Smp76, a Scorpine‑Like Peptide Isolated from the Venom of the Scorpion Scorpio maurus palmatus, with a Potent Antiviral Activity Against Hepatitis C Virus and Dengue Virus

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Abstract
Growing global viral infections have been a serious public health problem in recent years. This current situation emphasizes the importance of developing more therapeutic antiviral compounds. Hepatitis C virus (HCV) and dengue virus (DENV) belong to the Flaviviridae family and are an increasing global health threat. Our previous study reported that the crude venom of Scorpio maurus palmatus possessed anti-HCV and anti-DENV activities in vitro. We report here the characterization of a natural antiviral peptide (scorpion-like peptide Smp76) that prevents HCV and DENV infection. Smp76 was purified from S. m. palmatus venom and contains 76 amino acids with six residues of cysteine. Smp76 antiviral activity was evaluated using a cell culture technique utilizing Huh7it-1, Vero/SLAM, HCV (JFH1, genotype 2a) and DENV (Trinidad 1751, type 2). A potential antiviral activity of Smp76 was detected in culture cells with an approximate IC50 of 0.01 μg/ml. Moreover, Smp76 prevents HCV infection and suppresses secondary infection, by inactivating extra-cellular infectious particles without affecting viral replication. Interestingly, Smp76 is neither toxic nor hemolytic in vitro at a concentration 1000-fold higher than that required for antiviral activity. Conclusively, this report highlights novel anti-HCV and anti-DENV activities of Smp76, which may lay the foundation for developing a new therapeutic intervention against these flaviviruses.

Keywords Animal venom · Antiviral peptide · DENV · HCV · Scorpio maurus palmatus · Smp76
Introduction

Hepatitis C virus (HCV) is a single-stranded RNA virus that belongs to family Flaviviridae (Mohammed et al. 2013; Supanee et al. 2014). Around 150 million people worldwide are chronically infected with HCV and the annual mortality from HCV-related liver diseases reaches up to 700,000 individual (Ministry of Health and 2015; World Health Organization 2016; Jefferies et al. 2018). In the past decade, interferon-based therapy was the gold standard for HCV treatment with a sustained virological response (SVR) rate hovering around 50%. The recent approval of oral direct-acting antivirals (DAAs), like HCV NS3 protease inhibitors, NS5A inhibitors and NS5B RNA-dependent RNA polymerase inhibitors, for clinical use improved the SVR rates to more than 90% (Pawlotsky 2016; Falade-Nwulia et al. 2017). Nevertheless, cirrhosis patients remain at risk for severe complications. In addition, treatment with DAAs is not affordable for many patients and they are still not readily available around the globe. Therefore, uncovering novel HCV inhibitors is still a clinical priority.

Dengue virus (DENV) is another single-stranded RNA Flaviviridae virus that is transmitted by mosquitoes causing dengue fever (Rodenhuis-Zybert et al. 2010). DENV is currently endemic in more than 100 countries with the highest prevalence in South-East Asia, Africa and the Americas (Mackenzie et al. 2004; Malavige et al. 2004; Deen et al. 2006; Bhatt et al. 2013). Each year, there are around 390 million DENV infections are recorded worldwide and among them 50 to 100 million patients are presented with the clinical manifestations of dengue fever (Bhatt et al. 2013). Dengue fever leads to 20,000 annual deaths, mainly in young children (Rui-feng et al. 2008). To date, four DENV serotypes, DENV-1, DENV-2, DENV-3 and DENV-4, have been identified and infections with one serotype does not offer protection from infection with the remaining three serotypes (Weaver and Vasilakis 2009; Messina et al. 2014; Mustafa et al. 2015). A major impediment to the development of vaccines is that vaccines should have a tetravalent effect, i.e. sufficient protective immune responses against all four DENV serotypes. Owing to the absence of specific treatments against DENV and the limitations of the available vaccine (Dengvaxia® or CYD-TDV), the global burden of DENV infection is becoming enormous (Behnam et al. 2016). Therefore, the development of new antiviral compounds against DENV infections is urgently needed.

Scorpion venom is a rich source for drug discovery and prototyping (Ortiz et al. 2015; Ghosh et al. 2019). Scorpion venoms are highly complex mixture of nucleotides, enzymes, mucoproteins, biogenic amines, nucleotides, salts, as well as peptides and proteins (Omran 2003; Rodriguez de la Vega and Possani 2005; Ozkan et al. 2006a, b, c; Feng et al. 2008; Kanoo and Deshpande 2008; Ortiz et al. 2015). Antimicrobial peptides (AMPs), isolated from several venomous animals, exhibit a wide range of antibacterial and antiviral activity with direct or indirect microbicidal activity (Hv et al. 2006; Ortiz et al. 2015). Several studies demonstrated an antiviral effect for certain scorpion venom peptides (Carballar-Lejarazu et al. 2008; El-Bitar et al. 2015; Ortiz et al. 2015). In this study, we report the molecular and functional characterization of a new antiviral peptide (Smp76), a scorpion-like peptide derived from an Egyptian scorpion’s venom, S. m. palmatus. Our findings will broaden the currently known antiviral peptides and open a new avenue for the development of novel HCV and DENV therapeutics.

Materials and Methods

Collection of Scorpions and Venom Preparation

Adult S. m. palmatus scorpions were collected from the Western Coastal Mediterranean Desert (Alexandria Governorate, Egypt) and were housed individually in clear plastic containers. Scorpions were fed small insects and were given water. Crude venom was extracted using electrical stimulation (20 V) and the milked venom was collected and centrifuged for 20 min at 13,000 rpm/4 °C as detailed previously (Abdel-Rahman et al. 2013). Clear supernatants were pooled, freeze-dried and stored at −20 °C until use. Venom samples were dissolved in bi-distilled water and the total protein concentration was determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the standard protocols.

Cell Culture and Virus Production

The human hepatoma-derived cell line, Huh7it-1, was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako, Osaka, Japan) supplemented with non-essential amino acids (Invitrogen, Carlsbad, CA, USA), fetal bovine serum (Biowest, Nuaille, France), streptomycin (100 μg/ml) and penicillin (100 IU/ml) (Invitrogen) in a 5% CO2 incubator at 37 °C (Aoki et al. 2014). Huh7it-1 cells were infected with cell culture-adapted HCV (JFH1 strain of genotype 2a) and supernatants were collected at day 3 post-infection (Wakita et al. 2005; Yu et al. 2010). Next, supernatants were, concentrated by 100 K Amicon centrifugal filters and used for antiviral screening.

DENV type 2 (Trinidad 1751 strain) (Hotta et al. 1983; Hotta and Homma 1994) was infected into Vero/SLAM cells (Ono et al. 2001). Following an hour of virus adsorption, the
virus-infected cells were cultured with DMEM medium containing 10% fetal bovine serum at 37 °C in 5% CO₂. Supernatants were collected at 3 to 5 days post-infection and stored at –80 °C. Measles virus (K52 strain) was inoculated to Vero/SLAM cells and the culture supernatants was collected from the virus-infected cells as described previously (Otaki et al. 2006).

Cytotoxicity Assay

The cytotoxicity of Smp76 was estimated using WST-1 assay as described previously with a some modification (Deng et al. 2008). Briefly, Huh7it-1 cells seeded in 96-well plate (2.5 × 10⁴ cells/well) were treated with serial dilutions of Smp76 (0.1 to 10 µg/ml) or medium (control) for 48 h at 37 °C in 5% CO₂. Then, supernatants were discarded and replaced with fresh DMEM medium containing 10 µl of WST-1 reagent (Roche, Mannheim, Germany) and incubated for 4 h. The number of viable cells was quantified by using a microplate reader at 450 and 630 nm. For each dilution, the percentage of viable cells were compared to the control sample and used to calculate the 50% cytotoxic concentrations (CC₅₀) values according to the following formula:

\[
\text{Absorbance of sample/Absorbance of control} \times 100.
\]

Hemolysis Assay

Hemolytic activity of Smp76 was performed as previously described (Evans et al. 2013). Briefly, a total of 10 µl of Smp76 peptide was mixed with 190 µl of diluted human red blood cells (RBCs) to achieve a final dilution 1/20 of the original venom peptide 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 37 °C, the plate was centrifuged for 5 min 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 (HC₅₀) value was defined as the peptide concentration that can lyse 50% of the RBCs.

Antiviral Activities of the Venom Fractions

Huh7it-1 cells were grown on coverslips (13-mm in diameter; 1.9 × 10⁵ Cells/well) 1 day before viral infection. Different concentrations of the venom fractions were mixed with HCV at multiplicity of infection (MOI: 1) for 2 h at 37 °C. Then, the virus/venom fraction mixture was inoculated in Huh7it-1 cells for 2 h at 37 °C. Medium-treated virus and cells were used as controls. The percentage of inhibition for virus infectivity was compared to the control samples and the 50% inhibitory concentrations (IC₅₀) were calculated.

Virus Titration (Immunofluorescence Staining)

HCV infectivity was determined as described previously (Deng et al. 2008). In brief, Huh7it-1 cells, grown on glass coverslips, were incubated with tenfold serially diluted virus samples for 2 h; then, the cells were washed with fresh medium and cultured for another 24 h. Following fixation and permeabilization, Huh7it-1 cells were incubated for 1 h with the serum of HCV-infected patients, followed by FITC-conjugated goat anti-human IgG (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Finally, the cells were counterstained by Hoechst 33342 (Molecular Probes, Eugene, OR, USA) and mounted using Vectashield H-1000 reagent (Vector Laboratories, Inc. Burlingame, CA, USA). HCV antigen positive cells were counted under a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

For dengue virus infectivity, serially diluted venom fractions and Smp76 were mixed with fixed amount of DENV and incubated for 2 h at 37 °C. The virus/venom fractions mixture was inoculated for 2 h at 37 °C on Vero/SLAM cells. The cells were washed twice after the virus inoculation and incubated with a fresh medium for 24 h. The infected cells were incubated with mouse monoclonal antibody against dengue virus followed/Alexa Fluor A488 goat anti-mouse IgG (Life Technologies).

To determine the infectivity of measles virus, serially diluted Smp76 was mixed separately with fixed amount of measles virus and incubated for 2 h at 37 °C. Virus/venom fraction mixture was inoculated to Vero/SLAM cells for 2 h at 37 °C and the cells were washed twice then, incubated with fresh medium for 24 h. The plaques (virus-induced syncytia) forming on the infected monolayer cells were counted.

Virocidal Activity Assay

The Smp76 venom peptide was mixed with a fixed amount of HCV JFH1 for 2 h at 37 °C. Next, the virus/Smp76 mixture was inoculated to Huh7it-1 cells and incubated for 2 h at 37 °C. The cells were washed and cultured without Smp76 for 24 h. Finally, the cells were subjected to an indirect immunofluorescence assay as previously described (El-Bitar et al. 2015).

Immunoblot Analysis

Huh7it-1 cells were lysed in SDS sample buffer and equal amounts of protein were separated on a SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA, USA). The PVDF membrane was blocked by 5% skim milk.
and probed with anti-HCV NS3 antibody and anti-GAPDH antibody (Millipore). Followed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Invitrogen) as a secondary antibody and visualized using the enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

Real-Time Quantitative RT-PCR

The amounts of HCV RNA in the infected cells were determined as described previously (El-Bitar et al. 2015). RNA was extracted by RNA cell miniprep system ReliaPrep (Promega, Madison, WI, USA). The cDNA was transcribed from one µg total RNA using a GoScript Reverse Transcription system (Promega) with oligo(dT) primers. Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara, Kyoto, Japan) in a MicroAmp 96-well reaction plate. PCR was conducted on a ABI PRISM 7500 fast system (Applied Biosystems, Foster City, CA, USA) with specific primers used to amplify the NS5A region of the HCV genome 5′-AGACGT ATTGAGGTCCATGC-3′ (sense) and 5′-CCGCAGGCA CGGTGCTGATAG-3′ (antisense). The expression of GAPDH mRNA was also measured as a housekeeping gene using the 5′-GCCATCAATGACCCCTTCATT-3′ (sense) and 5′ TCT CGCTCCTGGAAGATGG-3′ primers.

RP-HPLC Fractionation of S. m. palmatus Venom

Chromatographic separation of S. m. palmatus venom was conducted using reverse phase high performance liquid chromatography (RP-HPLC; Waters, Milford, Massachusetts, United States) (Abdel-Rahman et al. 2013). A total of 4 mg scorpion venom was reconstituted in 200 ml 0.05% trifluoroacetic acid (TFA) and fractionated by a C18 RP-HPLC column (250 x 10 mm, 5 µm; Vydac, California, United States). A gradient of buffer A (0.12% TFA in MilliQ water) and sixty percent buffer B (0.10% TFA in acetonitrile) were used to separate scorpion venom in 1 h (1 ml/min flow rate). Individual venom fractions were collected manually according to the peak’s absorbance (at 230 nm). All collected fractions were dried using a rotary evaporator (Savant Speed Vac SC210A, Minnesota, United States). The active fraction eluted at retention time 36.4 min was further characterized using mass spectrometry and amino acids sequencing. In addition, recombinant and synthetic Smp76 derivatives (N-terminal 32 aa and C-terminal 44 aa) were prepared as described below.

Determination of Molecular Mass and N-Terminal Sequencing of Native and Recombinant Smp76 Peptides

The average molecular mass of native Smp76 peptide (8398 Da), the recombinant fusion protein Thioredoxine-Smp76 (22123 Da) and a recombinant C-terminal of Smp76 (4775.57 Da) were determined using ESI–MS, ESI LCQ FLEET spectrometer (Thermo Scientific, CA, USA). The sequence of native Smp76 (approximately 250 pmol) was determined using Edman degradation (Protein Sequencer PPSQ-31A, Shimadzu Scientific Biotech, Maryland, United States). Synthetic N, C-terminal and full-length Smp76 peptides were manufactured by GenScript Japan Inc.

Construction of Recombinant Smp76C-Terminal Peptide (44 aa)

Six oligonucleotides were designed to cover the C-terminal region of Smp76 (44 aa) (Supplementary Table 1). The oligonucleotides BHEK-Dir1 and scSMP-LW5 included the BamHI and XhoI restriction sites, respectively. Subsequently, this enabled the cloning into the pET22b-Thio-EK expression vector as detailed previously (Jiménez-Vargas et al. 2017; Vargas-Jaimes et al. 2017). PCR assembly of the C-terminal peptides was carried out using Vent DNA Polymerase (New England Biolabs, MA, United States). The final concentration of external primers BHEK-Dir1 and scSMP-LW5 was 0.2 pmol/µl while the concentration of internal oligonucleotides was 0.02 pmol/µl.

Expression and Purification of Recombinant C-Terminal (44 aa) Peptide of Smp76

In order to express the fusion protein Thioredoxine-C-terminal, pET22b-Thio-C-terminal plasmid was transformed into E. coli BL21 (DE3) using electroporation. The pellet was harvested and the fusion protein was purified using the Ni–NTA agarose resin columns (QIAGEN) as previously described (Vargas-Jaimes et al. 2017). HPLC purification was further performed using a C18 RP-HPLC column (250 x 10 mm, 5 µm; Vydac, California, United States). The purified fusion protein Thioredoxine-C-terminal was digested with enterokinase (New England Biolabs) in 200 mM Tris HCl (pH 8.0), 500 mM NaCl, 20 mM CaCl₂ for 16 h at 25 ºC. Then, the pure recombinant C-terminal of Smp76 was finally isolated and purified by HPLC as described above.

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM). The difference between data sets was determined by Student’s two-tailed t test. A P value of < 0.05 was considered to be statistically significant.
Results

Fractionation of Crude Venom Extract of *S. m. palmatus*

Since a whole *S. m. palmatus* soluble venom strongly inhibited HCV infectivity in vitro and displayed anti-HCV activity in cell culture (El-Bitar et al. 2015), the crude venom was fractionated to identify active molecule(s) with anti-HCV activity. Accordingly, 74 fractions from four milligrams of the venom were separated by HPLC analytical method (Fig. 1a; Table 1).

### Table 1 Anti-HCV and anti-DENV activities (IC$_{50}$) of selected fractions from the venom of the scorpion *S. m. palmatus*

| No. | RT (min) | Protein conc. (mg/ml) | HCV IC$_{50}$ (µg/ml) | DENV IC$_{50}$ (µg/ml) | No. | RT (min) | Protein conc. (mg/ml) | HCV IC$_{50}$ (µg/ml) | DENV IC$_{50}$ (µg/ml) |
|-----|----------|-----------------------|-----------------------|------------------------|-----|----------|-----------------------|-----------------------|------------------------|
| 1   | 3.3      | 0.496                 | >10.0                 | >10.0                  | 38  | 23.2     | ND                    | NT                    | NT                     |
| 2   | 3.9      | ND                    | NT                    | NT                     | 39  | 24.1     | 1.819                 | >10.0                 | >10.0                  |
| 3   | 4.5      | ND                    | NT                    | NT                     | 40  | 24.9     | ND                    | NT                    | NT                     |
| 4   | 5.2      | ND                    | NT                    | NT                     | 41  | 26.9     | 0.683                 | >10.0                 | >10.0                  |
| 5   | 5.7      | ND                    | NT                    | NT                     | 42  | 27.1     | 1.886                 | >10.0                 | >10.0                  |
| 6   | 6.9      | ND                    | NT                    | NT                     | 43  | 28.0     | ND                    | NT                    | NT                     |
| 7   | 8.4      | 1.037                 | >10.0                 | >10.0                  | 44  | 28.4     | 1.926                 | >10.0                 | >10.0                  |
| 8   | 9.0      | ND                    | NT                    | NT                     | 45  | 29.0     | 4.491                 | >10.0                 | >10.0                  |
| 9   | 9.2      | ND                    | NT                    | NT                     | 46  | 29.8     | ND                    | NT                    | NT                     |
| 10  | 9.5      | ND                    | NT                    | NT                     | 47  | 30.7     | 0.804                 | >10.0                 | >10.0                  |
| 11  | 9.7      | ND                    | NT                    | NT                     | 48  | 31.4     | 0.891                 | >10.0                 | >10.0                  |
| 12  | 10.5     | ND                    | NT                    | NT                     | 49  | 31.6     | 0.533                 | >10.0                 | >10.0                  |
| 13  | 11.4     | ND                    | NT                    | NT                     | 50  | 32.1     | 0.554                 | >10.0                 | >10.0                  |
| 14  | 11.7     | ND                    | NT                    | NT                     | 51  | 32.2     | ND                    | NT                    | NT                     |
| 15  | 12.3     | ND                    | NT                    | NT                     | 52  | 33.0     | ND                    | NT                    | NT                     |
| 16  | 12.5     | ND                    | NT                    | NT                     | 53  | 33.5     | ND                    | NT                    | NT                     |
| 17  | 13.0     | ND                    | NT                    | NT                     | 54  | 33.7     | ND                    | NT                    | NT                     |
| 18  | 13.2     | 1.273                 | >10.0                 | >10.0                  | 55  | 35.5     | 0.797                 | 0.10                  | ≤10                     |
| 19  | 13.4     | ND                    | NT                    | NT                     | 56  | 36.2     | 3.773                 | 0.05                  | NT                     |
| 20  | 13.7     | ND                    | NT                    | NT                     | 57a | 36.4     | 4.462                 | 0.01                  | 0.01                   |
| 21  | 13.9     | ND                    | NT                    | NT                     | 58  | 37.9     | 0.619                 | 0.10                  | NT                     |
| 22  | 14.7     | ND                    | NT                    | NT                     | 59  | 38.7     | 0.971                 | 1.0                   | 1.0                    |
| 23  | 15.0     | ND                    | NT                    | NT                     | 60  | 39.2     | 1.214                 | 1.0                   | 1.0                    |
| 24  | 15.5     | ND                    | NT                    | NT                     | 61  | 40.8     | 0.648                 | 1.0                   | 1.0                    |
| 25  | 15.7     | 4.446                 | >10.0                 | >10.0                  | 62  | 41.2     | ND                    | NT                    | NT                     |
| 26  | 17.0     | 5.034                 | >10.0                 | >10.0                  | 63  | 41.6     | ND                    | NT                    | NT                     |
| 27  | 17.4     | 2.132                 | >10.0                 | >10.0                  | 64  | 42.2     | 0.226                 | 1.0                   | 3.0                    |
| 28  | 18.0     | 3.209                 | >10.0                 | >10.0                  | 65  | 42.9     | ND                    | NT                    | NT                     |
| 29  | 18.9     | 2.371                 | >10.0                 | >10.0                  | 66  | 43.8     | 2.279                 | 6.0                   | >10.0                  |
| 30  | 19.5     | ND                    | NT                    | NT                     | 67  | 45.5     | ND                    | NT                    | NT                     |
| 31  | 20.0     | ND                    | NT                    | NT                     | 68  | 46.1     | 0.099                 | NT                    | NT                     |
| 32  | 20.2     | 0.209                 | >10.0                 | >10.0                  | 69  | 46.7     | ND                    | NT                    | NT                     |
| 33  | 20.5     | 1.692                 | >10.0                 | >10.0                  | 70  | 48.2     | ND                    | NT                    | NT                     |
| 34  | 21.1     | ND                    | NT                    | NT                     | 71  | 48.7     | 0.501                 | NT                    | NT                     |
| 35  | 21.5     | ND                    | NT                    | NT                     | 72  | 50.9     | 0.126                 | NT                    | NT                     |
| 36  | 21.9     | 0.687                 | >10.0                 | >10.0                  | 73  | 56.6     | 0.082                 | NT                    | NT                     |
| 38  | 22.6     | 1.821                 | >10.0                 | >10.0                  | 74  | 59.9     | 0.001                 | NT                    | NT                     |

*ND* not detected, *NT* not tested

*a* Venom fraction containing Smp76
Subsequently, the anti-HCV activity of fractions obtained from the venom of *S. m. palmatus* were tested against JFH1 strain of genotype 2a. Based on protein concentrations, 30 fractions were tested for anti-HCV activity. The other fractions showed very low protein concentrations and, therefore, it was not possible to check their antiviral activity (Table 1).

Each fraction was incubated separately with fixed amount of HCV for 2 h at 37 °C. Then, Huh7it-1 cells were infected with the virus/venom-fraction mixture (Fig. 2a) and virus infectivity was measured by infectious center assay. The anti-HCV activity started to appear from the retention time (RT) 35.5 until 43.8 min. The fraction at RT 36.4 min showed the most potent anti-HCV activity with IC50 being 0.01 μg/ml (Fig. 2b; Table 1).

Identification of Smp76 From the Active Fraction RT 36.4 min

In order to identify the bioactive compound(s) present in RT 36.4, LC–MS–ESI analysis was performed. Interestingly, the data of mass spectrometry showed that the active fraction contains a unique peptide with molecular mass of 8398 Da (Fig. 1b). To go further into the characterization of the active peptide, the amino acid sequence was determined (Fig. 1c). The sequence of this peptide contains 76 amino acids (GWINEKKMQQKIDEKIGNIIIIPGMAKAVIHKM – MAFQCVANVDTLGNCKKHCAKTTGEKGYCHGTKCKGIELSY). The obtained sequence belongs to the scorpion venom antimicrobial peptides and matched with the scorpine-like peptide Smp76, which was identified in the scorpion venom gland of *S. m. palmatus* using transcriptomic analysis (Abdel-Rahman et al. 2013). The amino acid sequence of Smp76 was confirmed until the amino acid number 40 by Edman degradation method and the molecular mass was confirmed by mass spectrometry resulting in 8398 Da (see “Materials and Methods”).

Cytotoxicity, Hemolytic Activity and Selectivity Index of Smp76

The cytotoxic activity of Smp76 against Huh7it-1 cells was tested using the WST-1 assay, and hemolytic activity was examined on human red blood cells. The CC50 and the HC50
were calculated. As shown in Table 2, CC$_{50}$ of Smp76 against Huh7it-1 cells and HC$_{50}$ were > 10 μg/ml. These results indicate that this peptide has no cytotoxic or hemolytic effects up to 10 μg/ml with selectivity index (SI) > 1000.

**Smp76 did not Inhibit the HCV Replication in Culture Cells**

Since Smp76 displayed a significant inhibitory effect at the early stage of HCV infection, we examined whether the Smp76 peptide can also inhibit HCV NS3 protein production and HCV RNA replication in the cells. Virus at multiplicity of infection of 2 pfu/cell was inoculated to the Huh7it-1 cells and cultivated for 24 h. a Schematic of infection assay. b Amounts of HCV infectious particles. The data represents Mean ± SEM of two independent experiments. *Below the detection limit; 1≤0.07%; 2<0.5%

Table 2 Cytotoxicity (CC$_{50}$), hemolytic activity (HC$_{50}$) and selectivity index (SI) of Smp76

| Venom peptide | CC$_{50}$ (μg/ml) | HC$_{50}$ (μg/ml) | SI   |
|---------------|------------------|------------------|------|
| Smp76         | >10              | >10              | >1000|

Fig. 2 Screening of anti-HCV activities of the venom fractions of S. m. palmatus. HCV (JFH1a strain) was treated with decreasing concentrations of the crude venom of S. m. palmatus and collected fractions (10, 1.0, 0.1 and 0.01 μg/ml) for 2 h or left untreated as a control (−) and then inoculated to Huh7it-1 cells and cultivated for 24 h. a Schematic of infection assay. b Amounts of HCV infectious particles. The data represents Mean ± SEM of two independent experiments. *Below the detection limit; 1≤0.07%; 2<0.5%

**Anti-DENV Activity of Smp76**

We previously showed that the crude venom of S. m. palmatus inhibits DENV (El-Bitar et al. 2015). Therefore, anti-DENV activity of the selected 30-fractions obtained from the crude venom of S. m. palmatus was tested. Each fraction was incubated separately with fixed amount of DENV for 2 h at 37 °C. After that, the virus/venom fractions mixtures were used to infect Vero/SLAM cells and virus infectivity was measured by infectious center assay. Interestingly, the results were consistent with the data of anti-HCV activity obtained in this study (Table 1). Also, the fraction identified at 36.4 min which contains Smp76 showed the potent anti-DENV activity with IC$_{50}$ being 0.01 μg/ml (Table 1 and Fig. 4b).

**Specificity of Antiviral Activity of Smp76**

To determine whether the antiviral activity of Smp76 peptide (previously described) was specific to HCV and DENV,
we tested its possible effects on another enveloped virus such as measles virus (Otaki et al. 2006). In this investigation, the virus was incubated with Smp76 (10–0.01 μg/ml) for 2 h or left untreated as a control (−) and then inoculated to Vero/SLAM cells. a Schematic of infection assay. b Amounts of DENV and measles virus infectious particles. The data represents Mean ± SEM of two independent experiments. §<0.01%; ‡≤0.07% of the control.

Lack of Antiviral Activity of Smp76 Synthetic and Recombinant Derivatives (N- and C-Terminals)

In an attempt to identify the active domain of Smp76, the antiviral activity (anti-HCV and anti-DENV) of synthetic N-terminal (32 aa) and C-terminal (44aa without disulfide bonds) were tested. Although, the purified native Smp76 showed strong antiviral activity with IC₅₀ of 10 ng/ml, there was no antiviral activity for both synthetic terminals (Table 3). Moreover, antiviral activity of recombinant C-terminal (44 aa) was examined. Also, no antiviral activity for recombinant C-terminal was detected (Table 3). These results indicate that the full-length of Smp76 may be required for its activity against HCV and DENV.

Lack of Antiviral Activity of Full-length Synthetic Smp76 Without Disulfide Bonds

The above mentioned results imply that the full-length of Smp76 may be required for its activity against HCV and DENV. The full-length Smp76 peptide (76 aa) was synthesized but without disulfide bonds. The antiviral activity of the synthesized Smp76 peptide was examined against HCV and DENV. These results showed no antiviral activity for the synthetic full-length peptide without disulfide bonds against HCV and DENV (Table 3).

### Table 3 Antiviral activity of Smp76 and its derivatives

| Source                | Smp76/derivatives     | IC₅₀ (ng/ml) Anti-HCV | IC₅₀ (ng/ml) Anti-DENV |
|-----------------------|-----------------------|-----------------------|-----------------------|
| Synthetic             |                       |                       |                       |
| N-terminal 32 aa      | > 10,000              | > 10,000              |
| C-terminal 44 aa (without disulfide bonds) | > 10,000              | > 10,000              |
| Full-length 76 aa (without disulfide bonds) | > 10,000              | > 10,000              |
| Recombinant           |                       |                       |                       |
| C-terminal 44 aa      | > 10,000              | > 10,000              |
| Native or purified Smp76 | Full-length 76 aa | 10                    | 10                    |
Discussion

Initial fingerprint analysis of the soluble venom of S. m. palmatus allowed the identification of at least 65 different components with molecular masses ranging between 413 and 14,009 Da (Abdel-Rahman et al. 2013). Most peptides showed a molecular weight ranging from 3 to 5 kDa. In the present study 74 fractions were obtained from 4 mg of the crude venom of S. m. palmatus. According to HPLC fraction peaks profile and the protein concentrations, we examined antiviral activity of 30 fractions against HCV and DENV to identify the active fraction. The fraction eluted at RT 36.4 min showed the most potent anti-HCV and anti-DENV activity with IC$_{50}$ being ~ 0.01 μg/ml. According to the data of mass spectrometry and amino acids sequencing, this active fraction contains only one peptide with molecular mass of 8398 Da and consists of 76 amino acids. The obtained sequence matches with scorpine-like peptide Smp76 (Abdel-Rahman et al. 2013).

Scorpine is firstly isolated from the venom of Pandinus imperator. The structure of scorpine is a hybrid between a cecropin and a defensin. The sequence of scorpine carboxyl terminal region is similar to that of β-KTx family, with cysteine-stabilized α/β fold, and three disulfide bridges. On the other hand, its amino-terminal region is identical to the cecropin family peptides (Conde et al. 2000). Scorpine has also amino acid sequences similar to AMPs and K$^+$ channel blocking peptides (Luna-Ramirez et al. 2015). Scorpine homologs were thereafter identified from the venom of various scorpions such as Opistophthalmus carinatus (Zhu and Tytgat 2004), Heterometrus laoticus (Uawonggul et al. 2007), H. gertschi (Schwartz et al. 2007), S. m. palmatus (Abdel-Rahman et al. 2013), genus Vaejovis (Quintero-Hernandez et al. 2015) and Urodacus yaschenkoi (Luna-Ramirez et al. 2015). Importantly, all these peptides possess anti-malaria as well as antimicrobial activities (Conde et al. 2000; Carballar-Lejarazu et al. 2008) and act also as potassium channel blockers (Diego-Garcia et al. 2007). The present data clearly showed that smp76 inhibits the ability of HCV virus to infect the host cells. Indeed, our previous study demonstrated that the crude venom of S. m. palmatus venom prevents HCV infection with direct virucidal activity (El-Bitar et al. 2015). On the other hand, we cannot rule out the possibility that, smp76 peptide might has an independent effect on the receptor complexes of host cell components or interacts with components that inactivate viral entry. This possibility will be further investigated by the incubation of smp76 with cells in a free-virus condition prior to HCV infection. However, it worth to mention that the incubation of S. m. palmatus crude venom with cells prior to the HCV infection of cells did not impair the viral infectivity (El-Bitar et al. 2015). Thus, the possible effect of smp76 on the host cell to abrogate HCV infection is unlikely. In the present study, Smp76 prevents the early stages of life cycle of HCV and DENV most probably through interacting with viral particles. The viral particle can be neutralized by targeting the envelope of the HCV or host factors related to the mature viral particle (Zeisel et al. 2013). Notably, it has been reported in various studies that the structure of biological membranes could be altered by AMPs (Zasloff 2002; Harrison et al. 2016).

Currently, the new approach for HCV infection treatment probably based on the combination of several drugs (Pereira and Jacobson 2009; Sarrazin and Zeuzem 2010; Zeisel et al. 2011; Qian et al. 2016). Therefore, the use of Smp76 with anti-HCV drugs for treatment of HCV infection may have synergistic effect. However, further experiments are needed to check this possibility.

The present study reported distinctive data on the ability of Smp76 peptide to protect cellular systems from attack of DENV and neutralize viral infection. Currently dengue virus considered as one of the most important arthropod born viral disease worldwide (Botta et al. 2018). Despite the global efforts, there is no antiviral therapy against DENV infections clinically approved and only symptomatic treatment and hospital supportive care setting are available for infected people (Behnam et al. 2016). It was shown that recombinantly expressed scorpine (RScrp) inhibited DENV-2 replication in C6/36 mosquito cells. Also, it was suggested that the development of transgenic mosquitoes that overexpress and correctly secrete RScrp and could eventually break the dengue fever transmission (Carballar-Lejarazu et al. 2008). On the other hand, Smp76, as an infection inhibitor, has some advantages compared to antiviral drugs that target the viral replication stages inside the target cell. Smp76 can inhibit DENV infection before viral entry and is, therefore, helpful for treatment of DENV viraemia.

The prospective pharmaceutical potential of Smp76 cannot be neglected, especially considering its potent antiviral activity. However, Smp76 isolation from natural sources is ineffectual and time-consuming. Synthetic Smp76 without disulfide bonds showed no antiviral activities against HCV and DENV. One possibility is that the synthetic Smp76 peptide without disulfide bonds did not have the properly folded structure necessary for activity. Recently, recombinant scorpine with antimarial and antibacterial activities was produced by different fusion technology using small ubiquitin-related modifier (SUMO) (Zhang et al. 2014) and maltose binding protein (MBP) (Zhang et al. 2016). These methods have improved efficiency and reduced the cost of producing scorpine and can contribute to the future production of active recombinant Smp76. Interestingly, the recombinant Smp76 was shown to inhibit DENV and ZIKV infections in cultured cell lines and primary mouse...
macrophages. However, rSmp76 did not inactivate the viral particles directly but suppressed the established viral infection by upregulating the expression of IFN-β (Ji et al. 2018). This mechanism is significantly different from the virucidal effect of native Smp76 peptides. The exact mechanism by which Smp76 exerts its antiviral activity against HCV and DENV to inhibit infecting their target cells need further studies. In vivo studies should also assess the future role of Smp76 in managing HCV and DENV infections.

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Compliance with Ethical Standards

Conflict of interest Drs. Hak Hotta, Moustafa M Sarhan, Alaa MH El-Bitar, Lourival D. Possani and Mohamed A. Abdel-Rahman have a patent (anti-virus drug: PCT/JP2017/286) pending containing some of the information described in this work.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animal performed by any of the authors.

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