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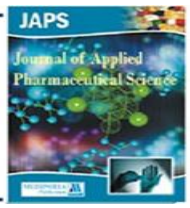

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Antiviral Activity of Freshwater Algae

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ABSTRACT

Five freshwater algal species were isolated from Nile River and studied for their biological (cytotoxic and antiviral activity) in order to test their benefit in the Egyptian drinking water source. The algal species were isolated and identified as: *Anabaena sphaerica, Chroococcus turgidus, Oscillatoria limnetica,* and *Spirulina platensis* **(**blue – green algae, Cyanobacteria) and *Cosmarium leave* (green algae). They were cultivated using a Photobioreactor and purified using BG11 media. Twenty five grams of each of the five powdered algal species were extracted with MeOH till exhaustion to give five methanolic extracts for ***A****nabaena sphaerica,* ***Ch****roococcus turgidus,* ***O****scillatoria limnetica,* ***S****pirulina platensis* and ***Co****smarium leave* respectively. The residues left were extracted with distilled H_2O at 50^oC to give five aqueous extracts respectively. The cytotoxicity of all the extracts was tested on Hep-2 cell line and their antiviral assays were tested on Adenovirus Type 40 as a preliminary testing. Nested PCR was carried out for confirmation of Adenovirus. Antialgal inhibitory effect on algal community was carried out. Results revealed that the non toxic concentrations for all the extracts were 2mg/ml and *Spirulina platensis* methanol and water extracts were active alga as antiviral (50% and 23.3% of reduction respectively).

Keywords: freshwater, algae, cytotoxic, antiviral.

INTRODUCTION

The prevalence of diseases such as cancer, human immunodeficiency virus (HIV)–acquired immune deficiency syndrome, hematological and autoimmune disorders is increasing rapidly. In recent years, the pharmaceutical industry focusing on the discovery of new bioactive compounds as antiviral agents and the main focus in recent decades for pharmaceutical discovery from natural products has been on microbial sources (bacterial and fungal), dating back to the discovery of penicillin from the mould fungus *Penicillium notatum* in the first half of the twentieth century (Singh *et al.,* 2011). The first investigation of the antibiotic activity of algae was carried out by **(**Pratt *et al.,* 1944).

Evidence of phycochemical and pharmacological studies on algae is available in the literature with special references to terpenoids and steroids (Parameswaran *et al.*, 1944; Patterson, 1968). Several screening studies have been carried out over the past years with the aim to discover new antibiotic or cytotoxic metabolites of microalgae especially green algae and cyanobacteria **(**Piccordi *et al.*, 2000; Ördög *et al.*, 2004) as well as to discover cyanobacteria which were toxic to other cyanobacteria or green algae (Schlegel *et al.*, 1998; Rainer, 2005) and as enzyme inhibitions (Cannell *et al.*, 1987; Sveshnikov *et al.*, 1997). Cyanobacteria have unique food storage compounds, myxophycean starch and cyanophycin. Besides the immune effect, blue-green algae improves metabolism, where blue green algae have cholesterol - lowering effect in animals and humans. The level of the total cholesterol in rate serum was reduced when a high cholesterol diet was supplemented with blue-green algae (Iwata *et al.*, 1990). In addition, substances controlling growth and behavior of aquatic microorganism's allelopathic substances (Honjo and Asakawa, 1990) and antitumor diglycosyl diacylglycerols (Tokuda *et al.*, 1996) have been reported. The aim of this work is to evaluate the benefit of methanol and water extracts of the five fresh water algal species as cytotoxic and antiviral activities.

MATERIAL AND METHODS

Isolation and Purification of microalgae Species

Five algal species *Anabaena sphaerica, Chroococcus turgidus, Oscillatoria limnetica, Cosmarium leave and Spirulina platensis* were isolated from phytoplankton community structure of River Nile. Algal identification has been carried out according to the keys of identification. Algal isolation and purification took place using BG11 media **(**Sayda *et al.*, 2010).

Cultivation of the Isolated Strains

Cultivation was carried out in sterilized 5 L. conical shoulder flasks containing 3 L. of the corresponding culture medium under continuous aeration and continuous illumination. The cultivation time differed from one strain to another depending on the optimum growth rate and it always ranged between 10-15 days.

Preparation of Algal Extract for biological Tests

Twenty five grams of each of the five powdered algal species were extracted several times with methanol till exhaustion to give five methanolic extracts for ***A****nabaena sphaerica, Chroococcus turgidus****,*** *Oscillatoria limnetica****, S****pirulina platensis* and ***Co****smarium leave* respectively. The residues left were extracted with distilled H_2O at 50^oC to give five aqueous extracts. All the extracts were used in biological evaluation tests.

Cytotoxicity assay of algal extracts on Hep-2 cell line

0.1 g of each of methanol and water extracts of the five algae dissolved in 500µl ethanol and then in 500µl water respectively. 12µl of 100x of antibiotic, antimycotic mixture was added. Ten fold dilutions of decontaminated samples were inoculated in Hep-2 cell line to estimate the non toxic dose of the algal extracts. Cytotoxicity assay was carried out using cell morphology evaluation by inverted light microscopy as described by Simões *et al.*, 1999 and by cell viability test trypan blue dye exclusion method as mentioned by (Walum *et al.*, 1990).

Antiviral Effect of Algal Extracts on Adenovirus Type 40

Non toxic dilutions were mixed (100µl) with 100µl of different doses of adenovirus type 40 ($1X10^4$, $1X10^5$, $1X10^6$). The mixture was incubated for 1/2 hr in 37°C. The inoculation of (100µl) 10 fold dilutions of treated and untreated Adenovirus was carried out into Hep 2 cell line in 12 multi well- plates. After 1 hr of incubation for adsorption at 37°C, 1 ml of media (Dulbecco´s Modified Eagle Medium, Gibco- BRL (DMEM) was added to each well. The cell line was observed daily for one week under the inverted microscope until cytopathogenic effect (CPE) appeared followed by three times freezing and thawing for tested plates. Nested PCR was performed for confirmation of adenovirus (presence/ absence) in each well According to Puig *et al.*, 1994.

The external primers hexAA 1885 (5-GCCGCAGTG GTCTTACATGCACATC-3) and hexAA1913 (5-CAGCACGC CGCGGATGTCAAAGT-3) were used in the first 30 cycles of amplification, and 1 µl was further added to a new batch of 50 µl of PCR mixture containing each nested primer pair, nehexAA1893 (5-GCCACCGAGACGTACTTCAGCCTG-3) and nehexAA1905 (5-TTGTACGAGTACGCGGTATCCTCGCGGTC-3) at 0.16 µM in a new 30 cycles amplification.

Antialgal Inhibitory Effect of Algal Extract

For testing the inhibitory effect of the algal extracts on the growth and community structure of phytoplankton assemblages of River Nile were used. The natural phytoplankton assemblages were collected from El-Gezira site (Cairo district) and concentrated using phytoplankton net (80µ mesh). Three main algal groups namely, green algae, blue-green algae and diatoms were present in the phytoplankton assemblages. The algal extracts were diluted in proportion with distilled water enriched with nutrient media suitable for algal growth as cited in National eutrophication Research Program, Pacific Northwest Environmental Research Lab, 1971.

RESULTS

The results of cytotoxicity assay revealed that the non toxic concentration for methanol and water extracts of all algal species was 2mg/ml. Data presented in Table (1) indicated that methanol extract of *Spirulina* showed a considerable antiviral activity in reduction of viral titre reached 50% in comparison with other species which showed lower antiviral activities. The reduction of viral titer amounted to 10% for methanol extracts of *Anabaena*, *Chroococcus*, *Oscillatoria* and 0% for *Cosmarium.*

The antiviral activity of water extracts of algal species is presented in table 2. water extract of *Spirulina* reached 23.3% while that of *Chroococcus* and *Oscillatoria* showed very low antiviral activity (3.3%) and water extract of *Anabaena* and *Cosmarium* had no activity (0%).

Table. 1: Antiviral activities of Methanol extracts of selected algal species on Adenovirus Type 40.

Algal species	Initial dose	Final dose	% Reduction	Average
Anabaena sphaerica	$1x10^4$	$8x10^3$	20%	10%
	$1x10^5$	$9x10^4$	10%	
	$1x10^6$	$1x10^6$	0%	
Chroococcus turgidus	$1x10^4$	$8x10^3$	20%	10%
	$1x10^5$	$9x10^4$	10%	
	$1x10^6$	$1x10^6$	0%	
Oscillatoria limnetica	$1x10^4$	$8x10^3$	20%	10%
	$1x10^5$	$9x10^4$	10%	
	$1x10^6$	$1x10^6$	0%	
Spirulina platensis	$1x10^4$	$4x10^3$	60%	50%
	$1x10^5$	$5x10^4$	50%	
	$1x10^6$	$6x10^5$	40%	
Cosmarium leave	$1x10^4$	$9x10^3$	10%	3.3%
	$1x10^5$	$1x10^5$	0%	
	$1x10^6$	$1x10^6$	0%	

Table. 2: Antiviral activities of water extract of selected algal species on Adenovirus Type 40.

Algal species	Initial dose	Final dose	% Reduction	Average
Anabaena sphaerica	$1x10^4$	$1x10^4$	0%	0%
	$1x10^5$	$1x10^5$	0%	
	$1x10^6$	$1x10^6$	0%	
Chroococcus turgidus	$1x10^4$	$9x10^3$	10%	3.3%
	$1x10^5$	$9x10^4$	10%	
	$1x10^6$	$1x10^6$	0%	
Oscillatoria limnetica	$1x10^4$	$9x10^3$	10%	3.3%
	$1x10^5$	$1x10^5$	0%	
	$1x10^6$	$1x10^6$	0%	
Spirulina platensis	$1x10^4$	$7x10^3$	30%	23.3%
	$1x10^5$	$8x10^4$	20%	
	$1x10^6$	$8x10^5$	20%	
Cosmarium leave	$1x10^4$	$1x10^4$	0%	0%
	$1x10^5$	$1x10^5$	0%	
	$1x10^6$	$1x10^6$	0%	

Data in Figure (1 A, B & C) showed that water extract of *Spirulina* showed a pronounced toxic effect on algal community structure. Algal species belonging to green and blue-green algae are highly affected by different concentrations used from water extracts Species of *Scenedesmus quadricuda*, *Selenastrum gracile* and *Scendesmus obliquus* (green algae) and species of *Oscillatoria limnetica*, *Microcystis aeruginosa* (blue-green algae) are continued to grow and give an increase in algal count up to the fifth day when algal culture treated with 25% water extract of *Spirulina*. Green and blue-green algal cells are highly affected and subjected to disintegration and completely disappeared from the algal culture.

Green and blue-green algal species are highly affected with other water extract of *Spirulina* concentrations (50% and 100%) and gave minimum detectable algal count starting from 3rd day (Fig. 1B and C) and up to the end of experiment period. Diatoms species are the most tolerant algal species to water extract of *Spirulina* at different tested concentrations. This may be due to that diatoms species having rigid silica frustules and also the filamentous form species are subjected to broken to small fragment. Diatoms species revealed detectable algal count up to 10th day such as *Melosira granulate*, *Cyclotella comta* and *Nitzschia linearis*.

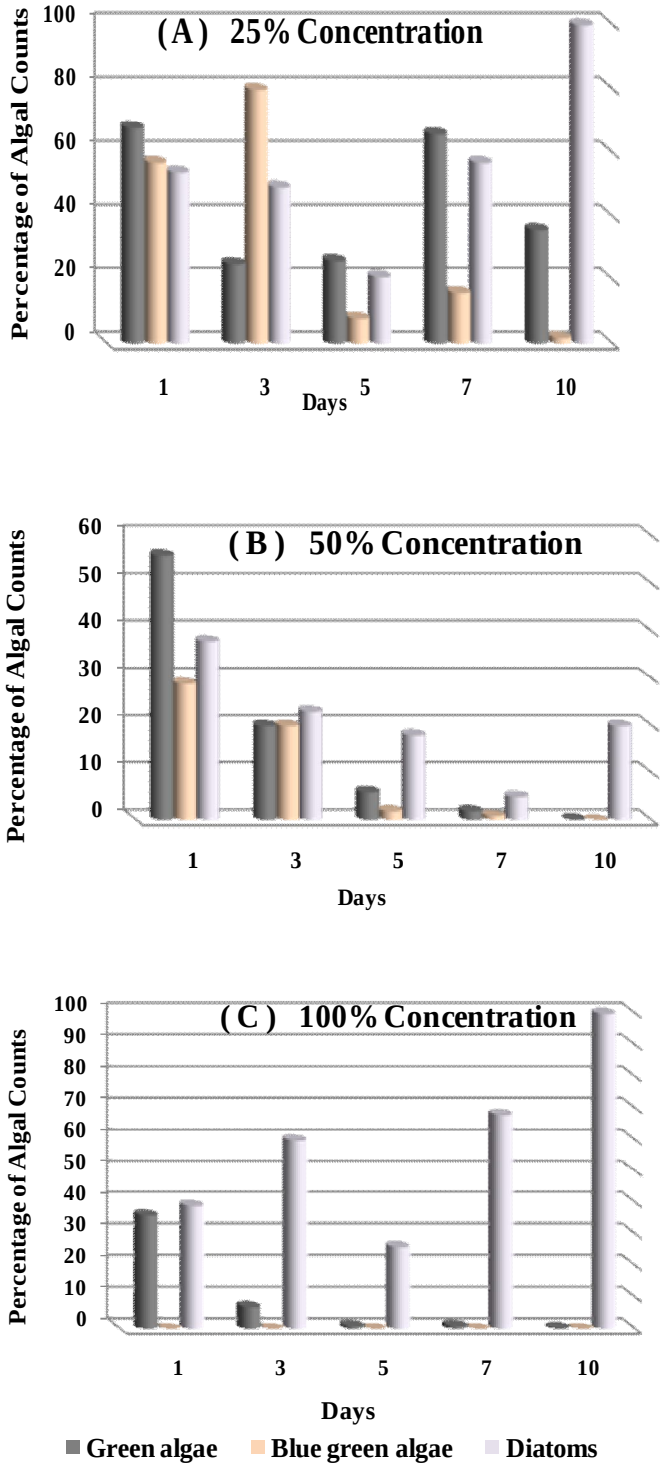

Fig. 1: Effect of water extract of *Spirulina platensis* on algal community structure as percentage of algal count.

DISCUSSION

Cytotoxicity assay revealed that the non toxic concentration for methanol and water extracts of all species was 2mg/ml. Methanol extracts of all selected species were analyzed for their antiviral activities on adenovirus Type 40.

A pronounced antiviral activity of Methanol extract of Spirulina was observed, where the reduction of viral titer reached 50%. These results are closely related to that reported by Chirasuwan et al. 2009, who observed that Methanol extract of Spirulina exerts antiviral effect on herpes simplex virus type 1 (HSV-1), with IC_{50} value of 25.1 µg/ml. They suggested that this effect is due to the presence of a compound called sulphoquinovosyl diacylglycerol (SQDG**)**. This compound contained palmitic acid and linoleic acid groups. It was reported that SQDG of some cyanobacteria (*Phormidium* and *Lyngbya*) possess both antiviral (human immunodeficiency virus type 1 (HIV-1) and antitumor activity (Shirahashi *et al.,* 1993; Loya *et al.,* 1998) which is in agreement with our previous publication which proved the presence of palmitic acid and linoleic acid with the percentages of 21.1% and 1.31% respectively in *S. platensis* **(**Sayda *et al.,* 2010).

Corona *et al.,* 2002, also reported that methanol extract of *Spirulina maxima* exhibited antiviral activity against HSV-2 with EC_{50} 6.9 mg/ml, and IC_{50} 0.13 mg/ml. They suggested that the antiviral activity could be due to highly polar compounds present in methanol extract.

The work of Ayehunie *et al.,* 1998, showed that water extract of *Spirulina platensis* inhibited human immunodeficiency virus type 1 (HIV-1) replication in human T-cell lines, peripheral blood mononuclear cells (PBMC). Extract concentrations ranging between 0.3 and 1.2 µg/ml reduced viral production by approximately 50% in PBMCs. The 50% inhibitory concentration (EC50) of extract for PBMC growth ranged between 0.8 and 3.1 mg/ml. In the present investigation, water extract of anabaena has no antiviral activity, while that of water extracts of *Chroococcus* and *Oscillatoria* have very low antiviral effect reached 3.3%. In contrast, water extract of *Spirulina* shows little inhibitory effect (23.3%) than that of methanol. This result is closely related to that reported by Corona *et al.,* 2002 who mentioned that hot water extract of *Spirulina maxima* inhibited the infection for adenovirus type 3 with a percentage less than 20%, with an IC_{50} 5.2 mg/ml. They also reported that the hot water extract of *Spirulina maxima* showed no cell growth inhibition at concentrations below 2 mg/ml. The antiviral activity of algal water extract might be attributed to the presence of sulfated polysaccharide **(**Singh *et al.,* 2011**;** Sayda *et al.,* 2010) where, for the last decade, algal sulfated polysaccharides have been extensively studied owing to their numerous biological activities including antiviral activities (Witvrouw and De Clercq, 1997; Zvyagimtseva *et al.,* 2000). It was reported that Spirulan (Sulphated polysaccharide composed of O-rhamnosyl-acofriose and O-hexuronosylrhamnose) showed activity against HIV-1 and HIV-2 (inhibit reverse transcriptase) HSV, influenza, They inhibit the reverse transcriptase activity of HIV-1 (like azidothymidine) (Singh *et al.,* 2011). Scytovirin is a 95 amino acid long was first isolated from the aqueous extract of *Scytonema varium*, it binds to the envelope glycoprotein of HIV and inactivates the virus in low nanomolar concentrations (Bokesch *et al.,* 2003).

It could be concluded that both methanol and water extracts of *Spirulina*, could be good sources as antiviral agents in comparison with the other algal extracts.

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