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Antiherpetic efficacy of aqueous extracts of the cyanobacterium *Arthrospira fusiformis* from Chad

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Natural substances offer interesting pharmacological perspectives for antiviral drug development with regard to broad spectrum antiviral properties and novel modes of action. Drugs currently used to treat cutaneous or genital herpetic infections are effective in limiting disease, but the emergence of drug-resistant viruses in immunocompromised individuals can be problematic. A nontoxic cyanobacterium *Arthrospira* strain from Chad has been characterized by sequence analysis of the intergenic spacer region of the phycocyanin gene. This cyanobacterium was identified as *Arthrospira fusiformis* by phylogenetic tree analysis. The antiherpetic activity of crude aqueous extracts from the Chad *A. fusiformis* isolate was determined. Antiviral efficacy against herpes simplex virus of cold water extract, hot water extract and phosphate buffer extract was assessed in plaque reduction assays and their mode of antiherpetic action was analysed. In virus suspension assays, cold water extract, hot water extract and phosphate buffer extract inhibited virus infectivity by 54.9%, 64.6%, and 99.8%, respectively, in a dose-dependent manner. The mode of antiviral action was determined by addition of cyanobacterial extracts separately at different time periods during the viral infection cycle. Extracts of *A. fusiformis* strain clearly inhibited herpesvirus multiplication before and during virus infection of host cells. The phosphate buffer extract of the *A. fusiformis* strain affected free herpes simplex virus prior to infection of host cells and inhibited intracellular viral replication. It is concluded, that *Arthrospira* compounds warrant further investigation to examine their potential role in the treatment of 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 even found to grow in heavily polluted lakes in Egypt (El Bestawy 1990). These lakes have a different climate than the lakes in Mexico, Kenya and Chad, from which most of the studied *Arthrospira* strains have been isolated. Previous reports demonstrated an antiviral activity of complex sulfated polysaccharides extracted from various species of marine algae and suggested that they interfered with the attachment of virions to host cells (Rechter et al. 2006). Natural products in their crude form containing bioactive compounds are sometimes more effective in their natural combination than isolated compounds (Astani et al. 2010, 2012).

Herpes simplex virus type 1 (HSV-1) is an important pathogen for humans and discovery of novel effective antiherpetic drugs without adverse effects is of great interest. The primary symptoms of herpes infection include a prodromal flu-like syndrome with fever, headache, malaise, diffuse myalgias, followed by local symptoms consisting of itching and painful papules. Gingivostomatitis and pharyngitis are the most frequent clinical manifestations of first episodes of HSV-1 infection. After establishing latency, HSV can reactivate, causing frequent recurrent infections in some patients, while most people experience few recurrences (Whitley and Roizman 2001). Recurrent herpes labialis is the most frequent clinical manifestation of reactivated HSV-1 infection. The clinical manifestation of the disease exhibits different severity in immunocompetent patients. However in immunocompromised patients and neonates, herpetic infections can cause serious systemic illnesses (Whitley et al. 1984). An antiviral treatment for herpes simplex virus infection is available since the introduction of acyclovir in the 1970 s which is still the most commonly used chemotherapy (Brady and Bernstein 2004). This nucleoside analogue functions as DNA chain terminators, ultimately preventing elongation of viral DNA (De Clercq 2004). The development of viral resistance towards antiviral agents enhances the need for new effective

compounds against viral infections. A number of essential oils and plant extracts have been shown to induce an anticancer, antibacterial and antiherpetic effect, acting before virus penetration and revealing different modes of action than the commonly used drugs (Efferth 2009; Fu et al. 2007; Schnitzler et al. 2008a, Nolkemper et al. 2010). They all represent promising antiviral agents for topical therapeutic application (Koch et al. 2008; Schnitzler et al. 2008b; Reichling et al. 2008). Still there is a need for antiviral agents with different or combined modes of action from natural sources.

Here we report the antiherpetic activity of different crude aqueous extracts from *A. fusiformis* isolated from Chad. The cyanobacterial strain was identified by sequence analysis of the PC-IGS phycocyanin gene. Antiviral activity of cold water extract, hot water extract and phosphate buffer extract from the cyanobacterium was assessed in plaque reduction assays and their mode of antiherpetic action was determined.

2. Investigations and results

DNA sequences of the phycocyanin intergenic spacer region (PC-IGS) gene of the Chad cyanobacterial strain were aligned using BIOEDIT software for molecular authentication. This analysis classified the Chad cyanobacterium as *Arthrospira fusiformis* as presented in the phylogenetic tree (Fig. 1). The PC-IGS phylogenetic tree shows a tight cluster including the investigated *Arthrospira* strain and other *Athrospira fusiformis* as well as *Arthrospira indica* strains. The whole cluster is supported by bootstrap value of 96%. *Spirulina subsalsa* and *Spirulina* substrains were grouped in a separate cluster relatively distant from the *Arthrospira fusiformis* strains.

Monolayer cultures of RC-37 cells were grown in extract-containing medium. After 3 days of incubation, cell viability was determined using a standard neutral red assay. Cold water extract, hot water extract and phosphate buffer extract reduced cell viability by 50% (TC_{50}) at concentrations of 2.8 mg/ml, 7.2 mg/ml and > 10 mg/ml, respectively. Maximum noncytotoxic concentrations of cold water and hot water extract were determined at 2.5 mg/ml, the phosphate buffer extract of the Chad cyanobacterium did not show any toxicity up to 10 mg/ml. 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 assay, a 100-fold dilution of the extract-treated viruses was performed 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 clearly dose-dependent antiviral activity of the extracts was demonstrated (Fig. 2). In all experiments infected cells without addition of extracts were used as control. Phosphate buffer extract of the cyanobacterium demonstrated the highest antiviral activity. The phosphate buffer extract of the cyanobacterium inhibited herpesvirus by 99.8%, whereas the cold water extract and hot water extract inhibited herpes simplex virus by 54.9% and 64.6%, respectively. The phosphate buffer seems to contain more antiviral substances than the cold water and hot water extracts.

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 of

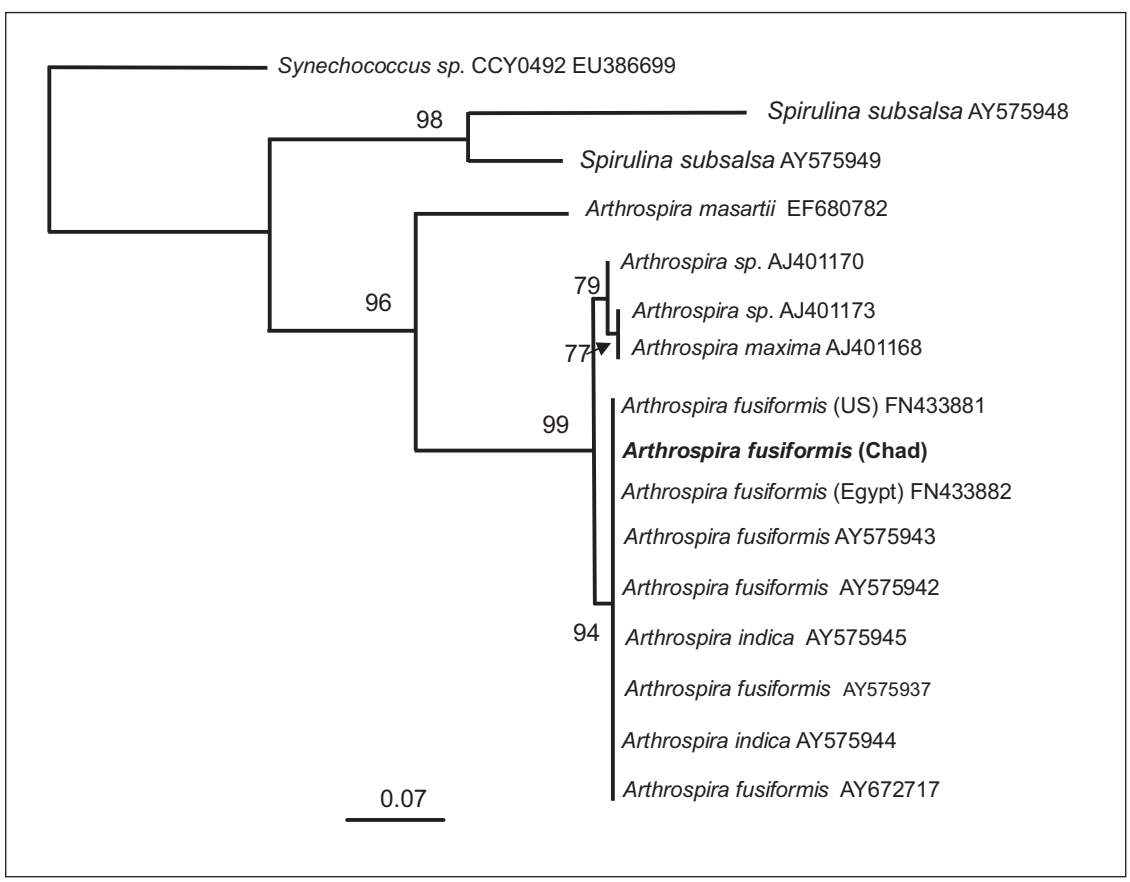

Fig. 1: Maximum likelihood tree based on PC-IGS sequences of 16 cyanobacterial strains, bootstrap values above 50 are included. The *Arthrospira* strain from this study is marked in bold, bar indicates 7% sequence divergence

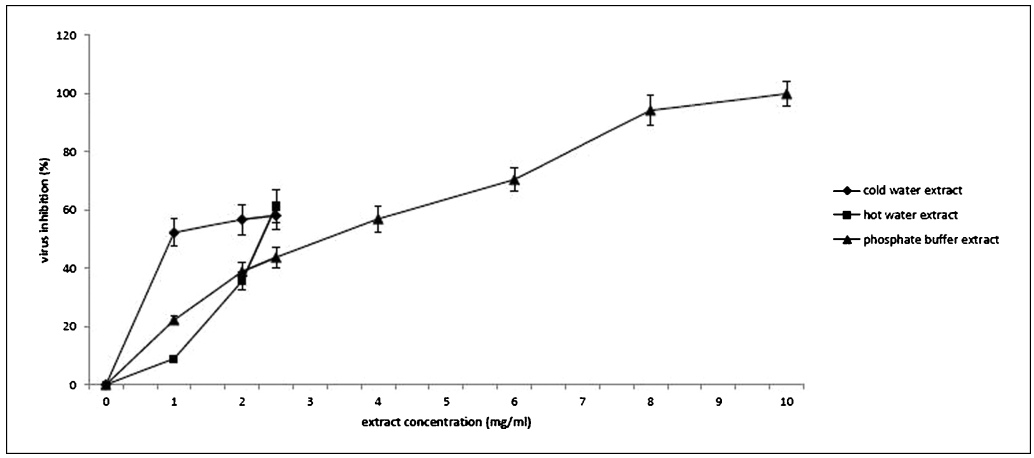

Fig. 2: Determination of the 50% inhibitory concentration (IC_{50}) of crude extracts of Chad *Arthrospira fusiformis* for herpes simplex virus. Viruses were incubated with increasing concentrations of the extracts for 1 h and inhibition of viral infectivity was tested in a plaque reduction assay. Data represented are the mean of three independent experiments

the extract. In all experiments the maximum noncytotoxic concentrations of the cyanobacterial extracts were used. Extracts of *Arthrospira* were effective at different phases of viral infection. When cells were pretreated with cyanobacterial extracts, no significant inhibitory effect was observed (Table). However, all extracts of the cyanobacterium demonstrated a significant inhibition of HSV when virus was pretreated with extracts (Table). A less pronounced antiviral effect was observed when cold or hot water extracts were added to infected cells during viral replication. In contrast, the phosphate buffer extract of *Arthrospira* inhibited viral infection nearly completely when HSV was pretreated with this extract and by 84.8% during intracellular replication. Only minor effects on viral infection were detected when cells or viruses were pretreated with acyclovir. This synthetic drug showed the highest antiviral activity when added during the replication period with inhibition of the viral replication. Acyclovir has been reported to inhibit specifically the viral DNA polymerase during the replication cycle when new viral DNA is synthesized.

3. Discussion

The pharmaceutical industry is increasingly targeting natural products with the aim of identifying lead compounds, focusing particularly on suitable alternative antiviral agents. Herpes simplex virus (HSV) is a member of the Herpesviridae family that causes general communicable infections in humans, the most common being genital and orolabial disease. The current treatments of herpetic infections are nucleoside analoges such as acyclovir, valacyclovir and famciclovir. Despite the safety and efficacy, extensive clinical use of these drugs has led to the emergence of drug-resistant viral strains, mainly in immunocompromised patients. Topical treatment of herpes labialis infection is standard, for the most part carried out with acyclovir creams, but also with phytotherapeutic preparations (Wölbling and Leonhardt 1994; Schnitzler and Reichling 2011; Vo et al. 2011).

In the present study, the antiherpetic effect of several aqueous extracts derived from a Chad strain of the cyanobacterium *Arthrospira fusiformis* were compared and their mode of antiviral action determined. This *Arthrospira* strain cluster tightly in the PC-IGS phylogenetic tree with other *Athrospira fusiformis* and *Arthrospira indica* strains indicating the molecular authentication. Experiments to assess the cytotoxicity of the aqueous cyanobacterial extracts for cultured eucaryotic cells indicate moderate toxicity for cold and hot water extracts and no toxicity for phosphate buffer extract 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 protein and its pigments as well. The phosphate buffer extract exhibited high levels of antiviral activity against HSV-1 in viral suspension tests. Plaque formation was inhibited nearly completely indicating a high dose-dependent antiviral activity. Pretreatment of the cells with all extracts of *Arthrospira* revealed only minor inhibition of herpesvirus. The phosphate buffer extract, however demonstrated high antiviral activity when viruses were pretreated with extract prior to cell infection and this extract revealed also a high antiviral activity during the intracellular replication phase of herpes simplex virus. These results indicate that extracts of the Chad cyanobacterium possess a broad spectrum of antiherpetic 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. Antiviral activity of aqueous extracts of *Arthrospira platensis, A. maxima* and an Egyptian *A. fusiformis* had been reported previously (Hernandez-Corona et al. 2002; Sharaf et al. 2010). It was found that sulfated polysaccharides were antivirally effective (Witvrouw and De Clercq 1997) by blocking virus

Table: Antiviral effect of *Arthrospira fusiformis* extracts against HSV-1 strain KOS

	Cold water extract	Hot water extract	Phosphate buffer extract	Acyclovir
Pretreatment cells	30.1 ± 3.8	24.7 ± 2.9	12.3 ± 1.0	0.9 ± 0.1
Pretreatment virus	54.9 ± 4.9	64.6 ± 6.1	99.8 ± 8.2	8.1 ± 0.9
Replication	36.2 ± 2.8	22.3 ± 2.5	84.8 ± 7.8	99.6 ± 6.4

Maximum noncytotoxic concentration of the extracts were used for all experiments. Data represent percentages of plaques compared to untreated controls. Experiments were repeated independently two times and data presented are the mean of three experiments.

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 *Arthrospira* strain could be interpreted as inhibition of protein synthesis as demonstrated previously (Lee et al. 2000). 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 (Ghosh et al. 2009; Harden et al. 2009; Bandyopadhyay et al. 2011; Saha et al. 2011). Adsorption of viruses to host cells is another specific target of polysaccharides (Bouhlal et al. 2011). 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 Chad *Arthrospira* interfered at two steps during viral infection, whereas the previously reported Egyptian *Arthrospira* strain only interfered with free viral particles. It is suggested that the tested extracts of the Chad strain most probably contain a number of antiherpetic compounds with different modes of action, some affecting free virus particles and some inhibiting intracellular virus replication. Similar results have been reported by Rechter et al. (2006), where spirulan-like substances exhibited strong antiherpetic effects by targeting primarily virus entry to host cells. 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 showed the highest antiviral effect.

The phosphate buffer extract of the Chad *A. fusiformis* strain revealed a high antiviral activity against herpes simplex virus type 1 *in vitro*. Considering the abundant availability and cheap production of *Arthrospira,* this cyanobacterium might be promising for future antiviral design.

4. Experimental

4.1. Cultivation of Arthrospira and preparation of extracts

Arthrospira 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. 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 bacterial 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.

4.2. Molecular identification of cyanobacteria by phylogenetic analysis

The cyanobacterial strain was 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 gentle 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 15 min. To separate from proteins 600 μl ice-cold 7.5 M ammonium acetate were added and the sample centrifuged for 8 min at 12 000 rpm. The supernatant was transferred into a new 2 ml Eppendorf tube. To precipitate DNA 700 μl isopropanol were added and the sample centrifuged for 60 min at 12 000 rpm. The pellet was twice washed with 80% ethanol and centrifuged for 5 min at 13 000 rpm 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 10x 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 3.1 v, 1.5 μl 5x 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 accession number is HF 536495. 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). The evolutionary substitution model was evaluated using the AIC criterion in Modeltest v.3.06 (Posada and Crandal 1998). TrN+I was found to be the best-fitting evolutionary model for the PC-IGS.

4.3. 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.

4.4. 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).

4.5. 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.

4.6. 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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