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Evaluation of the Cytotoxic and Antiviral Activities of Partially Purified *Naja haje haje* Venom

Elsayed A. Elsayed[1,2]*, Hamed A. El-Serehy[3,4], Walaa H. Salama[5] and Fahad Al-Misned[3]

[1]Bioproducts Research Chair, Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia.
[2]Natural and Microbial Products Department, National Research Centre, Dokki, Cairo, Egypt.
[3]Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia.
[4]Port Said University, Faculty of Science, Marine Science Department, Port Said, Egypt.
[5]Department of Molecular Biology, National Research Center, Dokki, Cairo, Egypt.

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Since their introduction, nucleoside analogues such as acyclovir are considered to be effective antiviral drugs against viral infections caused by herpes simplex. However, with the emergence of acyclovir-resistance, there is a growing need to develop an alternative antiherpetic drug. Within such context, snake venom could serve as a potential lead. In this study, the antiviral properties of *Naja haje haje* (*N. haje*) venom was evaluated against herpes simplex virus types 1 and 2 (HSV-1, 2) *in vitro*. HSV-infected Vero cells and cell-free virus suspensions were treated with *N. haje* crude venom phospholipase A2 (PLA2) as well as 3 different fractions, and the virus yield and infectivity were then quantified by direct plaque assay. The results of the present study showed that *N. haje* venom at a concentration of 1 μg/ml resulted in 72.1% inhibition of plaque of HSV-1 and 67.5% inhibition against HSV-2, while PLA2 and fraction 1 at a concentration of 25 μg/ml caused 18.4 and 56.3% inhibition against HSV-1 and 15.2 and 52.2 % against HSV-2, respectively. On the other hand, fraction 3 at a concentration of 150 μg/ml resulted in 61.1% inhibition against HSV-1 and 65.9% plaque inhibition against HSV-2. *N. haje* venom inhibited both HSV-1 and 2 and showed some virucidal activity. These properties suggest that this venom could provide advantages as a topical prophylactic/therapeutic agent for HSV infections.

Key words: *Naja haje haje*; Venom; Herpes simplex virus; Antiviral activity.

Snake venoms have been regarded for long time as excellent sources for drugs because of their structural diversity and the wide variability of their biological properties. Due to their broad range of pharmacological functions, these active components have been the subject of hundreds of scientific papers in different research fields[1,2].

* To whom all correspondence should be addressed.
E-mail: eaelsayed@ksu.edu.sa

Herpes simplex virus type 1 (HSV-1) is a widespread human pathogen that infects primarily epithelial tissues and causes severe diseases including mucocutaneous lesions in the oral mucosa (cold sores), encephalitis, meningitis, and blinding keratitis[3]. On the other hand, *herpes simplex* virus type 2 (HSV-2) is the primary cause of genital ulcer disease worldwide. In 2003, an estimated 536 million people aged 15–49 years were living with the infection, with seroprevalence varying widely across settings and populations. Most infected individuals are unaware of their infections. In symptomatic infections, the

virus causes painful ulcerative lesions that can take two to four weeks to heal in primary outbreaks, and recurrences can be frequent. The prevalence of HSV-2 infection in the general population ranges from 10 to 60 percent, with higher prevalence's in female sex workers, men who have sex with men (MSM), and certain regions of the world[4].

Currently no vaccine is available. The standard therapy for managing HSV infections depends on nucleoside analogues that target the viral DNA polymerase. These include acyclovir, penciclovir and their derivatives, valacyclovir, and famciclovir[5]. These antiviral drugs can be efficient in the treatment of clinical signs and symptoms of first and recurrent episodes, but their widespread use and the long term prophylactic therapy may be associated with relative high toxicity and emergence of drug-resistant viral strains especially in immunocompromised patients[6]. For these reasons, there is a great demand for the development of new antiviral drugs with novel mode of action. Many reports have been published describing the anticancer activities of venoms from different snake species[7-8]. Furthermore, it has been reported that certain fractions isolated from snake venoms showed cytotoxic activities on tumor cells, and they can even result in the initiation of apoptosis mechanism of tumor as well as normal cells[9-11].

In this context, natural products are very important sources of anti-HSV agents and several extracts and pure compounds from venoms have been reported to exert an anti-HSV activity[1]. To the best of our knowledge, there have been no reports dealing with the effect of venom isolated from the Egyptian Cobra (*Naja haje*) on HSV-1 and 2. This work was designed to evaluate the antiviral activity of *N. haje* venom of HSV-1 and 2.

MATERIALS AND METHODS

Viruses and cell lines

Vero cells were grown in Dulbecco-modified Eagle's Minimum Essential Medium (DMEM; Gibco, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco®, Brazil) and gentamicin (80 µg/mL). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The Herpes Simplex Virus type 1 and 2 were propagated in Vero cells, titrated on the basis of plaque forming units (PFU) count by plaque assay as previously described[12] and stored at -80°C until the experiments were performed.

Venom collection

Pooled venom samples from *Naja haje haje* (*N. haje*) were milked from several adults' specimens collected from Nile valley, delta, and Faiyum in Egypt. The venom samples were kept at -20°C, thawed, and were centrifuged before use. The venoms were freeze-dried and stored at -20°C until use.

Protein isolation

Resolution of the protein peaks was performed in a successive gel filtration chromatographic column. Crude *Naja haje* venom sample containing 150 mg protein was dissolved in 1 ml of 0.05 M Tris-HCl buffer, pH 7 and was loaded on top of a Sephadex G-50 (Pharmacia LKB, Sweden) column (1.6×90 cm) equilibrated and eluted with the same buffer. Fractions of 3 ml were collected at a flow rate of 30 ml/h at 4°C. The eluted protein fractions were measured at 280 nm using Shimadzu spectrophotometer and the protein fractions namely, F1, F2 and F3 were pooled, dialyzed, freeze-dried, and stored.

Cytotoxicity assay

Confluent Vero cells were exposed to different sample concentrations (1-1000 µg/ml) for 72 h. After incubation, cell viability was assessed by a MTT [3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay[13]. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced cell viability by 50% when compared to the untreated controls.

Antiviral activity assay

The 96-well plates containing confluent cell monolayers were pre-incubated for 1 h with increasing non-cytotoxic concentration of the venom or fraction. Six wells were used for each concentration. Afterwards, the cells were infected with HSV-1 or 2 (10 TCID50), and were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The infected cells were observed daily for cell cytopathic effects (CPE) using a light microscope. When CPE was observed in all virus control wells, the percentage of wells with CPE was determined for each treatment concentration, as previously described. Acyclovir at concentrations ranging from 0.05 to 2 µg/ml served as the positive control.

Viral plaque number reduction assay

This assay was performed following the procedures previously described[14], with minor modifications. Approximately 100 PFU of HSV types were adsorbed for 1 h at 37°C on confluent cells and overlaid with MEM plus 1.5% carboxymethylcellulose (CMC, Sigma®, St. Louis, MO, USA) either in the presence or the absence of different concentrations of the samples. After 72 h, the cells were fixed and stained with naphthol blue-black (Sigma) and plaques were counted. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that inhibited 50% of viral plaque formation when compared to the untreated controls. Acyclovir (Sigma®) was used as a positive control. Results were expressed as CC_{50} and IC_{50} values in order to calculate the selectivity index ($SI = CC_{50}/IC_{50}$) of each sample[15].

Statistical analysis

Data are expressed as the mean ± standard error mean (SEM) from at least three separate experiments.

RESULTS AND DISCUSSION

The resolution of the protein fractions of the *N. haje* venom (150 mg protein applied on G-50 sephadex gel filtration) showed three major protein peaks as shown in Fig. 1.

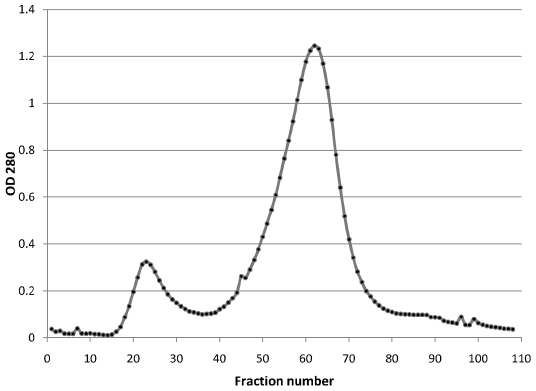

Fig. 1. Gel filtration of *N. haje* venom on Sephadex G-50. The venom (150 mg protein) dissolved in 0.05 M Tris-HCl buffer pH 7 was applied on the Sephadex G-50 column (2.6 ×50 cm) equilibrated by the same buffer. The column was eluted with the same buffer and 3 ml fractions were collected at a flow rate of 30 ml h^{-1}. F1, F2 and F3 protein peaks were collected from tubes (15-30), (47-60) and (60-80), respectively

The examination of the cytotoxic effect of crude venom and fractions was performed in concentrations up to 1000 µg/ml. The maximum non-cytotoxic concentrations were read individually from the obtained survival curves. According to the results of this experiment (Fig. 2), the crude venom showed cytotoxic effect at a concentration of 59.1µg/ml, the fraction of number 1 has cytotoxicity at 51.3µg/ml, the PLA2 was non-toxic up to 863.4 µg/ml, while the fraction number 3 has no cytotoxicity up to 1000µg/ml. The fraction number 2 was the most cytotoxic, with CC_{50} of 29.0 µg/ml.

The present study was carried out to test the antiviral activity of *N. haje* crude venom and 4 fractions isolated from the venom against herpes simplex virus types-1 and 2 using cytopathic

Table 1. Antiviral effects of *N. haje* crude venom and its different fractions on HSV-1 and 2 virus

Extracts	Cell Cytopathic Effect (CPE)	
	HSV-1	HSV-2
Crude venom	+	+
PLA2	+	+
Fraction 1	+	+
Fraction 2	+	+
Fraction 3	+	+

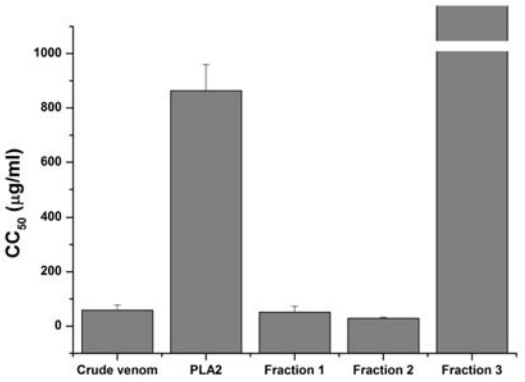

Fig. 2. Cytotoxic effects of *N. haje* crude venom and its different fractions on Vero cells. CC_{50} is the concentration of the 50% cytotoxic effect. Data are represented as Mean ± SEM of two independent experiments each performed in triplicate

inhibitory assay and plaque reduction assay (Table 1). On the basis of cytopathic effect (CPE) of the virus-infected confluent monolayer of Vero cells, all the samples did not show strong antiviral activity against HSV-1 or anti-HSV-2. In this manner, the cytopathic effect was shown with all samples at different concentrations (from 1/5 CC_{50} to 1 µg/ml).

Plaque inhibition assay was carried out to determine the IC_{50}. As shown in Fig. 3, *N. haje* venom at 1 µg/ml provided 72.1% inhibition of plaque of HSV-1 and 67.5% inhibition against HSV-2, while PLA2 and Fraction 1 at 25 µg/ml showed 18.4% and 56.3% inhibition against HSV-1 and 15.2% and 52.2% against HSV-2, respectively. Fraction 3 at 150 µg/ml provided 61.1% inhibition against HSV-1 and 65.9% plaque inhibition against HSV-2. Fraction 2 didn't show significant data due to its extreme toxicity.

Accordingly, the highest anti-HSV-1 and anti-HSV-2 activities detected in crude venom is possibly related to the synergic effects of these compounds, since there are many examples of natural compounds that exhibit synergistic effects based on multi-target mechanisms of action[16].

Animal venoms are complex mixtures of protein and non-protein components with several biological activities. Snake venoms represent an essentially unexplored source of bioactive compounds that may cure disease which do not respond to currently available therapies. The biomolecules that constitute snake venoms have great therapeutic potential[2]. Recently published studies report the existence of snake venom fractions with an antiviral effect; however, few studies have been conducted to evaluate Egyptian copra venoms.

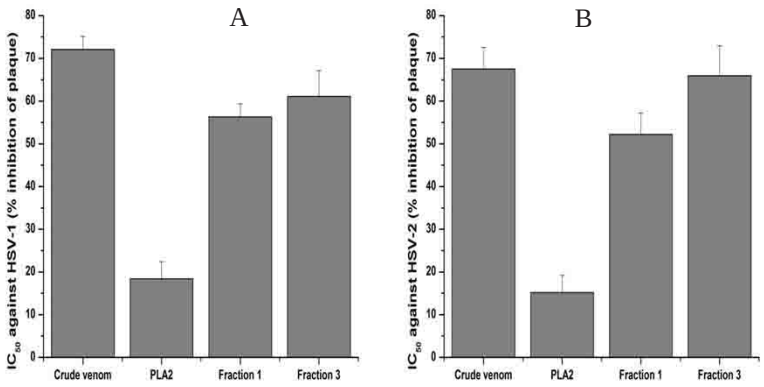

Fig. 3. The antiviral activities of *N. haje* crude venom and its different fractions onHSV-1 (A) and HSV-2 (B) virus. IC_{50} is the concentration of the sample required to inhibit 50% virus-induced CPE. Data are represented as Mean ± SEM of two independent experiments each performed in triplicate

Several reports had attempted to elucidate the mechanisms controlling the antiviral activity of snake venoms. In a study of Sundaram *et al.*[17], it was found that the homology between a short segment in Human immunodeficiency virus and the highly conserved amino acid residues of snake venom neurotoxins resulted in the competition between both the virus and venom for cell receptors, thus preventing viral entry into the host cell.

The present *in vitro* study provides evidence that *N. haje* venom has an anti-HSV effect, which can be further explored for establishing the exact mechanism of action and its possible therapeutic or prophylactic action. However, the substances responsible for the antiviral activity must be further isolated and chemically purified and characterized. Further analyses, including additional purification of the fraction, along with further antiviral testing, are currently being conducted.

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