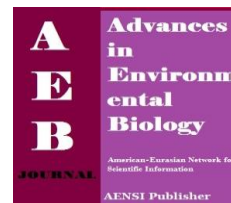




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### Implementation Antiviral Activity of Cyanobacteria Collected from Oil-Polluted Areas in South of Iran

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#### ABSTRACT

Cyanobacteria are worldwide distributed. They have potential applications such as nutrition, in agriculture and in wastewater treatment. In addition, they also produce wide variety of chemicals not needed for their normal growth (secondary metabolites) which show powerful biological activities such as strong antiviral, antibacterial, antifungal, antimalarial, antitumoral and anti-inflammatory activities useful for therapeutic purposes. In this study antiviral activity of cyanobacteria isolated from oil polluted area in south of Iran was evaluated. The aqueous extraction and methanolic extraction of *Leptolyngbya* ISC 40, *Anabaena* ISC 90, *Anabaena* ISC 88, *Anabaena* ISC 55 and *Leptolyngbya* ISC 25 was done. Their cytotoxic effects were evaluated on Lymphoblastoid Cell Line (LCL), HeLa and L929 cell lines by MTT assay. For antiviral activity, first LCL and HeLa cell lines which had EBV and HPV respectively, were treated by cyanobacterial extracts. Then DNA was extracted from treated cell lines and PCR was performed for capsid-coding region. Results were confirmed that cyanobacterial extracted had cytotoxic effects on LCL and HeLa cell lines. In addition viral load was significantly decreased in treated cells.

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#### INTRODUCTION

Cyanobacteria formerly known as green-blue algae are the oldest inhabitants of the Earth. They vitiate and are widely distributed throughout the world [1,2]. These species have noteworthy specific features. The investigation of cyanobacteria in different fields of science is of high significance. Being phototroph and not requiring organic matter in cyanobacteria cultivation are economic advantages to cyanobacteria in biological point of view which makes them superior over the other microorganisms. Besides, maintaining specific metabolic routes and limited information strengthen the possibility of acquiring compounds with new properties in this microorganism [3].

Their use in agriculture or wasted water recovery is among these consumptions. They also produce a wide range of secondary metabolites that even though they do not need them for growth, they have strong biological activities such as anti-viral, anti-bacterial and anti-fungal, anti-malarial, anti-tumor, and anti-inflammatory which are useful for therapeutic use [4,5]. Cyanobacteria Secondary metabolites have been used in medicine for more than two thousand years [6].

In recent years, several antimicrobial compounds have been isolated from cyanobacteria each of which has a wide range of effects on bacteria or fungi [7-11]. In addition, antineoplastic effects of cyanobacteria have also been investigated and it has been found that these properties concern with cyanobacteria secondary metabolites [12,13]. Studies have also shown the antiviral effects of some cyanobacteria species [14-17]. It has been specified that isolation environment, cultivation environment, Incobasion periods, temperature, and light intensity are the effective factors in cyanobacteria antimicrobial factors [18].

By the proliferation of the number of pathogenic microorganisms and drug-resistant cases, cyanobacteria are of great promise for the discovery and production of new drugs [19].

In this regard, the studies of Bechelli and his colleagues showed that the methanol extract of *Spirulina*, *Astaxanthin* (Ast), *Dunaliella salina* (Dun), *platensis* (Spir), *Aphanizomenon flos-aquae* (AFA) cyanobacteria on hematopoietic and leukemia cell lines has inhibitory effects [20]. Moreover, Kok and his colleagues reported the anti-cellular effects of methanol extract of *Synechococcus elongatus*, *Ankistrodesmus convolutes*, and *Spirulina*

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platensis cyanobacteria on three cellular lines of Burkitt's lymphoma (BL) including: Akata, B95-8, and P3HR-1 and also its anti-EBV effects [21]. Furthermore, Gustafson and his colleagues, profited by Tetrazolium-based microbial culture for examining the culturing aqueous cyanobacteria extract of *Lyngbia Lagrmy* and *Formydiom Teno* in controlling HIV-1 [22]. Ayehunie and his colleagues, revealed that the *Artrosyraplatnsis* aqueous extract at casual concentrations for human cells prevents the formation of Synsytom and the transcription of HIV-1 in T-cell lines of human, mononuclear cells of peripheral blood (PBMC), and Langerhans cells (LC).

Given that in recent years the antiviral properties of cyanobacteria have been proven and that they are of a wide range, there still remains a room for their investigation. On the other hand, the studied cyanobacteria in this research were isolated from the South and native oil-rich areas of the country (*Leptolyngbya* ISC 40, *Anabaena* ISC) that if their antiviral properties were examined, they would be very helpful and would be used as antiviral drugs in medicine and industry.

## Materials and Methods

### *Preparation of cyanobacteria strains:*

*Anabaena* samples used in this study numbered as ISC55, ISC88, and ISC90 and considered *Leptolyngbya* samples numbered as ISC40 and ISC25 purified as culture were provided from the special bank of culture and multiplication of microalgae in Shahid Beheshti University- Institute of Applied Sciences ...

### *Strain culture and proliferation:*

Among the specific culture mediums for *Anabaena* the BG-0 medium and for *Leptolyngbya* the BG-11 medium were used from the products of Merck company. Since the *Anabaena* type of cyanobacteria is capable of Nitrogen fixation, no Nitrogen source ( $\text{NaNO}_3$ ) was added to the culture medium. However, since the *Leptolyngbya* type of cyanobacteria is not capable of Nitrogen fixation, Nitrogen was added to the culture medium. Afterwards, the samples were incubated at the temperature of 25 to 30 degrees Celsius and the light intensity of 1500 to 2000 lux. Culture medium aerating was performed by aquarium pump.

### *Preparation of cyanobacteria aqueous extract:*

Under the laminar hood, the tested cells in the logarithmic phase (ISC55, ISC88, ISC90, ISC40 and ISC25) were sampled and each sample was poured in separated 1/5 ml micro-tubes and was centrifuged at 6000 g turn in 10 minutes and the scheme was discarded. Then 0/2 g of the sample was weighed on scale and was transferred to new 1/5 ml micro-tubes. Next, 1 ml PBS 1X and some [10] glass willows were added to them, respectively in order to be placed in the freezer for 1 hour. After an hour, all the micro-tubes were vortexed for 2-3 minutes and were then placed in the freezer for an hour repeatedly. This was repeated for 3-4 times until the samples became quite slippery. Finally, they were sterile by Syringe filter in order to be ready for the next step.

### *Preparation of cyanobacteria methanol extract:*

Under the laminar hood, the tested cells in the logarithmic phase (ISC55, ISC88, ISC90, ISC40 and ISC25) were sampled and were kept in Four at the temperature of 50 degrees Celsius for 24 hours. The completely dried and powdered-like cultured samples were then weighed in order to pour 0/12 g of each sample into Falcon. Later, 6 ml methanol and 10 glass willows were added to each falcon to be vortexed for 10 minutes. Again, 4 ml methanol was added to the falcons and the suspension was placed in ice for 2 hours. Then, the falcons were centrifuged at 16000 g turn at the temperature of 4 degrees Celsius for 15 minutes. The scheme was then transferred to another falcon and 2 ml deionized water per 8 ml methanol was added to it in a ratio of volume-volume. Culturing the human cell strains

Among the above cell lines, LCL (Lymphoblastoid Cell Line) concerns with the suspended cell line and HeLa concerns with the adherent cell line. These cell lines were obtained from Tehran Pasteur Institute. Since LCL and HeLa cell lines contain EBV and HPV viruses in their genomes respectively, they are called immortal. The cells were cultured in RPMI1640 medium which is the product of Merck company and which contains 10% FBS. Then the cells were transferred to a 96 wells flask.

### *The impact on cells:*

The experiment was repeated three times for each group. The prepared aqueous extracts were added to the wells at doses of 10, 20, 30, 50, 60, 70, 80, 90, and 100 mM and the prepared methanol extracts were added to the wells at doses of 1, 3, 5, 7, 9, 10, 20, 30, 40, 50, 70, 90, and 100 mM. Three wells containing 100  $\mu\text{l}$  cell and 100  $\mu\text{l}$  RPMI + FBS 10% culture medium were selected as control groups. The total volume of the whole tested wells reached to 200  $\mu\text{l}$  by adding RPMI + FBS 10%. Then the plate was placed in incubator of  $\text{CO}_2$  for 24 hours in order the desired material to have its effect on cells.

*Examining the Cytotoxicity:*

After the incubation, M.T.T test was used to measure the cell toxicity.

*Examining the antiviral effects:*

After examining the cytotoxicity, the methanol extract both alone and together with nanoparticles in a dose that not only was effective on but also was cultured the cells was injected to a flask in a greater volume (10 ml) in order to be placed in the incubator of CO<sub>2</sub> for 24 hours.

After 24 hours, in order to examine the antiviral properties of cyanobacteria the flask was taken out of the incubator and its cell DNA was extracted using DNA extraction kit produced by Fermentaz Company. Then the PCR reaction was performed using specific primers against the genes encoding the viral coat.

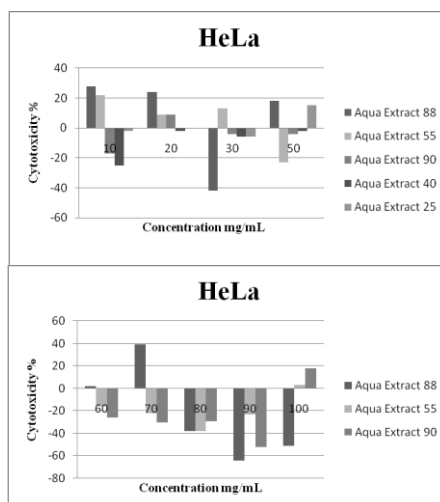
**Table 1:** Primer sequences used in this study.

Primer	Sequence	Gene
EA-1F	GGA GAT ACT GTT AGC CCT G	Epstein-Barr Virus
EA-2R	GTG TGT TAT AAA TCT GTT CCA AG	
MY11	GCMCAGGGWCATAAYAATGG	Human Papilloma Virus
MY09	CGTCCMARRGGAWACTGATC	

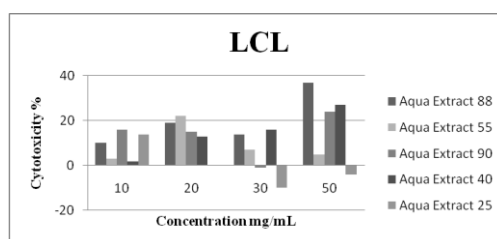
After PCR, the produced electrophores was studied in order to identify bands with the dimensions of Primer products, that is for HPV, 450 bp and for EBV, 208 bp.

*Results:*

The obtained results of the cytotoxicity testing of aqueous extracts of the samples on HeLa cell line showed that the highest cytotoxicity concerns with Anabaena 88 at the dose of 10 mg / mL. (Figure 1). These results are also obtained for LCL cell line related to Anabaena 88 at the dose of 50 mg / mL. (Figure 2).



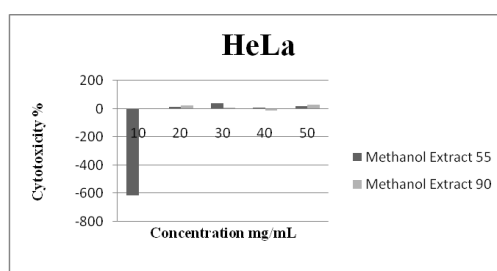
**Diagram 1:** The effect of aqueous extract samples of Anabaena ISC 90, 88, 55 and Lepto Lyngbya ISC 40, 25 on HeLa cells at a wavelength of 492 nm.



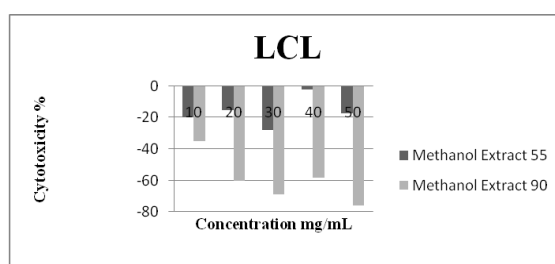
**Diagram 2:** The effect of aqueous extract samples of Anabaena ISC 90, 88, 55 and Lepto Lyngbya ISC 40, 25 on LCL cells at a wavelength of 492 nm.

However, the obtained results of the cytotoxicity testing of methanol extracts on HeLa cell line unraveled that the highest cytotoxicity relates to Anabaena 55 at the dose of 30 mg / mL. (Figure 3). Furthermore, the results of LCL cell line revealed that the sample did not have any cytotoxicity effect. (Figure 4).

The results also indicate that Anabaena ISC 90 and Anabaena ISC 55 samples could effectively reduce the expression of virus capsid gene. (Figure 1) According to the obtained results concerning HPV virus, the best result relates to Anabaena 55 which effectively could reduce the LOAD of the virus. The results concerning EBV virus relates to Anabaena 90 which could effectively reduce the LOAD of the virus. (Figure 2)



**Diagram 3:** The effect of methanol extract samples of Anabaena ISC 90, 55 on HeLa cells at a wavelength of 492 nm for 24 hours.



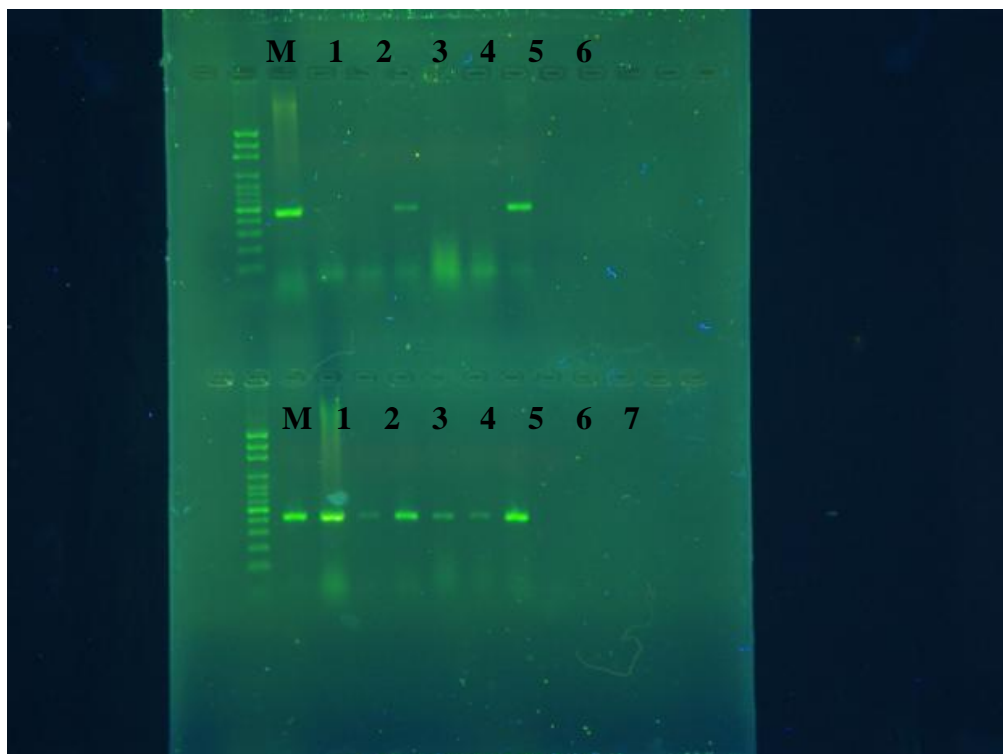
**Diagram 4:** The effect of methanol extract samples of Anabaena ISC 90, 55 on LCL cells at a wavelength of 492 nm for 24 hours.

#### Discussions and Conclusions:

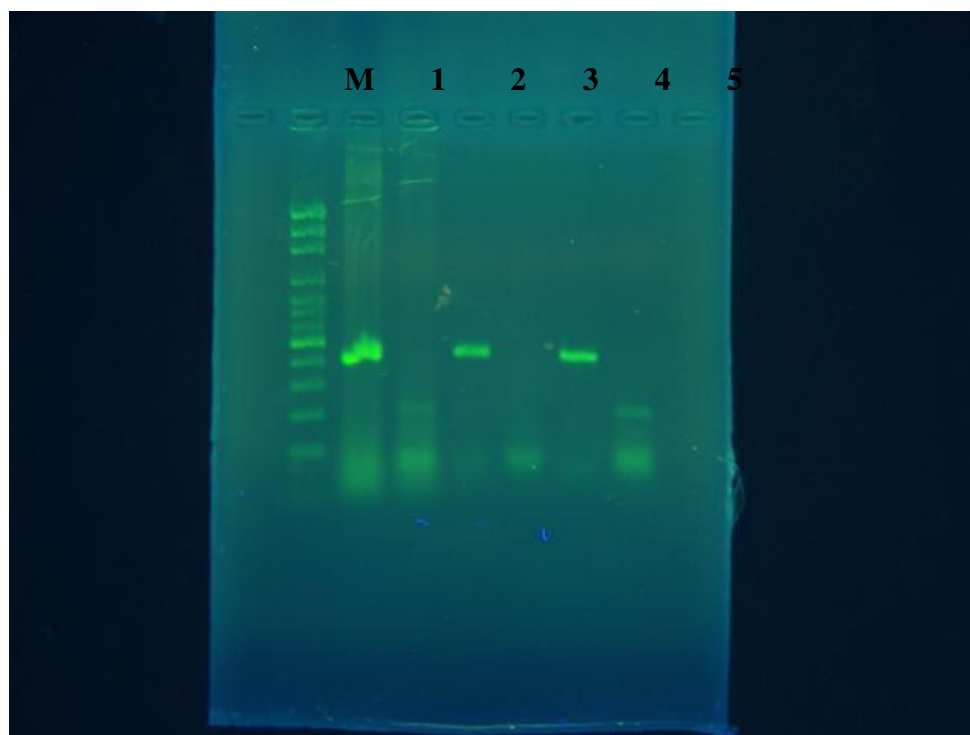
Investigating the effect of methanol extract of Astaxanthin (Ast), Dunaliella salina (Dun), Spirulina platensis (Spir), and Aphanizomenon flos-aquae (AFA) cyanobacteria on hematopoietic and leukemia cell lines, Bechelli and his colleagues, showed that Ast and AFA have inhibitory effects on HL-60, MV-4-11 (human leukemia cell lines), and primary CLL cells [20]. This study is somehow close to our method of study in that we similarly observed a decrease in cell survivals by increasing the concentrations of methanol and aqueous extract of the samples. Reviewing the impact of methanol extract of Ankistrodesmus convolutes, Synechococcus elongatus, and Spirulina platensis cyanobacteria on three cellular lines of Burkitt's lymphoma (BL) including Akata, B95-8, and P3HR-1 using the MTT assay methods (for investigating the cytotoxicity) and eventually the RT PCR testing (for studying the Epstein-Barr virus and Primer BamH1-W), Kok and his colleagues, reported that the methanol extract of Synechococcus elongatus had the greatest antiviral effect on B95-8 and P3HR-1 cell lines, but had no effect on Akata cell line [21]. This study is also very similar to our method of study in that the methanol and aqueous extracts of the samples which were investigated in cytotoxicity way revealed that among the samples, Anabaena ISC 90 and Anabaena ISC 55 samples of the methanol extract and Anabaena 88 sample of the aqueous extract had a greater impact on HeLa cell lines.

Ayehunie and his colleagues, indicated that the Artrosopyrplatisis aqueous extract at casual concentrations for human cells prevents the formation of Synsytom and the transcription of HIV-1 in T-cell lines of human, mononuclear cells of peripheral blood (PBMC), and Langerhans cells (LC) [22]. This research is also similar to ours in that the obtained extracts from the cyanobacteria samples inhibit transcription and ultimately control Epstein-Barr and papilloma viruses.

Hayashi and his colleagues, studied the extracts of 49 algae in order to inspect the anti-HSV and anti-HIV activities. The anti-HSV activity was observed in 25 aqueous extracts. In addition, 4 extracts had inhibitory capability. The anti-HIV transcription activity was found in 8 aqueous extracts [22]. This research is like our study in that it has derived the aqueous extracts of different cyanobacteria species and has examined their impact on different viruses. Likewise, the present study extracted the 5 species of cyanobacteria containing both aqueous and methanol extracts and examined their impact on two EBV and HPV viruses.



**Fig. 1:** Samples loaded in the wells from left to right are, respectively: 1 - Control HeLa, 2 - Dose of 150 mM + dose of 150 mM nanoparticles of the sample Anabaena 55, 3 - Dose of 450 of the sample Anabaena 55, 4 - Dose of 450 of the sample Anabaena 90, 5 - Dose of 350 of the sample Anabaena 90, 6 - Dose of 250 of the sample Anabaena 90, 7 - Dose of 150 of the sample of Anabaena 90. The top row is the PCP products of HPV primers and the bottom row is the PCR products of HGH primers (control).



**Fig. 2:** Samples loaded in the wells from left to right are, respectively: 1 - HGH control LCL, 2 - EBV control LCL, 3 - HGH sample of Anabaena 90, 4 - EBV sample of Anabaena 90, 5 - HGH sample of Anabaena 55, 6 - EBV sample of Anabaena 55.

## ACKNOWLEDGMENTS

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