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ANTIVIRAL EFFECT OF EDAPHIC CYANOPHYTES ON RABIES AND HERPES-1 VIRUSES

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Five cyanophyte species *(Amorphonostoc punctiforme, Gloeocapsa turgidus, Sphaeronostoc coeruleum, Stratonostoc linckia* f. *spongiaeforme* and *Synechococcus cedrorum)* were isolated and identified from sandy Egyptian soils. Polysaccharides extracted from these species showed a pronounced antiviral activity against Rabies and Herpes-1 viruses represented by the absence of the characteristic cytopathic effects of these viruses. It was found that 100 µg polysaccharide/ml induced 100% inhibition of the two viruses which, depending on the polysaccharide concentration. Both of *Gloeocapsa turgidus* and *Synechococcus cedrorum* showed higher antiviral activity against rabies virus than that against herpes-1 virus. *Amorphonostoc punctiforme* showed nil to weak antiviral activity against both viruses. It was suggested that polysaccharides of such species of cyanophyte react against human and animal viruses. So, it could be concluded that there is a need for further studies to explain the mode of action of these substances on the replication of different viral origins to know how one deals with cyanophyte polysaccharides as antiviral substances in the most suitable and effective manner.

Keywords: Cyanophytes polysaccharide – antiviral activity – soil cyanophytes cultures – Rabies virus and Herpes-1 virus – viral drug development

INTRODUCTION

Most of the past therapeutic works used the higher plants and edaphic (soil) microbes as sources of medicines throughout history, but algae have been largely unexamined. Now microalgae are particularly attractive as natural sources of bioactive molecules which enable the production of structurally complex molecules which are difficult or impossible to produce by chemical synthesis. Thus the recent biotechnological researches on microalgae (especially cyanophytes) and macro- algae have received ever increasing interest for utilization of their extracts and derivatives as natural sources of a wide variety of antibacterial, antiviral, antifungal and pharmacologically active compounds [2, 26].

Cyanophytes (blue-green algae) are among the oldest photoautotrophic and dominant prokaryotic organisms in soil. Edaphic cyanophytes occur in nearly all terres-

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trial environments both on and beneath soil surfaces [20]. Commonly, they are among the prokaryotes that can exist as anhydrobiotic cells [30] and they were the first organisms to evolve oxygenic photosynthesis, and so changed the Earth's atmosphere from anoxic to oxic [13, 24]. They were begun to be studied widely since many can be cultured. Their cultivation without organic substrates can be an economical advantage over macro-algae. An optimized production of relevant compounds under controlled culture conditions is conceivable [18]. Recently, the screening of blue-green algae extracts and derivatives for therapeutics and pharmacologically active compound have been carried out. Cyanophytes polysaccharides of aquatic and soil taxa have found applications in many industrial sectors; agricultural treatment as a soil conditioner; in pharmaceutical applications and in biotechnological interest [1, 5, 23, 34]. Now it is possible to produce a large number of antiviral compounds (most of them are polysaccharides) which could not have been discovered earlier from many cultured blue-green algae and other microalgae [8, 15, 26]. Bacteria and fungi are much more productive than algae at this time, but algae generate complex and unique polysaccharides. Under optimum conditions, 15 to 55 percent of the weight of the microalgae can be extracted as polysaccharides [3].

A number of biological and synthetic sulfated polyanions, such as heparin, inhibit the replication of various mammalian viruses [36]. Similarly other sulfated algal polysaccharides selectively inhibit reverse transcriptase (RT) enzyme of human immunodeficiency virus (HIV) and its replication *in vitro* [21]. Sulfolipids of the blue–green algae *(Lyngbya, Phormidium, Oscillatoria* and *Anabaena)* have been used as AIDS substances to prevent viruses from either attaching to or penetrating into cells [17]. Compounds and extracts from blue–green algae, as well as other microalgae, showing HIV inhibitory activity are often active against other retroviruses such as herpes simplex virus types 1 & 2, simian immunodeficiency virus (SIV), cytomegalovirus, measles virus, mumps virus and influenza A virus [10].

The present work was designed to study the *in vitro* antiviral activity of polysaccharides extracted from various edaphic cyanophytes against rabies and herpes virus 1.

MATERIALS AND METHODS

Isolation and identification of algal organisms

In this work, the concerned cyanophyte taxa were isolated from Egyptian reclaimed sandy soils. In clean air-tight plastic bags soil samples were collected from the surface strata down to a depth of about 17 cm [12] from Al-Arish valley (North Sinai); South of El-Sheakh Zowaid village (North Sinai); Siwa and El-Bohyrat Almora soils. Samples of soils were brought immediately to lab. for culturing, identification, and isolation of algae.

For culturing, isolation and purification of algae, sterilized liquid and solid blue-green Rodhe's media [35] were used. Under aseptic conditions, about 0.1 g from the

investigated sandy soils was inoculated in 50 ml of sterilized liquid medium and incubated at 25 °C under continuous shaking and continuous florescent, white daylight with intensity of 4000 lux. After 15 days, and for each studied cyanophyte taxa, microscopic examination; identification and uni-algal isolation were done using different serial dilution on sterilized solid media (20 g agar-agar/one liter of liquid medium). Microphotographs also were obtained for them. Axenic culture of the investigated uni-algal isolates was obtained by repeating of uni-algal sub-culturing together with changing in pH (range from 4–8) of the previously used medium using NaOH and HCl buffer solution. pH of growth medium was measured by the laboratory pH meter (Knick digital pH meter, model: 643). When the algal growth of the tested species start to appear (after about one week), washing three times with sterilized distilled water was done. Finally microscopic examination was conducted by bacterial Gram-staining method (it was done three times for each inoculums) to detect the presence or absence of bacteria (whatever dead or life) in their cultures [22]. These were followed by the inoculation and incubation the obtained axenic algal inoculums into the previous growth medium and under previous conditions for two weeks.

Polysaccharide extraction

For isolation of intracellular polysaccharides of the different algal species, one g fresh weight of algal biomass was suspended in 2 ml ultrapure water and heated for 1 h at 100 °C. The aqueous extract was centrifuged for 30 min at 10,000 × *g*, dialyzed (using cellulose membrane) against deionized, ultrapure water for two days and then lyophilized. Dry material was dissolved in 10% trichloroacetic acid and soluble material was dialyzed again. Extracellular polysaccharides were isolated from culture supernatant by centrifugation of culture broth, lyophilization and dialysis against deionized and ultrapure water. Substances contained in the primary extract were further purified by precipitation with 80% ammonium sulfate and dialysis of the centrifuged supernatant. Both intra and extra polysaccharide mixed together then subjected for further investigation. An adequate amount (10 ml) of anthrone (0.1 g anthrone in 76% H_2SO_4) was added, and then boiled in water bath for 15 min. The tubes were cooled and measured at 620 nm. The concentration of the polysaccharide were determined finally from the standard curve of glucose and calculated as mg/g fresh weight [9].

Cells and viruses

Baby hamster Kidney (BHK-21, ATCC CCL 10) and Vero cell lines (ATCC, CCL 81 supplied from Egyptian Organization for Biological Products and Vaccines, VACSERA) were cultured using Eagle's minimum essential medium supplemented with 5% fetal bovine serum. These cells were used to estimate the cytotoxicity and antiviral activity of the tested samples. Both cell culture attenuated rabies virus (ERA-strain) propagated in BHK cell [7] and herpes virus 1 were supplied by the

Department of Pet Animal Vaccine Research, Veterinary serum and Vaccine Research Institute, Cairo. Virus titration was carried out using the microtiter technique [32]. Both viruses had a titer 10^7 $TCID_{50}$/ml.

Cellular toxicity assay

Each polysaccharide extract of different used algal species was diluted in Eagle MEM without serum to a final concentration of 100 µg/ml. These extracts were sterilized by filtration using 0.22 µm Millipore (Gelman Sciences) filter. The cytotoxicity assay was carried out on both BHK-21 and Vero cell lines using 0.1 ml cell suspension containing 10,000 cells seeded in each well of 96-well microtiter plate. Fresh media containing polysaccharide extract of each algal species were diluted twofold and added after 24 h of all seeding. Control cells were incubated without tested samples. The micro titer plate was incubated at 37 °C with 5% CO_2 for 72 h. The morphology of the cells was inspected daily for microscopically detectable alterations [14].

Cell infection and determination of the anti-rabies and herpes-1 viral activity

For rabies virus reduction assay, Confluent monolayer of BHK-21 in 96-well plates was inoculated with {25 µl of rabies virus ($100TCID_{50}$) and 25 µl of (100 µg/ml) of the test algal polysaccharides extracts}. Similarly, the confluent monolayer of Vero cells were inoculated with {25 µl of herpes virus 1 ($100TCID_{50}$) and 25 µl of the same test algal polysaccharides extracts}. The anti-rabies and anti-herpes activities were determined by the cytopatheic effect (CPE) inhibitory assay as described by [14], using untreated infected cells as control.

RESULTS

Algal identification

Mass algal growth of the studied algal cyanophytes appeared during two weeks from the incubation time. The investigated edaphic blue-greens were identified according to Desikachary [6] as: *Amorphonostoc punctiforme* (Kutz.) Elenk; *Gloeocapsa turgidus* (Kutz.) Nag.*; Sphaeronostoc coeruleum* (Lyngb.) Elenk; *Stratonostoc linckia* f. *spongiaeforme* (Ag.) Kutz.; and *Synechococcus cedrorum* Sauv. Synonyms (Syn.) and cultural description which accompanied by microscopic photographs were shown as follows.

Amorphonostoc punctiforme (Kutz.) Elenk (Syn. *Nostoc punctiforme* (Kutz.) Hariot). Isolated from Al-Arish valley soil (Fig. 1A). Cells were short barrel-shaped,

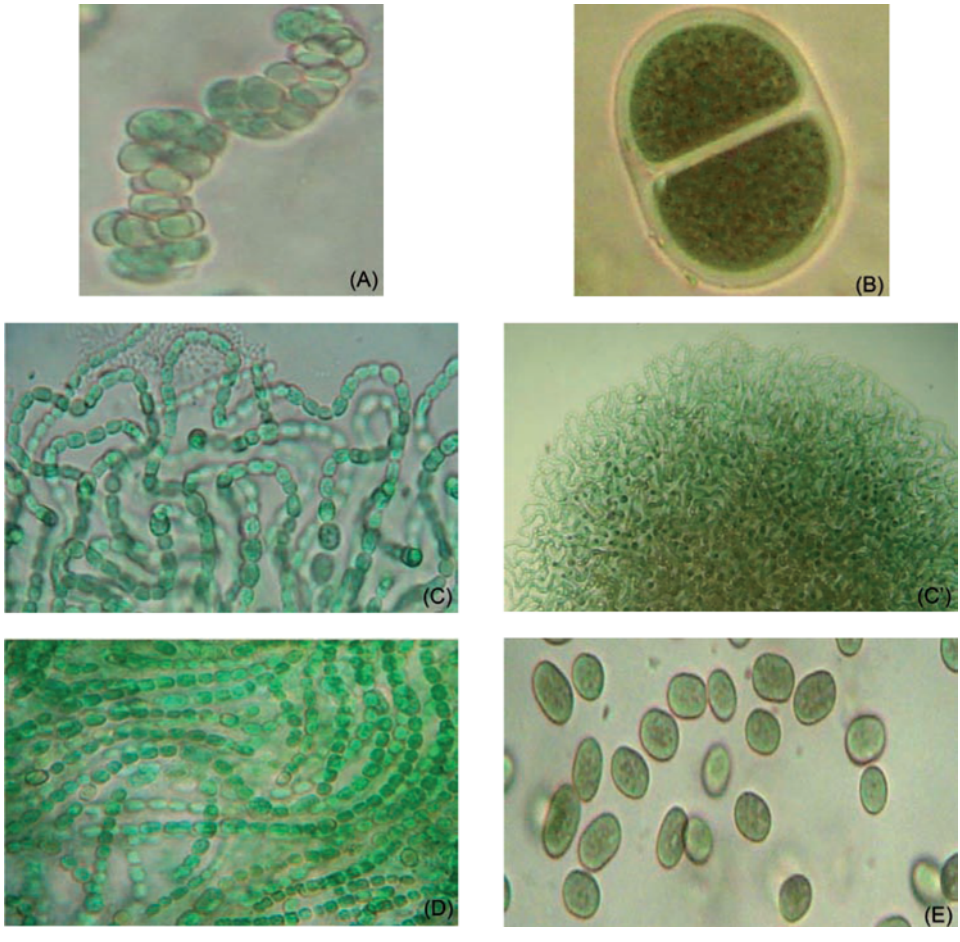

Fig. 1. (A) *Amorphonostoc punctiforme* (Kutz.) Elenk (3 μ broad and 4 μ long. Heterocysts, 4 μ in diameter. Spores are sub-spherical with 5 μ width). (B) *Gloeocapsa turgidus* (Kutz.) Nag. (colonies give 8–32 μ in diameter) (C and C') *Sphaeronostoc coeruleum* (Lyngb. Elenk (colonies are 5 mm in diameter and cell with 4 μ width). (D) *Stratonostoc linckia* f. *spongiaeforme* (Ag.) Kutz. (cells cylindrical and short, with 4 μ width. Heterocysts, 5 μ in diameter. Spores oblong, 5–6 μ broad and 7 μ long). (E) *Synechococcus cedrorum* Sauv. (cells with 2–4 μ width and 6 μ length)

3 μ broad and 4 μ long. Heterocysts, 4 μ in diameter. Spores were sub spherical and 5 μ broad. They became pure and axenic at (pH = 4).

Gloeocapsa turgidus (Kutz.) Nag. (Syn. *Chroococcus tugidus* (Kutz.) Nag.). Isolated from West of El-Bohyrat Almora soil (Fig. 1B). Cells are microscopic, coccoied spherical and single or in groups of mostly 2–4 cells. Colonies, 8–32 μ in diameter with colorless sheath. They became pure and axenic at (pH = 5).

Sphaeronostoc coeruleum (Lyngb.) Elenk (Syn. *Nostoc coeruleum* Lyngb). Isolated from Al-Arish valley soil (Fig. 1C and C'). Trichomes were densely entangled, cells were short barrel-shaped with 4 µ broad. They became pure and axenic at (pH = 4).

Stratonostoc linckia f. *spongiaeforme* (Ag.) Kutz. (Syn. *Nostoc spongiaeforme* Ag.). Isolated from El-Sheakh Zowaid village (North Sinai) soil (Fig. 1D). Trichomes were densely entangled. Cells were short, 4 µ broad, cylindrical in shape. Heterocysts 5 µ in diameter. Spores were oblong, 5–6 µ broad and 7 µ long. They became axenic only by repeating its sub-culturing.

Synechococcus cedrorum Sauv. (Syn. *Synechococcus cedrorum* Sauv.) isolated from Siwa soil (Fig. 1E). Cells were ellipsoid or slightly curved, solitary, microscopic, coccoied with light blue green colure (2–4 µ broad and 6 µ long). Sometime cells united pole to pole to form colony consisting of two cells. They became pure and axenic at (pH = 5).

Microalgae polysaccharides

All the tested microalgal species showed to contain polysaccharides but in different concentrations according to the species. The data represented in Figure 2 showed that *Gloeocapsa tugidus* give highest polysaccharide concentration followed by *Synechococcus cedrorum,* then *Amorphonostoc punctiform.* Both *Sphaeronostoc coeruleum* and *Stratonostoc linckia* f. *spongiaeforme* have approximately similar and low polysaccharides content (99.0 and 100.2 µg/ml) comparing to the previous species.

Antiviral effects of algal polysaccharides on human viruses

Cytotoxicity assay of the five polysaccharides algal extracts was performed before studying the antiviral activity. All algal extracts showed non-cytotoxic activity even at high concentrations. While the results of their polysaccharide extracts as antiviral

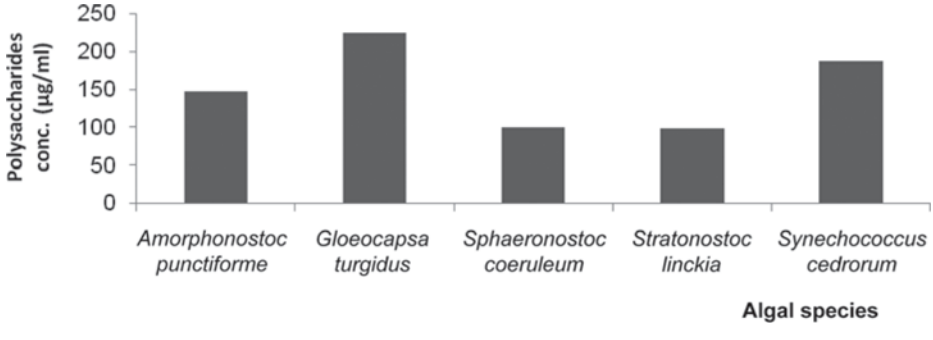

Fig. 2. The relation between polysaccharides concentration in µg/ml and the five tested soil cyanophytes species

Table 1
Antiviral activity of tested algal polysaccharide extracts on rabies and herpes viruses infectivity

Cyanophytes species	Conc. used for antiviral activity (µg/ml)	Percentage of anti-rabies inhibitory activity	Percentage of anti-herpes inhibitory activity
Amorphonostoc punctiforme (Kutz.) Elenk	100	100	–
	50	–	–
	25	–	–
	12.5	–	–
Gloeocapsa tugidus (Kutz.) Nag.	100	100	100
	50	100	100
	25	75	75
	12.5	75	–
Sphaeronostoc coeruleum (Lyngb.) Elenk	100	100	100
	50	50	50
	25	–	–
	12.5	–	–
Stratonostoc linckia f. *spongiaeforme* (Ag.) Kutz.	100	100	100
	50	75	75
	25	50	50
	12.5	–	–
Synechococcus cedrorum Sauv.	100	100	100
	50	100	100
	25	75	75
	12.5	75	–

activity against rabies and herpes viruses showed that both *Gloeocapsa turgidus* and *Synechococcus cedrorum* extracts exhibited high antiviral activity (75%–100%) even with serial polysaccharides dilutions and the rabies virus more affected than herpes virus (Table 1). *Sphaeronostoc coeruleum* and *Stratonostoc linckia* f. *spongiaeforme* extracts have moderate anti-rabies and anti-herpes activities (50%–100%) where this activity disappeared with decreasing polysaccharide concentration. They exhibited the same activity at the same concentrations against the two viruses (Table 1).The polysaccharide extract of *Amorphonostoc punctiforme* failed to show antiviral activity at all against herpes virus while, against rabies virus it exhibited high activity only at high polysaccharide concentration (100 µg/ml).

DISCUSSION

The studied terrestrial cyanophytes species are subjected to desiccation regularly in natural environments because they inhabit open areas of reclaimed sandy Egyptian soils and are completely air dried over periods of time. This extreme environment encompasses the investigated cyanoalgal species ability to produce conspicuous extra polysaccharides during the rehydration or culturing of desiccated cells and colonies [11, 33]. The present study revealed that of the polysaccharides cyanophytes content was depending on the algal species, where *Gloeocapsa turgidus* & *Synechococcus*

cedrorum have the highest content. The obtained results are morphologically unexpected due to the fact that all the used nostocales species *(Amorphonostoc punctiforme*; *Sphaeronostoc coeruleum* and *Stratonostoc linckia* f. *spongiaeforme)* are known to produce copious amounts of polysaccharides in the form of their jelly colonies; surrounding mucilaginous, gelatinous sheath and capsules. While chroococcales members *Gloeocapsa* give single spherical cells or in groups of mostly 2–4 cells with mucilaginous sheath and *Synechococcus* give oblong single cells or with colonies of two cells associated with very thin mucilaginous sheath. It was recorded that there is a relation between cell morphology and polysaccharides content where the cell surface ultra-structure change is followed by the changes of polysaccharide layer on cell wall surface [37]. The previous observation may refer to the cultured media and other growth conditions used in this study [25, 28]. Studying the effect of polysaccharide extracts of the tested edaphic nostocales and chroococcales against rabies and herpes viruses showed that, they did not exhibit cytotoxic effects when applied to cell culture at different concentrations. This is at least a pressive result for using these algal polysaccharides as antiviral agents. Studying the other microalgae toxicological activity based on certain biochemical components as Omega fatty acids of *Haemetococcus pluvialis, Tetraselmis suecica,* and *Chorella minutissima* exerted cytotoxic activity on MDCK, Vero, and Hella cell culture at 30:50 μg/ml, which limit the general use of these algae [31]. Martins et al. [19] reported also that both aqueous and methanol extracts of *Synechocystis* sp. exhibited strong apoptotic effects on HL-60 eukaryotic cell line.

The antiviral effect of these non-cytotoxic polysaccharides using different concentrations supported to the effectiveness of these polysaccharides in inhibiting both rabies and herpes viruses. However, polysaccharide concentrations were relatively parallel with the antiviral activity, when the polysaccharide and virus mixed before cell inoculation. Moreover, chroococcales members *(Gloeocapsa turgidus* and *Synechococcus cedrorum)* as antiviral agents extracts exhibited high antiviral activity (75%–100%) than the nostocales ones even with diluted polysaccharides, and the rabies virus was more affected than herpes virus. The present results are in agreement with the previous study which examining the lipophilic and hydrophilic extracts of 600 strains of cultured cyanophytes for antiviral activity against three pathogenic viruses [29], also recorded that the order chroococcales was commonly producing antiviral agents. The previous studies ruled out the possibility that part of the inhibitory effect was due to blocking some receptors, thus interferring with the penetration of the virus into the cell. There was a possibility of direct interaction between polysaccharides and virus particles reported before between herpes simplex virus and *Porphyridium* sp. Polysaccharides [15]. It has been suggested that these negatively charged molecules, including the sulfated algal polysaccharides, exert their inhibitory effect by interacting with the positive charges on the virus or on the cell surface and thereby prevent the penetration of the virus into the host cells. The microplate inhibition assay to study the antiviral effect of *Spirulina maxima* against several viruses (herpes simplex-2 virus, pseudorabies virus, human cytomegalovirus, adenovirus, measles virus, vesicular stomatitis virus, polio virus, and rota virus SA-11) [4]. They

reported highest antiviral activity with water and methanol extracts of this algal species. The acidic polysaccharides (Nostoflan) isolated from terrestrial algae (Nostoc flagelliforme) used as anti-herpes simplex virus. The results revealed that the inhibition of virus binding to but not penetration into host cell was responsible for anti-herpetic effect and suggested that this polysaccharide may be a potential anti-herpes agent [16]. Thus the current results suggested that, these polysaccharide extracts from soil cyanophytes (particularly, chroococcales members) are a good candidate as animal antiviral agents and further studies should be carried out to learn about their mode of action.

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