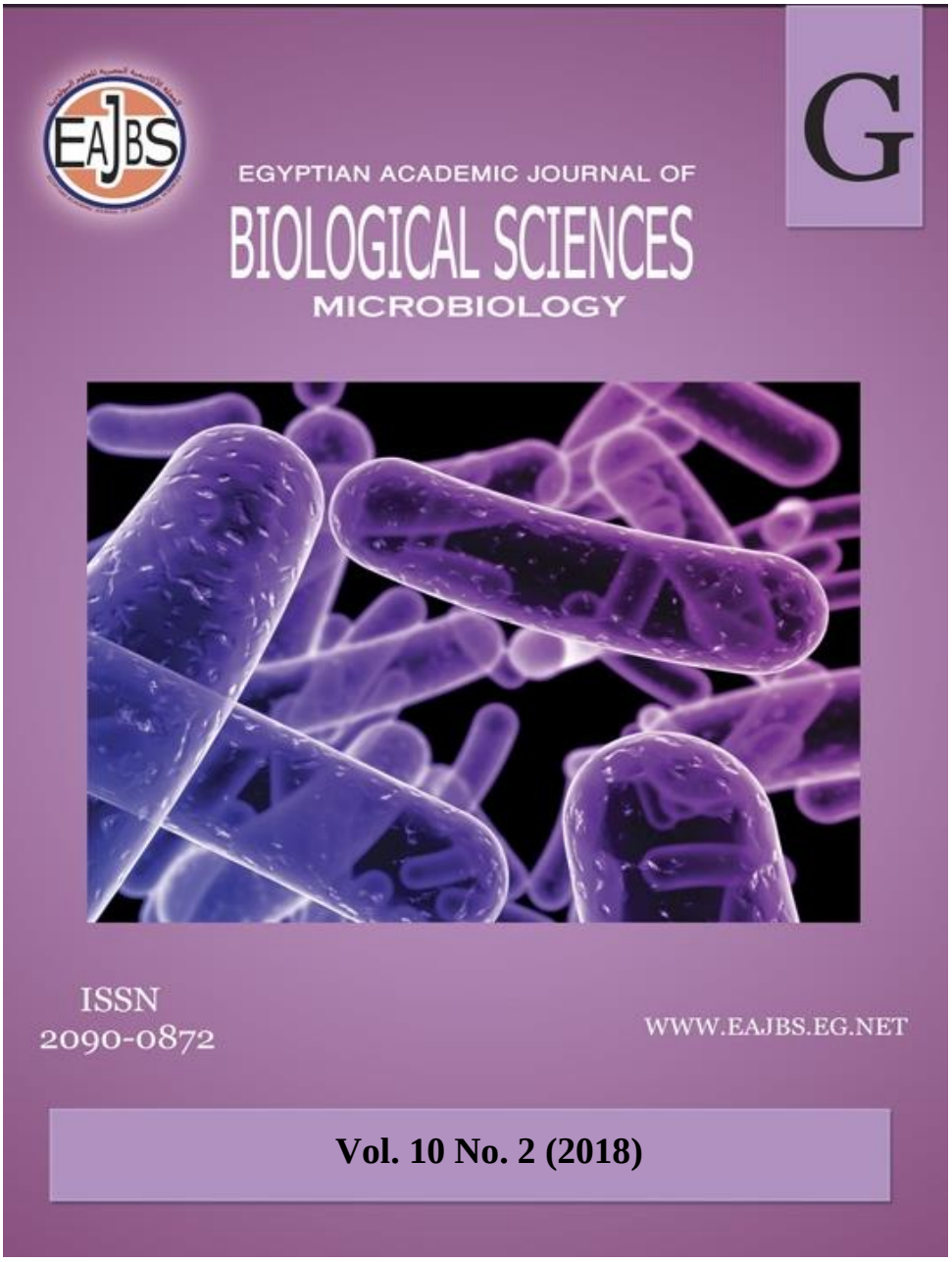

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Antiviral Activity of Egyptian Snake, *Cerastes vipera* Venom Against Hepatitis C Virus

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ABSTRACT

Background: The development of effective antiviral compounds has become public health emergency worldwide. Animal venoms, including snake venoms, are gaining increased attention as bioactive compounds with crucial therapeutic activities. The antiviral activity of snake venoms represents a new and promising therapeutic alternative against the resistance mechanisms developed by viruses. Hepatitis C virus infection (HCV) is a major worldwide health problem, and it is the foremost reason for progressive hepatic fibrosis and cirrhosis, with an elevated risk of hepatocellular carcinoma (HCC) development. The current treatment of HCV is still expensive and has side effects as, gene selectivity, low accessibility and resistance to mutated virus strains. For these reasons, achieving the discovery of more successful antiviral agents is always urgent.

Objective: the present study aimed to evaluate the antiviral activities of crude venom of *Cerastes vipera* against HCV.

Methods: The antiviral activity of crude venom of *Cerastes vipera* was evaluated by a cell culture technique using human hepatocellular carcinoma-derived cell line (Huh7.5) cells and the J6/JFH1-P47 strain of HCV.

Results: The results revealed that crude venom inhibited HCV infectivity with 50% inhibitory concentration (IC_{50}) of 1ng/ml in culture medium, through direct virucidal effect. The anti-HCV activity of this venom was not inhibited by a metalloprotease inhibitor or heating at 60°C. Interestingly, crude venom is neither toxic nor hemolytic *in vitro* at a concentration 1000-fold higher than that required for antiviral activity.

Conclusion: Conclusively, the obtained results indicate the therapeutic potential of crude venom of *Cerastes vipera* against the hepatitis C virus *in vitro* which many lay the foundation for developing a new therapeutic intervention against HCV.

INTRODUCTION

Animal secretions have been extensively studied in recent decades for treating several diseases, like Leishmania, hypertension, Alzheimer's diseases, congestive heart failure, as well as different types of cancer (Smith and Vane, 2003; Ciscotto *et al.*, 2009; Vyas *et al.*, 2013; Macedo *et al.*, 2015; Chan *et al.*, 2016). Snake venom is a secretion produced by their venom glands which are primarily used to paralyze and capture prey in order to feed or defend against predators.

The majority of venom components are a complex mixture of a number of peptides, toxins, peptides, enzymes, proteins and non-protein compounds(Leon *et al.*, 2011; Vyas *et al.*, 2013; Tasoulis and Isbister, 2017). Snake venoms contain a variety of cardiotoxic, neurotoxic, cytotoxic and other active chemicals with pharmacological significance (Pal S.K *et al.*, 2002; Doley and Kini, 2009; Cho Yeow Koh and Kini, 2012). This understanding has aided the development of biomedical applications such as therapeutic development, design of diagnostic tests and the study of the pathogenesis of the different diseases. Several anti-microbial active compounds have been discovered from snake venoms, some of them belong to the peptides which include cardiotoxins (or cytotoxins), crotamine, and cysteine-rich secretory proteins (CRISPs) and the other class is an enzyme which includes metallo-proteinases, L-amino acid oxidases, and phospholipase A_2 (Samy *et al.*, 2007; Samy *et al.*, 2010; Muller *et al.*, 2012; de Oliveira Junior *et al.*, 2013).

Egypt as a desert land comprises different species of venomous animals including snakes. In Egypt, 1000–10,000 incidences of snake envenomation per year with about 11–100 death cases annually (Kasturiratne *et al.*, 2008). Cerastes is a small genus of vipers found in North Africa and the Middle East. *Cerastes cerastes* and *Cerastes vipera* belong to the family Viperidae and the genus Cerastes. *Cerastes vipera* (Sahara sand viper, 25–50 cm) is one of the most important venomous viper species endemic to the deserts of North Africa and the Sinai Peninsula (Soslau *et al.*, 1988; Lifshitz *et al.*, 2000). Viper venoms are rich sources of pharmacologically active peptides and proteins (Lewis and Garcia, 2003; Izidoro *et al.*, 2014). The snakes belonging to the Cerastes genus are poisonous vipers whose lethality is mainly attributed to the highly active enzymatic component, phospholipase A2 (PLA) that hydrolyzes cellular phospholipids thereby releasing arachidonic acid. The venom of Cerastes species is a low-complexity proteome composed of 25–30 toxins belonging to 6 protein families, mainly targetting the hemostatic system (Bazaa *et al.*, 2005; Fahmi *et al.*, 2012). Previous studies on the venom proteome of Cerastes showed the presence of disintegrins, procoagulant snake venom serine proteinase (svSP) cerastocytin, phospholipase A2 (PLA2), C-type lectin-like proteins (CTL), L-amino acid oxidase enzyme isoforms and metalloproteinases (svMP) (Dekhil *et al.*, 2003; Bazaa *et al.*, 2005; Fahmi *et al.*, 2012; El Hakim *et al.*, 2015; Calvete *et al.*, 2017). Previous studies revealed these most of these compounds have an antimicrobial effect. However, the antiviral features of genus Cerastes have not been well examined and its mode of actions is not completely understood.

Viral diseases remain a major health burden worldwide, and more efforts are undoubtedly required to search for new molecules with antiviral actions that could lead to novel therapeutic options (El-Bitar *et al.*, 2015). One of the foremost bases of chronic liver diseases is the hepatitis C virus (HCV), a hepatotrophic RNA virus. Hepatitis C virus (HCV) causes a serious health problem worldwide, with no effective vaccine available. HCV infection is a major cause of hepatocellular carcinoma (Chen and Morgan, 2006; Yang and Roberts, 2010; Tinkle and Haas-Kogan, 2012; El-Bitar *et al.*, 2015; Wong and Gish, 2016). Recently, the U. S. Food and Drug Administration (FDA) approved direct-acting antivirals (DAAs) for HCV treatment, but shortly after DAA approval, an observed increase in early occurrence or recurrence of hepatocellular carcinoma after HCV eradication with DAA has been reported (Reig *et al.*, 2016). In view of the importance of the development of novel and safe compounds with improved efficacy is still required for HCV treatment. This study describes the potential antiviral activity against HCV *in vitro* of crude venom extract of *Cerastes vipera*.

MATERIALS AND METHODS

Crude Venom Collection*:*

Cerastes vipera snakes were collected from the wild by professional Egyptian hunters. The venom was collected separately from the adult snake of *Cerastes vipera*, by holding the head of the snake and trying to bite on the rubberized synthetic sheet, stretched and tied at the mouth of a sterilized glass beaker (Willemse *et al.*, 1979). The collected venom, filtered, lyophilized and stored at 4°C until use. Protein concentrations of the samples were determined using BCA Protein Assay Kit (Price Biotechnology, USA).

Cell Culture and Viruses*:*

Huh7.5 cells and the plasmid pFL-J6/JFH1 to produce the J6/JFH1 strain of HCV genotype 2a (Lindenbach *et al.*, 2005) were kindly provided by Dr. C. M. Rice, the Rockefeller University, New York, NY, USA. Huh7.5 cells were cultivated in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with fetal bovine serum (Biowest, Nuaille, France), non-essential amino acids (Invitrogen, Carlsbad, CA, USA), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Invitrogen). Cells were grown at 37°C in a 5% CO_2 incubator. The J6/JFH1-P47 strain (Lindenbach *et al.*, 2005) of HCV genotype 2a propagated in Huh7.5 cells was used in this study.

WST-1 Assay:

WST-1 assay was performed for cytotoxicity check as described by El-Bitar et al., (2015). In brief, Huh7.5 cells seeded in a 96-well plate were treated with serial dilutions (0.001 to 20 µg/ml) of crude *Cerastes vipera* venom or complete medium as a control for 48 h at 37 °C. WST-1 reagent (Roche, Mannheim, Germany) was added to the cells and incubated for 4 h. The number of living cells in each well was determined using a microplate reader. The absorbance was measured at 562 nm. Percent cell viability compared to the control was calculated for each dilution of the *Cerastes vipera* venom or peptide and CC_{50} values were determined by SPSS probit analysis in SPSS software (SPSS Inc., Chicago, IL).

Hemolysis Assay*:*

Hemolytic activity of *Cerastes vipera* crude venom extract was performed as described (Evans *et al.*, 2013). Briefly, a total of 10 µl of serial dilutions (0.001 to 20 µg/ml) of crude venom was mixed with 190 µl of diluted human red blood cells (RBCs) to achieve a final dilution of 1/20 of the original venom per well. Alternatively, the RBCs were incubated with 200 µl of 0.5% Triton X-100 or PBS to serve as both positive and negative controls, respectively. After an hour incubation period at 37º C, the plate was centrifuged for 5 minutes at 500×g and 100 µl of supernatant was transferred to a clear 96-well plate. The released hemoglobin was measured on a microplate reader at 400:541 nm. The percentage of hemolysis was calculated relative to the positive control (0.5 % Triton X100). The hemolysis concentration (HC_{50}) value was defined as the crude venom concentration that can lyse 50% of the RBCs.

Analysis of Antiviral Activities of Crude Venom:

Huh7.5 cells were seeded in 24-well plates (1.6×10^5 cells/well). A fixed amount of HCV was mixed with serial dilutions of *Cerastes vipera* crude venoms (0.001 to 1 µg/ml) for one hour and inoculated to the cells. After 2 hr, the cells were washed with medium to remove the residual virus and further incubated in a medium containing the same concentrations of the crude venoms as those used during virus inoculation. Culture supernatants were obtained and titrated for virus infectivity. Virus and cells treated with medium served as controls. Percent inhibition of virus infectivity by the samples was calculated by comparing with the controls and 50% inhibitory concentrations (IC_{50}) were determined.

Determination of Viral Yield in Cell Supernatant (Virus titration):

HCV infectivity was determined as described previously (El-Bitar *et al.*, 2015). In brief, virus samples were diluted serially 10-fold in a complete medium and

inoculated onto Huh7.5 cells seeded on glass coverslips in a 24-well plate. After virus adsorption for 2 hr, the cells were washed with medium to remove the residual virus and cultured for 24 hr. The virus-infected cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After being washed three times with PBS, the cells were incubated with HCV-infected patient's serum for 1 hr, followed by incubation with FITC-conjugated goat anti-human IgG (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). The cells were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 5 min and HCV-infected cells were counted under a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Immunoblot Analysis*:*

Cells were lysed with SDS sample buffer and equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked by incubation with 5% skim milk and incubated with the respective primary antibodies. The primary antibodies used were mouse monoclonal antibodies against HCV NS3 and GAPDH (Millipore). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Invitrogen) was used to visualize the respective proteins by means of an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

Neutralization of the Proteinase Activities of Crude Venom by Heating and A Metalloproteinase Inhibitor:

Crude venom was heated at 60°C for 30 minutes or treated with a metalloprotease inhibitor (1,10-phenanthroline; 5 mM) (Naves de Souza *et al.*, 2011; Wahby *et al.*, 2012; Abdel-Aty and Wahby, 2014; El-Bitar *et al.*, 2015; Cordeiro *et al.*, 2018). The treated venom or untreated control was mixed with HCV for 2 hr at 37°C. The virus/venom mixture was then inoculated to Huh7.5 cells for 2 hr at 37°C. After the virus inoculation, the cells were washed three times and incubated with a medium without venom. After 48 hr, culture supernatants were collected and virus infectivity was titrated. The virus-infected cells were subjected to immunoblot analysis to check the level of HCV protein accumulation, as described above.

Statistical Analysis:

Data are representative of at least 2 independently repeated experiments and presented as mean ± SEM. CC_{50} values were determined by SPSS probit analysis in SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Cytotoxicity Assay of *Cerastes vipera* crude Venom*:*

The measurement of cell proliferation and cell viability has become a key technology in the life sciences. Proliferation assays have become available for analyzing the number of viable cells by the cleavage of tetrazolium salts add to the culture medium. The tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. To check the cytotoxic effect of crude venom, Huh7-5 cells seeded in a 96-well plate were treated with serial dilutions (0.001 to 20 µg/ml) of *Cerastes vipera* crude venom extract or complete medium as a control for 48 h at 37 °C. The number of living cells in each well was determined using a microplate reader and percent cell viability was calculated. Hence, no significant decrease in cell viability with respect to control was observed after 48 hours. These results indicate that this crude venom has no cytotoxic up to 1000ng/ml with a selectivity index (SI) greater than 1000-fold (Table1 and Fig.1 A).

Table 1: Antiviral activity (IC_{50}) against HCV, cytotoxicity (CC_{50}) and selectivity index (SI) of crude venom of ***Cerastes vipera*** snake tested in this study

Species	IC_{50} (ng/ml) [a]	CC_{50} (ng/ml) [a]	HC_{50} (ng/ml) [a]	SI
Cerastes vipera	1	1000	>10,000	1000

[a]: Data represent means of the data obtained from two independent experiments using the J6/JFH1-P47 strain of HCV.

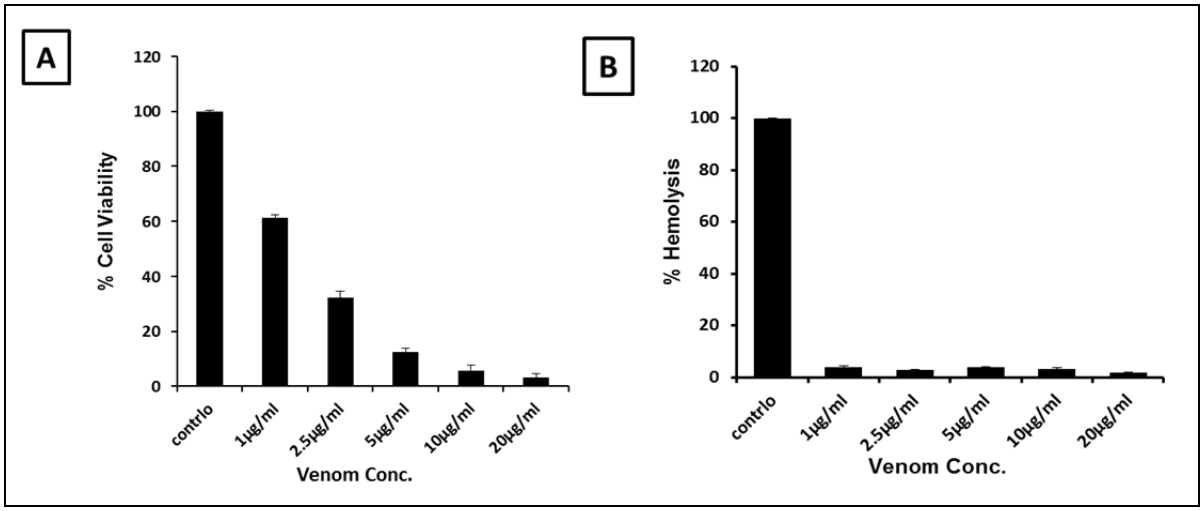

Fig. 1. Huh7-5 cells seeded in a 96-well plate were treated with serial dilutions (0.001 to 20 µg/ml) of *Cerastes vipera* crude venom extract or complete medium as a control for 48 h at 37 °C. **(A) Determination** of 50% cytotoxic concentration (CC50). The CC50 values are expressed as percentages of treated vs. untreated cells. Each value is the means ± SD of two experiments. **(B) Determination** of 50% hemolysis concentration (HC50), serial dilution (0.001 to 20µg/ml) of crude venom was mixed with diluted human red blood cells (RBCs). The HC50 values are expressed as percentages of RBCs treated with venom vs. Triton X100. Each value is the means ± SD of two experiments.

Hemolysis Assay of Cerastes vipera Venom:

To check the hemolytic activity of *Cerastes vipera*, the crude venom extract was performed as described. Serial dilution (0.001 to 20µg/ml) of crude venom was mixed with diluted human red blood cells (RBCs). Alternatively, the RBCs were incubated with Triton X-100 or PBS to serve as both positive and negative controls, respectively. The released hemoglobin was measured on a microplate reader and the percentage of hemolysis was calculated. The results showed this crude venom has no hemolytic activity up to 10 µg/ml with a selectivity index (SI) greater than 10000-fold IC_{50} **(**Fig.1 B and Table1).

Screening of Anti-HCV Activities of *Cerastes vipera* Venom:

Anti-HCV activities of crude venom of Egyptian snake *Cerastes vipera* were tested. To check whether crude venom has any possible anti-HCV activity, serial dilutions of crude venom (0.1 to1,000 ng/ml) with a fixed amount of HCV were mixed and inoculated to the Huh7.5 cells. The cells were washed with medium after 2 h, and further incubated with the same concentrations of crude venom. The culture supernatants at 2 dpi were titrated for virus infectivity. Results showed that crude venom inhibited HCV with an IC_{50} of 1 ng/ml and CC_{50} of 1000 ng/ml. The data demonstrated that crude venom exhibited a remarkably high selectivity index (SI) of 1000-fold than IC_{50} (Table 1 and Fig. 2).

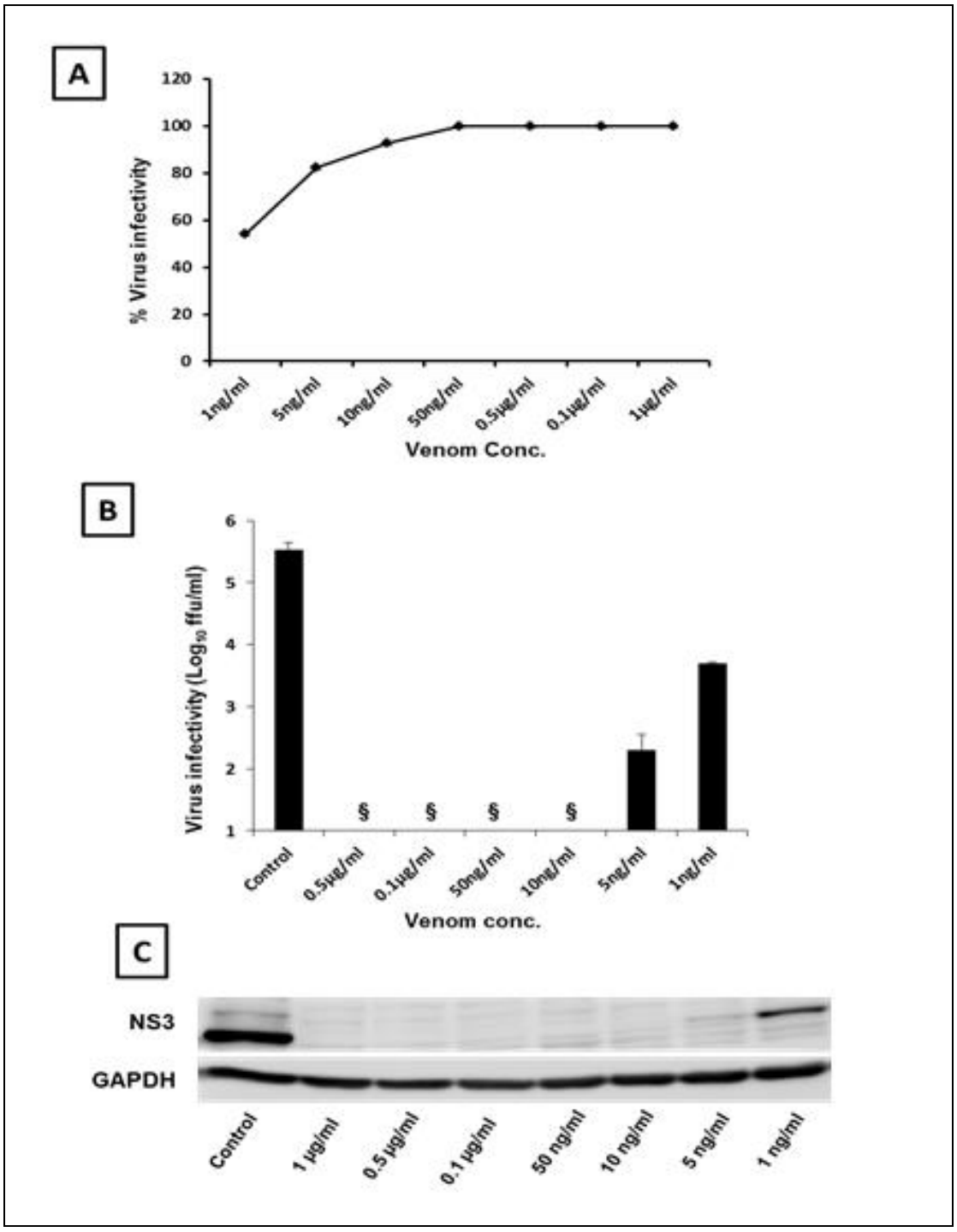

Fig. 2. Serial dilutions of *Cerastes vipera* crude venom were mixed with a fixed amount of HCV and inoculated to Huh7.5 cells at a multiplicity of infection of 2 pfu/cell. The cells were incubated in medium containing the same concentrations of venom for 46 h. **(A) Dose dependent curve** of *Cerastes vipera* crude venom**. (B) Amounts of** HCV infectious particles in the supernatants. **(C) Accumulation** of HCV NS3 protein inside the cells. Virus- infected cells were analyzed by immunoblot using an anti-HCV NS3 antibody. GAPDH was used as an internal control. Data obtained from two independent experiments and represented as means ± SEM; §, below the detection limit.

Dose-Dependent Manner of Anti HCV Activity of *Cerastes vipera* Venom*:*

To explore whether *Cerastes vipera* venom inhibits HCV virus particles in a dose-dependent manner serial dilutions of crude venom (0.1 to1,000 ng/ml) with a fixed amount of HCV at a multiplicity of infection of 2 pfu/cell was mixed and inoculated to the Huh7.5 cells. The culture supernatants at 2dpi were titrated for virus infectivity and the cells were harvested and subjected to immunoblot analyses. The results showed that the virus infectivity of supernatant had a significant dose-dependent manner of anti-HCV activity of crude venom as shown in (Figure 2A&B). Also, HCV NS3 protein accumulation in the cells was inhibited (Fig. 2C). The above results suggest that the *Cerastes vipera* venom directly affects HCV particles and/or host cells in the culture medium to inhibit the viral infection.

Effects Of Neutralization of The Proteinase Activity by Heating And/Or A Metalloproteinase Inhibitor:

In order to investigate whether the anti-HCV activity of *Cerastes vipera* venom involves an enzymatic activity, we treated the venom with heating at 60°C or a metalloproteinase inhibitor, 1, 10-phenanthroline (5 mM) to inactivate them, as reported by other investigators (Naves de Souza *et al.*, 2011; Wahby *et al.*, 2012; Abdel-Aty and Wahby, 2014; El-Bitar *et al.*, 2015; Cordeiro *et al.*, 2018). The treated venom was added to HCV and incubated for 2 hr at 37°C. Then, the virus/venom mixture was inoculated to Huh7.5 cells and virus replication was analyzed. The results obtained revealed that the treated venom, either treated with heating at 60°C or the metalloproteinase inhibitor, or both at the same time, still markedly suppressed production of HCV infectious particles in the culture to the same extent compared to the untreated control (Fig. 3A). Consistent with this observation, the accumulation of intracellular HCV NS3 protein was also inhibited (Fig. 3B).

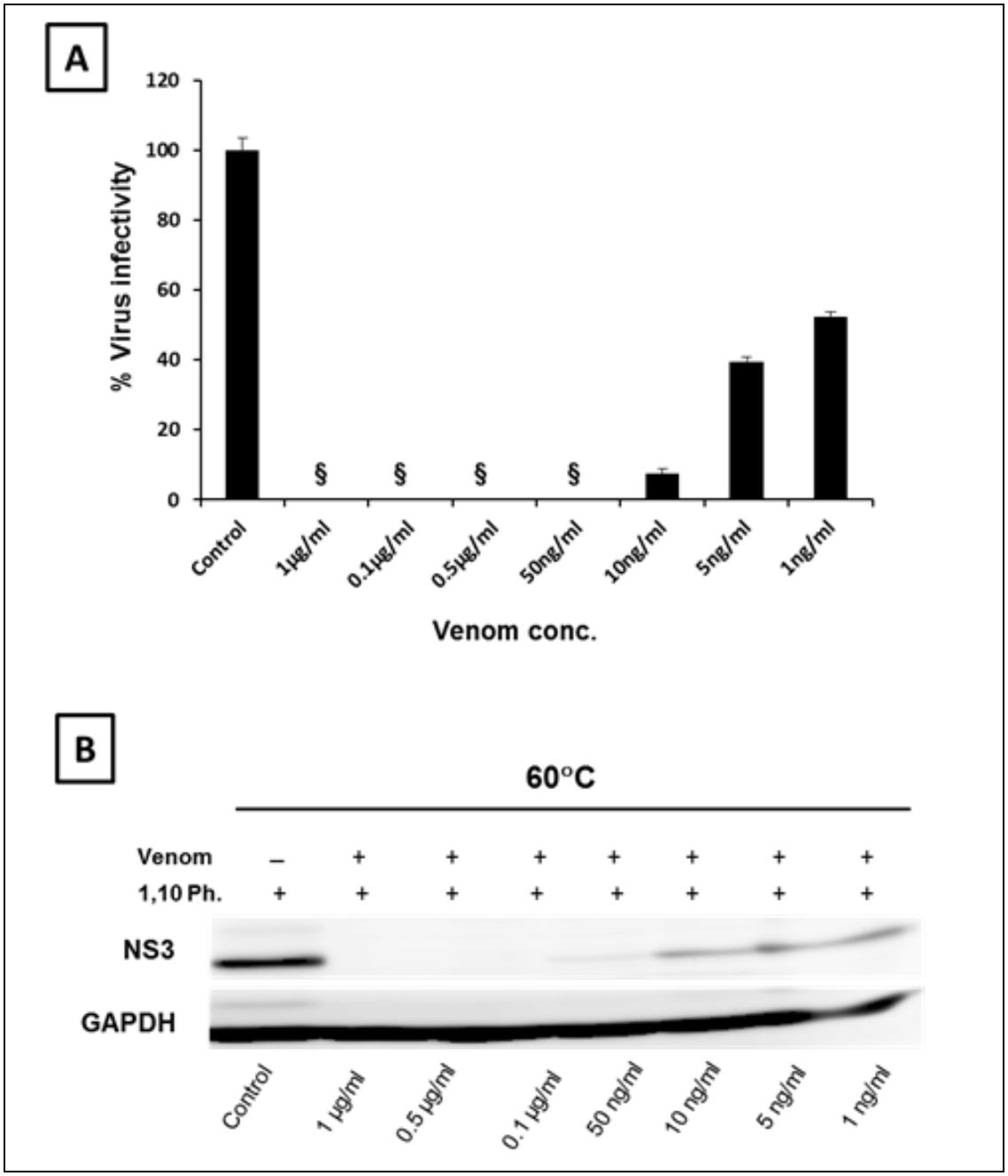

Fig. 3: Effects of neutralization of the proteinase activities of the virucidal effects of *Cerastes vipera* venom against HCV. *Cerastes vipera* venom was treated with a metalloproteinase inhibitor (1, 10-phenan-throline; 5 mM) at 60°C for 30 min. The treated venom was mixed with HCV for 1 hr at 37°C and the mixture was inoculated to Huh7.5 cells for 2 hr at 37°C. The cells were cultivated in the absence of the venom for 46 h. **(A) Amounts of** HCV infectious particles in the supernatants **(B) Accumulation** of HCV NS3 protein inside the cells. GAPDH was used as an internal control. Data represent means ± SEM of the data obtained from two independent experiments. 1,10 Ph., 1,10-phenanthroline; §, below the detection limit.

DISCUSSION

Snake venoms are complex combinations of toxins and enzymes that have a variety of biological actions. Snake venoms have become a rich source of novel bioactive proteins and peptides for drug development with a number of therapeutics derived from snake venom either in clinical use or in development (Smith and Vane, 2003; Ciscotto *et al.*, 2009; Vyas *et al.*, 2013; Macedo *et al.*, 2015; Chan *et al.*, 2016). This is because venom proteins exert a variety of biological effects, often with high potency and specificity for their target. Snake venoms have been shown to present antibacterial (Wang *et al.*, 2009; Samy *et al.*, 2010), antiparasite (Deolindo *et al.*, 2010), antifungal (Magaldi *et al.*, 2002), and antiviral activities (Borkow and Ovadia, 1992; Fenard *et al.*, 1999; Petricevich and Mendonça, 2003; Meenakshisundaram *et al.*, 2009; Muller *et al.*, 2012; Cecilio *et al.*, 2013); thus, representing a promising source of antiviral compounds. The antiviral activity of snake venoms represents a new and promising therapeutic alternative against the resistance mechanisms developed by viruses. The present study is designed to evaluate the crude venom obtained from Egyptian snake *Cerastes vipera* for their possible anti-HCV activities. The results showed that the crude venom extract of *Cerastes vipera* possessed anti-HCV activities, with their IC_{50} values being 1ng/ml. Interestingly, crude venom is neither toxic nor hemolytic *in vitro* at a concentration 1000-fold higher than that required for antiviral activity. Also, the crude venom exhibited a significant dose-dependent manner of anti-HCV activity**.** The present results suggested that *Cerastes vipera* venom maybe acts directly on HCV particles in culture supernatants to inhibit the viral infectivity, suggesting the inhibition at the entry steps is the first step of HCV life cycle. Previous studies on the venom proteome of Cerastes genus showed the presence of disintegrins, (svSP) cerastocytin, (PLA2), (CTL), (LAO) and (svMP) (Dekhil *et al.*, 2003; Bazaa *et al.*, 2005; Fahmi *et al.*, 2012; El Hakim *et al.*, 2015; Calvete *et al.*, 2017). In this sense, several studies have demonstrated the antiviral activity of these compounds such as svPLA2s and LAO from different snake venom against a variety of viruses, including DENV, YFV, HCV, HIV and others. Some svPLA2s or their products have been shown to interfere with a viral infection, mainly by inhibiting the replication of HIV-1 and HIV-2 (Gunther-Ausborn and Stegmann, 1997; Fenard *et al.*, 1999) *In vitro*, the svPLA2s can also modulate cell adhesion and cell proliferation and have anti-angiogenic properties (Bazaa *et al.*, 2009; Bazaa *et al.*, 2010; Khunsap *et al.*, 2011). Moreover, previous studies have shown that sPLA2 isolated from bee and snake venom inhibits the entry of HIV-1 in human primary blood leukocytes, and therefore the replication, through a mechanism linked to the binding of sPLA2 to cells (Fenard *et al.*, 1999). Chen et al., (2017) reported that CM-II-$sPLA_2$ and its derivatives are good candidates for the development of broad-spectrum antiviral drugs that target viral envelope lipid bilayers derived from the ER membrane. In addition, Petricevich and Mendonça (2003), showed that crude venom of *Crotalus durissus terrificus* inhibit the replication of measles virus on VERO cells At concentrations below 100 μg/mL, the Cdt venom showed no cell cytotoxicity. This inhibition occurred at the initial steps of the replication cycle, independently of the virucidal effect. Although, Muller *et al.*, (2012) reported that, the higher antiviral activity observed against DENV-2 and YFV was in the virucidal activity of sPLA2. Thus, it is likely that *Cerastes vipera* crude venom contains a compound(s) that induces anti-HCV by virucidal activity. Most available antiviral therapeutic compounds block replication processes shared by the virus and infected target cell and hence are toxic, mutagenic, and/or teratogenic and can potentially induce drug-resistant viral mutant substrains. Therefore, the identification of new anti-viral compounds, particularly those with new

mechanisms of action as antiviral peptides, is important (Petricevich and Mendonça, 2003). In the present study the results exhibited that, treatment of the *Cerastes vipera* venom with 1,10-phenanthroline and/or heating at 60°C for 30 min did not impair its anti-HCV activity. These results suggest that the anti-HCV activity of the *Cerastes vipera* venom is independent of its proteinase activities. Previous studies stated that, several SVMPs are heat labile and lose most of their activities above 60 °C (Peichoto *et al.*, 2007; Naves de Souza *et al.*, 2012; Wahby *et al.*, 2012; Abdel-Aty and Wahby, 2014).Also, from the above results it could be the *Cerastes vipera* venom is a relatively thermo-stable. These results consistent with previous study, *Naja naja* snake venom presents abundant thermostable peptides. Many of them possess useful pharmacological activity that may be employed for drug development (Binh *et al.*, 2010; Muller *et al.*, 2012). In conclusion, consistent with those results, we observed that *Cerastes vipera* crude venom inhibited infectivity of HCV particles, suggesting direct virocidal activity of the venom. Interestingly, crude venom is neither toxic nor hemolytic *in vitro* at a concentration 1000-fold higher than that required for antiviral activity. Further studies using Bioactivity-guided fractionation and purification analyses are needed to identify an active compound(s) responsible for this antiviral activity.

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