacterial culture for phage infection

To determine exponential phase of each bacterial host its growth was measured photometrically by a colorimeter (GENWAY 6051, UK) at 600nm, and the bacterial counting CFU/ml was determined as described by Hall *et al.,* 2013.To ensure that bacterial culture was in mid exponential phase and showed the same behavior in each experiment, it was monitored for a couple of generation before the infection where the measured optical density (OD) was proportional to the number of cells per milliliter (cells/ml) (Carlson, 2005).

2.6. Virus enrichment

The strains of *E. coli* ATCC-15597 and ATCC-13706 were cultured twice, first and second cultivations were incubated at 37°C for overnight and 4 hrs, respectively (Klieve, 2005). The 4 hrs-aged bacteria were inoculated with the viruses (MS-2 and ΦX-174, respectively), and incubated overnight at 37°C. After lyses, the viruses harvested by centrifugation at 4000 rpm for 30 min at 4°C, and the supernatant filtered through 0.45μm syringe filter and used or stored at -20°C as a stock (Maillard *et al.,* 1994).

2.7. Phage quantification (antiviral activity assay)

2.7.1. Plaque reduction assay

Plaque reduction assay was carried out by using the overlay technique (double agar layers) to confirm the antiviral activity of the extracts. Briefly, phage was added to different concentrations of the crude aqueous extract (0.08, 0.32, 1.28, 5.12, 20.48 and 81.92 mg/ml) in a ratio of (1:1), incubated for 1hr at 4°C (Harden *et al.,* 2009). The mixture was added to soft agar containing bacteria at its exponential growth phase, vortex and overlaid on a solidified plate. The top agar allowed to solidify and then incubated overnight at 37°C (Shin *et al.,* 2003). The plaques were counted and the log10 reductions in the active phages were determined. Purified C-PC (0.0125, 0.025, 0.125, 0.25 and 0.5 mg/ml), ribavirin and acyclovir (0.008, 0.032, 0.128, 0.512, 2.048 and 8.192 mg/ml) were used as positive controls (Li *et al.,* 2005; Wang *et al.,* 2007 and Murugan and Radhamadhavan, 2011). Each treatment was run in triplicate and replicate at least twice.

2.7.2. Virions titration

Briefly, phage was added to different concentrations of the extracts in a ratio of (1:1), incubated for 1hr at 4°C (Harden *et al.,* 2009) and the mixture was added to the bacteria in a broth, vortex and incubated overnight at 37°C (Shin *et al.,* 2003).

Phage concentration (virions titer) was determined by use the formula developed by Day and Wiseman (1987). The measurement of concentration depends on absorbance at λ =269 and 320 nm using Unico UV–2000, UV–Vis spectrophotometer,(Day and Wiseman, 1987):

$$\text{Phage particles /ml} = \frac{(A_{269} - A_{320}) \times 6 \times 10^{16}}{\text{No. of bases per virion x vol. used}} \times 100 \times \text{dilution}$$

No. of bases/ phage for MS-2 = 3569 (O'Connell *et al.,* 2006); while that of ΦX-174 = 5386 (Campbell, 2007). Finally, the reductions in the activity were determined as percentage and as mentioned before positive controls were used and each treatment was run in triplicates.

3. Results

3.1. Growth curve of *Synechococcus cedrorum* Sauvageau

Synechococcus cedrorum Sauvageau (BIRD-70) was described as blue-green elongated, ellipsoidal finely rounded single cells or two cells together, 3-4μ broad and 5-10μ long and 1-2 time as long as broad.

It is found that *Synechococcus cedrorum* Sauvageau (BIRD-70) has the short life cycle; where its decline phase begins after the 7th day of inoculation. The average dry weight during the incubation period was representative for the algal growth, (Figure 1).

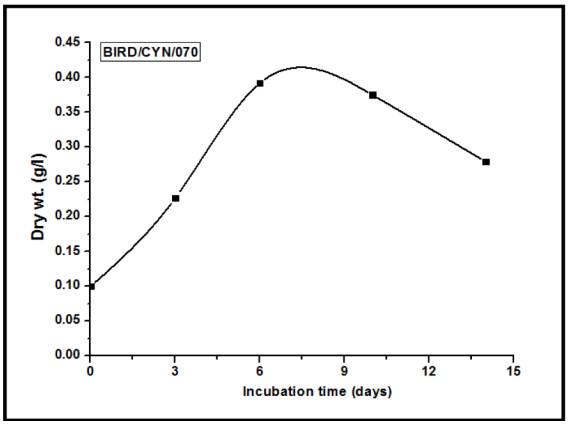

Fig. 1. *Synechococcus cedrorum* Sauvageau (BIRD-70) growth curve reflects its short life cycle.

3.2. Analysis of phycobiliprotiens

Concentration measurements of the tested extract is listed in Table (1) that illustrates the concentrations of C-PC, allophycocyanin (APC) and phycoerthrin (PE) as well as the purity of C-PC obtained from the crude extract.

Table 1. Absorbance of C-PC, APC and PE at 620nm, 652nm and 562 nm respectively, their concentrations in mg/ml and the purity ratio of C-PC:

	Conc. (mg/ml)				Purity
	PC	APC	PE	Tot. PBS	
BIRD-70	0.169	0.013	0.014	0.196	0.79

3.3. Antiviral activity assays

The effect of the algal extract was detected by plaques reduction assay and clarity method. This effect was compared to positive controls (pure PC, ACV and RIB) and negative one (bacteriophage without treatment), Table (2) and Table (3).

Table 2. Effects of different concentrations (mg/ml) of the crude aqueous extract, pure C-PC, acyclovir and ribavirin on the reduction of MS-2 phagetiters.

Treatment	Diff. conc. mg/ml	Overlay (PFU)			Clarity			
		PFU/ml (x 10^9)	Log $_{10}$ PFU	LR	A_{320}	A_{269}	Virions /ml (x 10^{11})	%
Crude Ex.	81.92	1.33	9.125	0.31	0.089	1.122	5.79	50.95
	20.48	1.39	9.145	0.29	0.067	1.158	6.11	48.20
	5.12	1.60	9.205	0.23	0.150	1.380	6.89	41.60
	1.28	1.64	9.215	0.22	0.185	1.458	7.13	39.55
	0.23	1.72	9.235	0.20	0.139	1.464	7.43	37.08
	0.08	1.80	9.255	0.18	0.055	1.440	7.76	34.24
Mean			9.169					41.94
pure C-PC	0.5	0.715	8.855	0.58	0.155	0.714	3.13	73.46
	0.25	0.767	8.885	0.55	0.322	0.912	3.31	71.98
	0.125	1.21	9.085	0.35	0.164	1.098	5.23	55.65
	0.025	1.24	9.095	0.34	0.055	1.022	5.42	54.08
	0.0125	1.68	9.225	0.21	0.048	1.254	7.30	38.18
Mean			9.029					58.67
ACV	8.192	1.63	9.213	0.222	0.196	1.458	7.07	40.08
	2.048	1.65	9.219	0.216	0.038	1.320	7.18	39.13
	0.512	1.68	9.225	0.210	0.112	1.410	7.27	38.37
	0.128	1.87	9.273	0.162	0.062	1.512	8.13	31.15
	0.032	1.99	9.299	0.136	0.002	1.542	8.63	26.88
	0.008	2.62	9.418	0.017	0.121	2.148	11.4	3.75
Mean			9.274					29.89
RIB	8.192	1.63	9.212	0.223	0.042	1.374	7.06	40.17
	2.048	1.65	9.217	0.218	0.442	1.716	7.14	39.51
	0.512	1.66	9.220	0.215	0.086	1.374	7.20	38.98
	0.128	1.67	9.224	0.211	0.017	1.278	7.26	38.51
	0.032	1.74	9.242	0.193	0.011	1.362	7.57	35.90
	0.008	1.90	9.280	0.155	0.010	1.464	8.26	30.01
Mean			9.232					37.18
Control		2.72	9.435	-	0.042	2.148	11.8	-

Abbreviations: PFU: Plaque Forming Unit, LR: Log10 reduction factor, C-PC: Cyanobacterial Phycocyanin, ACV: Acyclovir, RIB: Ribavirin

Table 3. Effects of different concentrations (mg/ml) of the crude aqueous extract, pure C-PC, acyclovir and ribavirin on the reduction of ΦX-174 phage titers.

Treatment	Diff. conc. mg/ml	Overlay (PFU)			Clarity			
		PFU/ml (x 10^5)	Log $_{10}$ PFU	LR	A_{320}	A_{269}	Virions /ml (x 10^{11})	%
Crude Ex.	81.92	0.242	4.384	1.75	0.528	0.750	0.824	98.23
	20.48	0.782	4.894	1.24	0.910	1.626	2.66	94.28
	5.12	1.79	5.254	0.88	0.282	1.932	6.13	86.82
	1.28	1.92	5.284	0.85	0.186	1.968	6.62	85.76
	0.23	1.97	5.294	0.84	0.360	2.190	6.80	85.38
	0.08	7.14	5.854	0.28	0.276	6.792	2.42	47.95
	Mean		5.16					83.07
pure C-PC	0.5	7.32	5.865	0.269	0.276	7.014	25	46.17
	0.25	11.96	6.078	0.056	0.528	11.520	40.8	12.19
	0.125	12.12	6.084	0.05	0.150	11.310	41.4	10.85
	0.025	12.37	6.093	0.041	0.186	11.565	42.3	9.10
	0.0125	12.46	6.096	0.038	0.204	11.685	42.6	8.28
	Mean		6.043					17.32
ACV	8.192	11.58	6.064	0.07	0.342	10.890	39.2	15.74
	2.048	11.85	6.074	0.06	1.032	11.820	40.1	13.82
	0.512	12.12	6.084	0.05	0.810	12.060	41.8	10.13
	0.128	12.40	6.094	0.04	0.882	12.240	42.3	9.27
	0.032	12.99	6.114	0.02	0.432	12.435	44.6	4.11
	0.008	13.60	6.134	0	0.082	12.600	46.5	0.0
	Mean		6.1					8.84
RIB	8.192	11.76	6.083	0.063	0.612	11.430	40.2	13.58
	2.048	12.09	6.087	0.051	0.906	12.030	41.3	11.14
	0.512	12.20	6.091	0.047	0.600	11.835	41.7	10.25
	0.128	12.32	6.096	0.043	0.630	11.955	42.1	9.53
	0.032	12.46	6.107	0.038	0.612	12.075	42.6	8.43
	0.008	12.78	6.083	0.027	0.108	11.880	43.7	5.96
	Mean		6.092					9.81
Control		13.60	6.134	-	0.082	12.600	46.5	-

Abbreviations: see table 2.

4. Discussion

The main objective of this study was to use an alternative approach in antiviral assays, by using bacteriophage models instead of mammalian viruses and the crude aqueous extract of *Synechococcus cedrorum* Sauvageau (BIRD-70) as a natural product to overcome ever evolving viral drug resistance.

One of the most important requirements for obtaining phycobiliprotiens from blue green algae is selection of extraction protocol where the extraction of these interest pigments performed via different way on the laboratory scale according to the selected organism al-

though sonication is the commonly used method with *Synechococcus* (Boussiba and Richmond 1979; Vernet *et al.,* 1990; Jeffrey and Mantoura, 1997; Abalde *et al.,* 1998 and Doke, 2005).

The results show that, *Synechococcus cedrorum* aqueous solution extracted with very simple and efficient thawing and dethawing methodyielded0.196 mg / ml with 0.79 C-PC purity which is reported as the better choice of extraction. This finds appeared to be in agreement with that found by Sarada and his coworkers who used homogenization of biomass in a virtimixer or in a mortar and pestle in the presence of distilled water or sodium phosphate buffer for cell breakage and phycobiliproteins extraction, they also found that the water extraction was the slowest process comparable to cell freezing-thawing and homogenization methods, as well as Abalde *et al*. found that the extraction with freezing at -21ºC and thawing at 4ºC in a buffer solution resulting high yield(Abalde *et al., 1*998 and Sarada *et al.,* 1999).

ΦX-174 and MS-2 were used as bacteriophage models for Enterovirus (EV71), HIV and HCV as water and blood-borneviruses are difficult to be cultured *in vitro* (Aranha-Creado and Brandwein, 1998 and Dennehy, 2009). So the present study used these phages as models for mammalian cell culture which is expensive, unsafe and time consumer (Hu and Aunins, 1997and Dennehy, 2009).

In general, the crude extracts were more effective in reducing the both models i.e. ΦX-174 and MS-2 when compared to Purified C-PC, ribavirin and acyclovir this observation seem to be similar to that obtained by Kok *et al.,* 2011. The plaque reduction assay shows that the antiviral activities of the crude aqueous extract of BIRD-70 achieved a dose-dependent inhibition on the viral models; where the highest conc. 81.92 mg/ml reduced titer by 1.75 and 0.31, while the lowest conc. 0.08 mg/ml gives 0.28 and 0.18 for ?X-174 and MS-2, respectively.

5. Conclusion

This study suggests the microalga *Synechococcus cedrorum* Sauvageau could be a potential source of antiviral compounds that can be used against enteroviruses, lentiviruses as well as blood-borne viruses.

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الملخص العربى

النشاط الضد فيروسي للمستخلص المائي لـ Phycobiliprotiens من طحلب *Synechococcus cedrorum* Sauvageau باستخدام لاقمات البكتيريا ΦX-174 / MS-2 كنموذج بديل

عـادل أحمـد المرسـى محمد إسماعيل عبد الحميد
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تستخدم الطحالب الخضراء المزرقة في العديد من تطبيقات التكنولوجيا الحيوية خاصة كمضادات للنشاط الفيروسي، ونظرا لمقاومة بعض الفيروسات للعقارات المستخدمة لعلاجها تعد المستخلصات الطحلبية من أهم البدائل المقترحة. استُخدِم طحلب *Synechococcus cedrorum* Sauvageau (BIRD-70) في هذه الدراسة كمصدر للمستخلصات الطحلبية ال (phycobiliproteins) تم الاستخلاص بطريقة التجميد والإذابة ل 1جم من الكتلة الحيوية للطحلب مذابة في 100مل من محلول فوسفت الصوديوم المنظم (pH=7) بتركيز 0.1 مولار يحتوي على 0.1 مليمولار من أزيد الصوديوم. كما استخدم كلا من MS-2 و ΦX-174 كبدائل للفيروسات الحيوانية، حيث يستخدم MS-2 كبديل للفيروسات المعوية والالتهاب الكبدي الوبائي (أ) (HAV) في اختبارات النشاط الضد فيروسي ,أما ΦX-174 فيستخدم كبديل لفيروس نقص المناعة (HIV) والالتهاب الكبدي الوبائي ب,ج (HCV, HBV) نظرا لتشابه كل منهم في الشكل. تم تعيين النشاط الفيروسي للمستخلصات الطحلبية عن طريق اختبار اختزال الروائق ال (plaques) باستخدام تقنية الآجار المزدوج. كذلك تم تقدير تركيز الفيروسات البكتيرية المعالجة بالمستخلص بكل من ال C-PC المنقى ،عقار الأسيكلوفيير وعقار الريبافرين كضوابط ايجابية والفيروسات الغير معاملة كضوابط سلبية. تبين من ذلك أن معاملات ال phycobiliproteins قللت تركيز ال MS-2 و ΦX-174 بنسبة 5.16 , 9.197 على التوالي والذي أكدته بدورها الطريقة اللونية حيث كانت نسبة الإختزال لكل منهما 83.07 و 41.94 %

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Anti-hepatitis Viruses of Phycobiliprotiens Aqueous Extract of the Cyanobacterium *Synechococcu scedrorum* Sauvageau Using Bacteriophages MS-2 / ΦX-174 as Model Systems

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