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Molecular authentication and characterization of the antiherpetic activity of the cyanobacterium *Arthrospira fusiformis*

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In recent years there has been an increasing interest for application of natural products as antiinfectives and concerns about the safety of synthetic compounds have encouraged more detailed studies of natural resources. Two different strains of the nontoxic cyanobacterium *Arthrospira* from the United States and Egypt have been characterized by sequence analysis of the intergenic spacer region of the phycocyanin gene. Both cyanobacteria were identified as *Arthrospira fusiformis* by phylogenetic tree analysis. The antiherpetic activity of crude aqueous extracts from the US and the Egyptian *A. fusiformis* isolates was determined. Antiviral activity against herpes simplex virus of cold water extracts, hot water extracts and phosphate buffer extracts from the American and the Egyptian strains was assessed in plaque reduction assays and their mode of antiherpetic action was analysed. In virus suspension assays, all extracts of the American cyanobacterium and the phosphate buffer extract of the Egyptian cyanobacterium inhibited virus infectivity by >90% in a dose-dependent manner. Phosphate buffer extract and hot water extract of the US cyanobacterium demonstrated the highest antiviral activity at low extract concentrations with high selectivity indices of 7464 and 542, respectively. The mode of antiviral action has been determined by addition of cyanobacterial extracts separately at different time periods during the viral infection cycle. Two extracts of the US *A. fusiformis* strain clearly inhibited herpesvirus multiplication before and after virus infection of host cells. In contrast, extracts of the Egyptian *A. fusiformis* strain affected only free herpes simplex virus prior to infection of host cells by direct inactivation of virus particles. In this study different *Arthrospira* crude extracts showed a significant antiviral effect and might be applied in recurrent herpetic infections.

1. Introduction

Arthrospira is a non-toxic cyanobacterium, well known as food additive and animal feed. It was shown to be rich in vitamins, minerals, proteins and fatty acids, activating immune response (Balachandran et al. 2006) and to produce anticarcinogenic (Schwartz et al. 1988) as well as antiviral substances (Hernàndez-Corona et al. 2002). *Arthrospira* was found to grow in Lake Maryut, a lake at southwest of Alexandria, Egypt (El Bestawy 1990), that contains brackish water and is heavily polluted. This lake has a different climate than the lakes in Mexico, Kenya and Chad, from which most of the studied *Arthrospira* strains have been isolated. This cyanobacterium excretes polysaccharides with antiviral effect (Rechter et al. 2006).

Herpes simplex virus type 1 (HSV-1) is a widespread human pathogen, which causes epidermal lesions in and around the mouth. The latent virus is reactivated spontaneously or is induced to reactivate by a variety of stimuli. During the reactivation process, the virus is transported through the nerve cells axons to the original peripheral infection site, where HSV replication occurs (Whitley and Roizman 2001). Antiviral agents currently licensed for the treatment of herpesvirus infections include acyclovir and derivatives, nucleoside analogues which function as DNA chain terminators, ultimately preventing elongation of viral DNA. Some of these antiviral agents might produce toxic side-effects. In addition, the emergence of virus strains resistant to commonly used anti-herpesvirus drugs is a growing problem, particularly in immunocompromised patients (Chakrabarti et al. 2000; Chen et al. 2000). A number of essential oils and plant extracts have been shown to induce an antiherpetic effect, acting before virus penetration, revealing a different mode of action than the commonly used drugs. They all represent promising antiviral agents for topical therapeutic application (Koch et al. 2008; Schnitzler et al. 2008a, 2008b; Reichling et al. 2008). Still there is a need for antiviral agents with different or combined modes of action from natural sources.

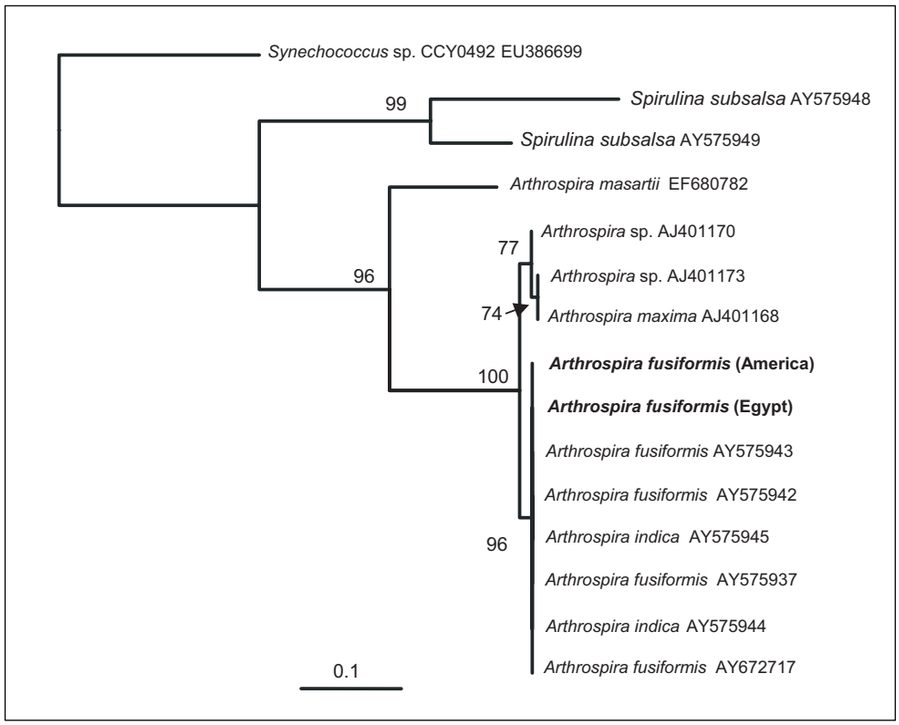

Fig. 1: Maximum likelihood tree based on PC-IGS sequences of 15 cyanobacterial strains, bootstrap values above 50 are included. Strains from this study are marked in bold, bar indicates 10% sequence divergence

Here we report the antiherpetic activity of different crude aqueous extracts from *A. fusiformis* isolated from Lake Maryut, Egypt, in comparison with A. fusiformis produced in California, United States, and commercially marketed. The identification of both cyanobacterial strains was performed by sequence analysis of the PC-IGS phycocyanin gene. Antiviral activity of cold water extracts, hot water extracts and phosphate buffer extracts from the Egyptian and the American cyanobacteria was assessed in plaque reduction assays and their mode of antiherpetic action was determined.

2. Investigations and results

Egyptian *Arthrospira* was isolated from Lake Maryut, Alexandria, Egypt, the American cyanobacterium was purchased from Greenvalley (San Diego, CA, USA). DNA sequences of the phycocyanin intergenic spacer region (PC-IGS) gene of both cyanobacterial strains were aligned using BIOEDIT software for molecular authentication. This analysis classified both, the US and the Egyptian cyanobacterium as *Arthrospira fusiformis* as presented in the phylogenetic tree (Fig. 1). The PC-IGS phylogenetic tree shows a tight cluster including both investigated *Arthrospira* strains and other *Athrospira fusiformis* and *Arthrospira indica* strains. The whole cluster is supported by bootstrap value of 96%. The PC-IGS tree supports the molecular genetic identification of both investigated strains as *Arthrospira fusiformis*. *Spirulina* strains were grouped in a separate cluster relatively distant from the *Arthrospira* strains.

Monolayer cultures of Rita cells were grown in 0.01–10% extract-containing medium and after 3 days of incubation, cell viability was determined using a standard neutral red assay. Extract concentrations which reduced cell viability by 50% (TC_{50}) were not detected for any of the extracts except for cold water extract of the US cyanobacterium ($TC_{50} = 5.5\%$). Maximum non cytotoxic concentrations of cold water, hot water and phosphate buffer extracts of the US cyanobacterium were 4%, 7% and 9% respectively. Extracts of the Egyptian cyanobacterium did not show any toxicity up to 10%.

The potential inhibitory effect of the extracts against free herpesvirus was determined by pretreatment of viruses with different extracts for 1 h at room temperature. In this virucidal assay, a 100-fold dilution of the extract-treated viruses was prepared and subsequently cells were infected. Increasing extract concentrations were applied and extract concentrations inhibiting virus infectivity by 50% (IC_{50}) were determined from dose-response curves. A clear dose-dependent antiviral activity of the extracts was demonstrated for the US (Fig. 2) and the Egyptian cyanobacterium (Fig. 3). In all experiments

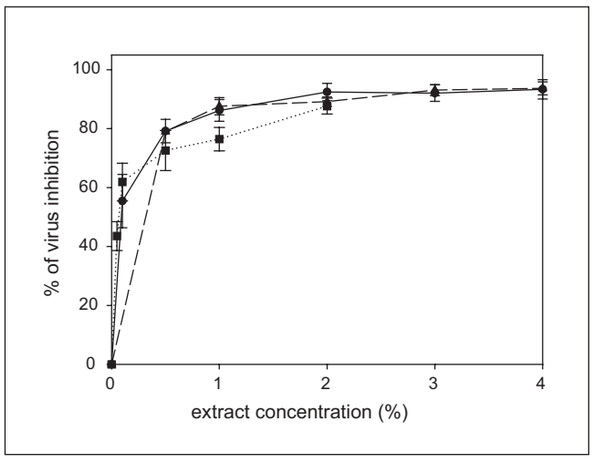

Fig. 2: Determination of the 50% inhibitory concentration (IC_{50}) of crude extracts of US *Arthrospira fusiformis* for herpes simplex virus. Viruses were incubated with increasing concentrations of the extracts for 1 h and inhibition of viral infectivity was tested in a plaque reduction assay. Data represented are the mean of three independent experiments. (—●—) cold water extract, (– –▲– –) hot water extract, (···■···) phosphate buffer extract

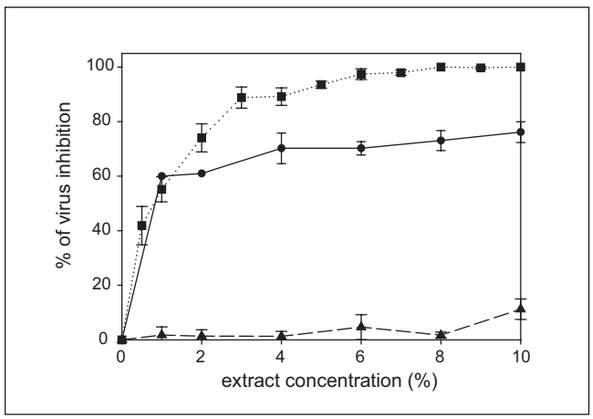

Fig. 3: Determination of the 50% inhibitory concentration (IC_{50}) of crude extracts of Egyptian *Arthrospira fusiformis* for HSV. Viruses were incubated with increasing concentrations of the extracts for 1 h and inhibition of viral infectivity was tested in a plaque reduction assay. Data represented are the mean of three independent experiments. (—●—) cold water extract, (– ▲ –) hot water extract, (····■····) phosphate buffer extract

infected cells without addition of extracts were used as control. Phosphate buffer extract and hot water extract of the US cyanobacterium demonstrated the highest antiviral activity at low extract concentrations, with high selectivity indices of 7464 and 542, respectively. All extracts of the American cyanobacterium inhibited herpesvirus by >90%, whereas the cold water extract and phosphate buffer extract of the Egyptian strain inhibited herpes simplex virus by 75% and >90%, respectively. The phosphate buffer seems to contain more antiviral substances when compared with the cold water extract. In contrast, the hot water extract of the Egyptian cyanobacterium did not reveal any antiviral activity against herpesvirus, which might be due to a thermal sensitivity of the active compound.

To identify the step at which viral multiplication might be affected by cyanobacterial extracts, cells were infected with HSV-1 after preincubation of cells with extracts, after pretreatment of the virus with extracts prior to infection, or extracts were added during the intracellular replication period. In all experiments infected cells without addition of extracts or in the presence of the synthetic herpesvirus inhibitor acyclovir were used as control. The inhibition of viral multiplication was calculated relative to the amount of virus produced in the absence

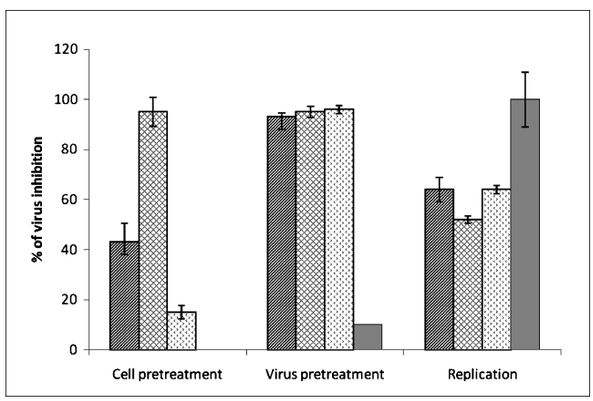

Fig. 4: Antiviral effect of US *Arthrospira* extracts in comparison with acyclovir against herpes simplex virus by incubation at different periods of time during infection. Cells were pretreated with the extracts prior to virus infection (cell pretreatment), viruses were pretreated with the extract for 1 h (virus pretreatment) or extracts were added 1 h after the penetration of viruses into the cells (replication). Data represented are the mean of three independent experiments. (▨) cold water extract, (▩) hot water extract, (▣) phosphate buffer extract, (▩) acyclovir

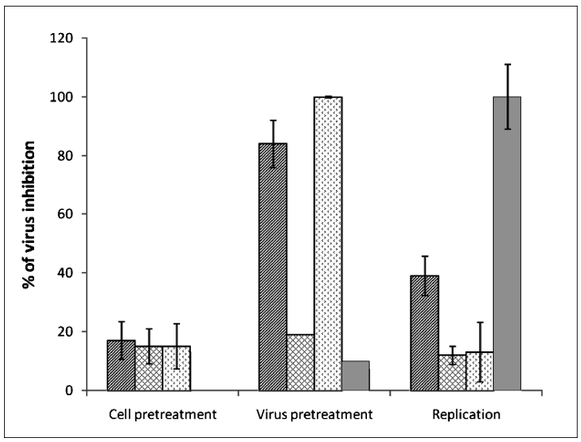

Fig. 5: Antiviral effect of Egyptian *Arthrospira* extracts in comparison with acyclovir against HSV by incubation at different periods of time during infection. For details see Fig. 4. Data represented are the mean of three independent experiments. (▨) cold water extract, (▩) hot water extract, (▣) phosphate buffer extract, (▩) acyclovir

of the extract. In all experiments the maximum noncytotoxic concentrations of the US cyanobacterial extracts or 10% nontoxic concentrations of the Egyptian cyanobacterial extracts were used. Extracts of US *Arthrospira* were effective at different phases of viral infection. When cells were pretreated with US cyanobacterial extracts, only the hot water extract revealed an influence on viral infection and inhibited virus multiplication by 90% (Fig. 4). However, all extracts of the American cyanobacterium demonstrated a >90% inhibition of HSV when virus was pretreated with extracts (Fig. 4). A less pronounced antiviral effect was observed when different US cyanobacterial extracts were added to infected cells during viral replication. In contrast, only the cold water extract and the phosphate buffer extract of the Egyptian *Arthrospira* inhibited viral infection when HSV was pretreated with these extracts (Fig. 5). Extracts of the Egyptian cyanobacterium did not show an antiviral effect during other phases of viral infection. Acyclovir showed the highest antiviral activity when added during the replication period with inhibition of the viral replication of >98%. This drug inhibits specifically the viral DNA polymerase during the replication cycle when new viral DNA is synthesized. However, only minor effects on viral infection were detected when cells or viruses were pretreated with acyclovir.

3. Discussion

The pharmaceutical industry is increasingly targeting natural products with the aim of identifying lead compounds, focusing particularly on suitable alternative antiviral agents. Several drugs are currently available for the management of HSV infections such as acyclovir. Acyclovir and related synthetic nucleosides interfere with viral DNA replication through activation by viral thymidine kinase. Incidence and severity of disease produced by herpes simplex virus have been increasing in recent years, especially in the immunocompromised host where viral resistance to acyclovir represents a particular problem. Topical treatment of herpes labialis infection is standard, for the most part carried out with acyclovir creams, but also with phytotherapeutics preparations containing lemon balm oil (Wölbling and Leonhardt 1994).

In the present study, the antiherpetic effect of several aqueous extracts derived from an American and an Egyptian strain of the cyanobacterium *Arthrospira fusiformis* were compared and their mode of antiviral action determined. Both *Arthrospira* strains cluster tightly in the PC-IGS phylogenetic tree with other

Athrospira fusiformis and *Arthrospira indica* strains indicating the molecular authentication of both strains. Experiments to assess the cytotoxicity of the aqueous cyanobacterial extracts for cultured eucaryotic cells indicate low toxicity in cell cultures. Besides the commonly used cold water and hot water extracts, an additional extraction procedure with a phosphate buffer was performed in order to extract phycocyanin pigments as well. With the exception of the hot water extract of the Egyptian cyanobacterium, all other tested extracts of both *Arthrospira* strains exhibited high levels of antiviral activity against HSV-1 in viral suspension tests. Plaque formation was significantly reduced by >90% for most crude extracts indicating a high dose-dependent antiviral activity. Pretreatment of the cells with hot water extract of the American *Arthrospira* revealed an inhibition of herpesvirus by >90%, whereas both other aqueous extracts had only minor influence on virus multiplication. However, the cold water extract, hot water extract and phosphate buffer extract demonstrated high antiviral activity when viruses were pretreated with extracts prior to cell infection and these extracts revealed also a moderate antiviral activity during the intracellular replication phase of herpes simplex virus. These results indicate that extracts of the American cyanobacterium possess a broad spectrum of antiviral activity during different phases of the viral multiplication cycle. In contrast, the widely used antiviral drug acyclovir is only effective during the intracellular replication of herpes simplex virus. Extracts of the Egyptian *Arthrospira* affected herpesvirus mainly when viruses were incubated with cyanobacterial extracts prior to infection. Thus for the cold water extract and the phosphate buffer extract of the cyanobacteria from Lake Maryut in Egypt a virucidal activity could be clearly demonstrated, a mode of action which is quite different from the acyclovir antiviral mechanism.

Antiviral activity of aqueous extracts of *Arthrospira platensis* as well as *A. maxima* have been reported previously (Hernandez-Corona et al. 2002). It was found that sulphated polysaccharides were antivirally effective (Witvrouw and De Clercq 1997) by blocking virus adsorption and/or penetration (Schaeffer and Krylov 2000; Dey et al. 2000; Huleihl et al. 2001). Through further purification of *Arthrospira* extracts calcium spirulan was isolated and was shown to inhibit virus penetration (Hayashi et al. 1996; Hernandez-Corona et al. 2002). The inhibition of intracellular replication by the US *Arthrospira* strain could be interpreted as inhibition of protein synthesis as demonstrated previously (Lee et al. 2000). In addition the hot water extract of the American cyanobacterium showed a high virucidal effect with a selectivity index superior to the reported isolated and purified calcium spirulan, which is considered to be the most effective compound in this extract (Hayashi et al. 1996). Combined modes of antiviral action displayed by polysaccharide compounds are mainly determined by molecular weight, structure, and sulfation, affecting viral receptor binding, entry and cell-to-cell spread and only in some cases with additional virucidal activity (Gosh 2009). The sulfate groups as well as the carboxyl groups of polysaccharides have negative charges which can react with the basic amino acids of viral proteins and block the interaction with cellular components. The difference in antiviral action between the two *Arthrospira* isolates might be explained partially by differences in cultivation. The US strain was grown in big lagoons, harvested at the log phase and dried by spray-drying, whereas the Egyptian strain was grown in a laboratory, harvested in the stationary phase and dried by lypholization. It is suggested that the tested extracts of the US strain most probably contain a number of antiherpetic compounds having different modes of action, some acting as strong virucidal and some inhibiting intracellular virus replication or cell-to-cell spread. Allophycocyanin extracted and purified from *A. platensis* was found to inhibit enterovirus 71-induced apoptosis, also suggested to interfere with a very early stage of viral replication such as virus adsorption and penetration (Shih et al. 2003). Since allophycocyanin showed antiviral effects, it was suggested that the other pigments, such as c-phycocyanin, might also be effective, therefore the anti-herpetic effect of a phosphate buffer extract, which is supposed to extract pigments, was also tested in this study. The phosphate buffer extract in both strains, showed the highest virucidal effect. As purified c-phycocyanin did not reveal any antiherpetic effect (data not shown), the effective compound might be allophycocyanin (Shih et al. 2003). This extract is the only one with absolutely no effect on cell pretreatment. Natural products containing bioactive compounds are sometimes more effective in their natural combination, rather than in a pure concentrated effective compound with identified composition (Astani et al. 2008). Considering the abundant availability and cheap production of *Arthrospira*, it is a promising topical therapeutic agent in the treatment of recurrent herpes infection.

4. Experimental

4.1. Cultivation of Arthrospira

Egyptian *Arthrospira* strain isolated from Lake Maryut, Egypt, was grown in Zarrouk medium at room temperature. The biomass of this cyanobacterium was resuspended in distilled water, frozen at −80 °C and then lypholized. American *Arthrospira* cultivated in California was obtained from Greenvalley (San Diego, CA, USA).

4.2. Molecular identification of cyanobacteria by phylogenetic analysis

Both cyanobacterial strains were examined and confirmed microscopically. Since the number of variable positions is low in the 16S rDNA, the more variable non-coding sequence of the intergenic spacer region of the phycocyanin operon between *cpc*B and *cpc*A subunit (PC-IGS) was used for phylogenetic analysis (Neilan et al. 1995; Baurain et al. 2002). Sequence analysis of the phycocyanin gene (PC-IGS) was performed after DNA extraction and PCR amplification. Briefly, DNA was extracted from 1 ml cyanobacterial culture by addition of autoclaved 0.5 g zirconium beads, 600 µl 120 mM sodium phosphate buffer and 100 µl of a 25% solution of sodium dodecylsulfate (SDS). After vortexing of the mixture for 10 min, the suspension was centrifuged at 12 000 rpm for 6 min. The supernatant was transferred into a 2 ml Eppendorf tube. The pellet was washed with 500 µl sodium-phosphate buffer and vortexed thoroughly. The supernatant was added into the same Eppendorf tube and 200 µl lysozyme (10 mg/ml in TE buffer) were added. After incubation at 37 °C for 15 min, 150 µl 25% SDS and 10 µl proteinase kinase (20 mg/ml) were added, incubated at 60 °C for 15 min. After separation and ethanol precipitation of the extracted DNA, the pellet containing genomic cyanobacterial DNA was dissolved in 150 µl TE-buffer.

PCR amplifcation of PC-IGS was done using the Taq PCR Core Kit (Qiagen GmbH, Hilden, Germany) and a Peltier Thermal Cycler PTC 200 MJ Research, Inc. (San Francisco, CA, USA). The PCR reaction mixture contained 0.1 µl Taq DNA polymerase (5 units/µL), 0.5 µl dNTPs (10 mM), 2 µl 10× buffer, 1 µl forward primer (10 pmol/µl) cpc_arF 5′ TCG AAG ATC GTT GCT TGA ACG 3′, 1 µl reverse primer (10 pmol/µl) cpc_arR 5′ TTA GGT CCC TGC ATT TGG GTG 3′ and 1 µl genomic DNA in a total volume of 20 µl (Ballot et al. 2004). The following program was used for the PCR amplification of PC-IGS: an initial denaturation step of 3 min at 94 °C, 30 cycles at 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 1 min with a final elongation step of 72 °C for 5 min. PCR products were visualized by standard agarose (1.5%) gel electrophoresis and ethidium bromide (1 mg/ml) staining. The amplified PCR products were purified using QIAquick spin columns (QIAquick Gel Extraction Kit, Qiagen GmbH, Hilden, Germany) and both strands were sequenced on an ABI 3100 Avant Genetic Analyzer using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's manual. The reaction mixture contained: 1 µl BigDye Terminator 3.1v, 1.5 µl 5× sequencing buffer, 1 µl forward or reverse primer (10 pmol/µl), 4 µl of DNA template and 2.5 µl H_2O. The following program was used: 1 min at 96 °C, 25 cycles of 20 s at 96 °C, 20 s at 50 °C and 4 min at 60 °C. For sequencing of PC-IGS the same primers cpc_arF and cpc_arR as for the PCR were used. The sequence data were submitted to EMBL. Accession numbers are FN433881 (*Arthrospira fusiformis* ESCP, produced in California, United States) and FN433882 (*Arthrospira fusifomis* LM, isolated from Lake Maryut, Egypt). Phylogenetic analysis of *Arthrospira* strains was performed by comparison

of PC-IGS sequences with sequences in GenBank. The sequences were aligned using BIOEDIT, phylogenetic tree for PC-IGS was constructed using maximum likelihood algorithm in the program PAUP * v 10b (Swofford 2002). For maximum likelihood, TrN+I evolutionary model of substitution was evaluated using the AIC criterion in Modeltest v.3.06 (Posada and Crandal 1998).

4.3. Preparation of Arthrospira extracts

For cold water extract, 50 g dry *Arthrospira* were suspended in 500 ml cold water, stirred for 1 h then left for 24 h at 4 °C. The suspension was first centrifuged at 15 300 g for 1 h, then the supernatant was centrifuged at 24 000 g for 1 h and sterile filtered through a filter with 0.22 μm pore size. For the hot water extract, 50 g dry *Arthrospira* were suspended in 500 ml boiling water, the suspension was boiled for 1 h and left for 24 h at 4 °C. The suspension was centrifuged and filtered as mentioned in the previous extract. Besides the commonly used cold water and hot water extracts, an additional extraction of the cyanobacteria with a phosphate buffer was performed in order to extract pigments as well. The phosphate buffer extract was prepared as follows: 50 g dry *Arthrospira* were suspended in 500 ml phosphate buffer (61.1 mM K_2HPO_4, 38.9 mM KH_2PO_4), pH 7, stirred for 1 h then left for 24 h at 4 °C, centrifuged and filtered as mentioned above.

4.4. Cell culture and herpes simplex virus

RC-37 cells (African green monkey kidney cells) were grown in monolayer culture with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 100 μ/ml penicillin and 100 μg/ml streptomycin. The monolayers were removed from their plastic surfaces and serially passaged. Cells were plated out onto 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and propagated at 37 °C in an atmosphere of 5% CO_2. Herpes simplex virus type 1 strain KOS was used for antiviral assays (Schnitzler et al. 2008a). Acyclovir was purchased from GlaxoSmithKline (Bad Oldesloe, Germany), dissolved in sterile water and used as defined synthetic inhibitor of herpesvirus replication.

4.5. Cytotoxicity test and plaque inhibition assay

RC-37 cells were grown in monolayer cultures, and then plated onto 96-well plates. After 24 h cells were treated with a serial dilution of the extracts for 72 h. Cells were fixed with formalin then stained with neutral red. Cell viability was measured photometrically at 540 nm wavelength. For plaque inhibition assay, cells were plated onto 6-well plates, infected with herpes simplex virus type-1 (HSV-1) for 1 h and then incubated at 37 °C. Medium was removed after 72 h, cells were fixed with 10% formalin, stained with crystal-violet and plaques were counted. Extracts were added at different time periods to the viral infection (Schnitzler et al. 2008a).

4.6. Mode of antiviral activity

Cells and viruses were incubated with the extracts at different stages during viral infection cycle in order to trace the mode of antiviral action. Cells were pretreated with the extracts prior to infection with HSV, or viruses were incubated with the extract for 1 h at room temperature, and then diluted 100-fold before infection, or the infected cells were incubated for 1 h after penetration of HSV into host cells with the extracts for 72 h (Koch et al. 2008). Extracts were always used at the maximum non-cytotoxic concentration.

4.7. Statistical analysis

All experiments were performed in triplicate and statistical analysis was performed by SPSS software (SPSS for Windows, 11.0, 2001, SPSS Chicago, Illinois). The means and standard errors were recorded.

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