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# Bothropstoxins I and II as potent phospholipase A2 molecules from *Bothrops jararacussu* to impair Hepatitis C virus infection

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## ABSTRACT

Hepatitis C is a hepatological disorder induced by the Hepacivirus hominis (Hepatitis C virus, HCV), with approximately 170 million individuals estimated to be presently affected globally. The current treatment for infected patients primarily relies on direct-acting antivirals (DAAs). However, this treatment is marked by its high cost, numerous side effects, and documented instances of antiviral resistance. These challenges underscore the imperative for developing novel therapeutic strategies. In this framework, naturally occurring compounds have exhibited considerable medical significance attributable to their biological functionalities. Compounds extracted from snake venoms have evidenced antiviral efficacy against a variety of viral pathogens including *Orthoflavivirus denguei* (DENV), *Orthoflavivirus flavi* (YFV), *Orthoflavivirus zikaense* (ZIKV), and HCV. Here, the activity of 10 proteins isolated from snakes' venom of *Bothrops* genus were evaluated against HCV replicative cycle. The full-length JFH-1 HCV system was used to infect the Huh-7.5 cell. Cell viability was measured simultaneously through MTT assay. Eight compounds inhibited up to 99% of HCV infection, being the most potent inhibitory rates observed in BthTX-I and BthTX-II, with an SI of 13.5 and 1736, respectively, being able to block 84.7% and 96% of HCV infectivity, in the same order. BthTX-II also demonstrated a protective effect in cells treated prior to HCV infection of approximately 86.7%. Molecular docking calculations suggest interactions between the two

proteins with HCV E1-E2 glycoprotein complex. BthTX-II exhibited stronger interactions, indicated by 22 hydrophobic interactions. In conclusion, these compounds were shown to inhibit HCV infectivity by either acting on the virus particles or protecting the cells against infection.

**Keywords:** Hepatitis C virus; Hepacivirus, Phospholipase $A_2$.

## 1. Introduction

Hepatitis C is a disease caused by the *Hepacivirus hominis* (Hepatitis C virus, HCV), a member of the *Hepacivirus* genus and *Flaviviridae* family[1], and is an enveloped virus, with a positive single-sense RNA genome of approximately 10kb. The genome contains an open reading frame (ORF) that encodes a polyprotein of around 3,000 amino acids, which is subsequently cleaved into structural and non-structural viral proteins[2,3].

Patients chronically infected with HCV can develop HCV-related liver disease that may gradually advance from chronic hepatitis to liver cirrhosis and hepatocellular carcinoma (HCC), which have considerable morbidity and mortality rates [4–6]. The global prevalence of HCV was estimated to be 170 million people with 3-4 million new infection per year, and globally, approximately 71 million people are affected by chronic HCV infection. [7–15].

Since 2011, the treatment of HCV infection is mostly based on direct-acting antivirals (DAAs) which present Sustained Virological Response (SVR) rates higher than 90% [16]. However, the high cost of DAA-based treatment (around US$ 7,000 weekly for each DAA) and documented resistance-associated variants to these treatments demonstrate the need of searching for new therapeutic approaches [17–19].

Several pharmacologically active components from animal venom have been described in the literature. Among them, the bee venom from the honeybee *Apis Melifera* L. has various biological properties including antibacterial, antiparasitic, and antiviral[20,21]; compounds isolated from the *Scorpio maurus palmatus* [22] and *Heterometrus petersii* [23] scorpions venoms inhibited HCV *in vitro* through virucidal activity, by directly interacting with the viral membrane and decreasing the virus infectivity; and different proteins isolated from the venom of *Bothrops* and *Crotalus* species blocked the replication of enveloped viruses like *Influenza A virus* (IAV), Vesicular stomatitis virus (VSV), *Orthopneumovirus hominis* (HRSV), and *Simplexvirus humanalpha1* and *2* (HSV1), as well as inhibited the replication of non-enveloped viruses such as *Alphapapillomavirus 9* (Human papillomavirus, HPV), and *Enterovirus A* viruses: EnterovirusA71 (EV-A71) and Coxsackie virus (CVA) [24,25].

Due to its complexity and variety of compounds, snake venom is a vast source of molecules capable of exerting biological activities[26]. Of more interest, the venom of *Bothrops* snakes is a complex mixture of L-amino acid oxidases, hialuronidases, serinoproteases, metalloproteases, phospholipases, among others[27]. What is more interesting, the phospholipases A2 ($PLA_2$) isolated from the venom of *Bothrops jararacussu* snake have also been described as inhibiting several viruses such as *Orthoflavivirus denguei* (DENV), *Orthoflavivirus flavi* (YFV), Rocio virus (ROCV), *Orthobunyavirus oropoucheense* (OROV), *Mayaro virus* (MAYV), *chikungunya virus* (CHIKV), *Orthoflavivirus zikaense* (ZIKV), and HSV1[28–31]. Shimizu and collaborators showed that the treatment with toxins isolated from the venom of *Crotalus durissus terrificus* inhibited HCV entry, replication, and release[32]. Recently, another class of phospholipases was demonstrated to have strong antiviral activity against ZIKV, two bothropstoxins (BthTX-I and BthTX-II) isolated from *Bothrops jararacussu* venom[33]. In addition, the CM-II isoform of a secreted $PLA_2$, obtained from *Naja mossambica mossambica* snake venom (CM-II-s$PLA_2$) possess potent virucidal activity against HCV, DENV and *Orthoflavivirus japonicum* (Japanese encephalitis virus - JEV)[34], highlighting the potential of this selected compounds as antiviral drugs.

Therefore, considering the potential of molecules isolated from snake venoms, 10 proteins isolated from *Bothrops jararacussu*, *B. moojeni*, *B. alternatus,* or *B. jararaca* venoms investigated towards their activity in the HCV replicative cycle.

## 2. Material and Methods

### 2.1 Compounds isolated from snake venoms

The venoms of *Bothrops alternatus*, *B. moojeni,* and *B. jararaca* were purchased from serpentarium "Serpentário Proteínas Bioativas Ltda" of Batatais/SP, registered at the Ministry of the Environment, nº 471301. The venom of *B. jararacussu* was acquired from the serpentarium "Centro de Extração de Toxinas Animal", registered at the Ministry of the Environment, nº 3002678. Isolation and purification of the $PLA_2$s BthTX-I and BthTX-II from *B. jararacussu*; the phospholipases CM12 and CM14 from *B. moojeni*; the peptide pools PV and PIV from *B. alternatus* and the peptide pools Bjara H, Bjara I, and Bjara H2 from *B. jararaca* were carried out at the Laboratory of Toxinology of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, under the supervision of Profª Suely Vilela Sampaio, as previously described in details[31,35–39]. Briefly, crude venom (300 mg) from *Bothrops jararacussu* was separated by size-exclusion chromatography on a Shephacryl S-200

(100 x 2.6 cm; Amersham, GE Healthcare Life Science, Pittsburgh, PA, USA), previously equilibrated with 50mM ammonium bicarbonate pH 8. The eluted fractions at a flow rate of 22.8 mL/min, with fractions of 3 mL collected was monitored for absorbance at 280nm, pooled and lyophilized. The fraction referred as SPIV was identified by means of SDS PAGE (Laemmili 1970) by containing phospholipases and myotoxins which have a molecular mass of 14 kDa at 17kDa approximately. For further purification, SPIV was turned in to ion exchange chromatography on a CM Sepharose columm (40 x 2 cm; Amersham, GE Healthcare Life Science, Pittsburgh, PA, USA), previously equilibrated with 50 mM ammonium bicarbonate pH 8 (Buffer A). Elution began with the same buffer, followed by a linear gradient of 500 mM ammonium bicarbonate (pH 8, Buffer B). The process was carried out at a flow rate of 1.5 mL/min, collecting 4 mL fractions based on absorbance at 280 nm. The fraction corresponding to BthTX-I and BthTX- II were collected, lyophilized, and stored at 4 °C for subsequent analysis. The purity of BthTX-I and BthTX- II were also assayed by 12% (w/v) SDS-PAGE and analyzed using an HPLC system with a reverse-phase column and N-terminal amino acid sequencing via automated Edman degradation in a protein sequencer (PPSQ 33A, system, Shimadzu)[37–39]. The lyophilized compounds were dissolved in PBS (Phosphate-Buffered Saline), filtered, and stored at -80°C. Protein concentrations after dissolution were verified using a colorimetric assay with the Pierce BCA Protein Assay kit (ThermoFisher). Compounds were diluted in a complete medium immediately prior to the experiments. PBS was used as untreated control in all performed assays.

### 2.2 Cell Culture

The human hepatoma cell line Huh-7.5 (RRID:CVCL_7927) [40] was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) and supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, BR), 100 U/mL penicillin (Gibco Life Technologies, USA), 100 µg/mL streptomycin (Gibco Life Technologies, USA), 1% (v/v) non-essential amino acids (Gibco Life Technologies, USA), and 2% (v/v) HEPES (Gibco Life Technologies, USA). The cells were maintained at 37 ºC in a humidified 5% $CO_2$ incubator.

### 2.3 Cell viability assay

The cell viability of compounds was determined by the MTT [3-(4,5-dimeth- ylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Sigma-Aldrich, St. Louis, MO, USA) [41] as previously described [42]. A suspension of 5 x $10^3$ Huh-7.5 cells per well was transferred to 96-well microplates 24 h before the assay and incubated at 37 ºC with an atmosphere at 5% $CO_2$. The supernatant was replaced by fresh DMEM containing drugs at specific concentrations and

maintained for 72 h. After treatment, cells were treated with DMEM containing 1 mg/mL of MTT, incubated for 30 minutes, and then the medium was replaced with 100 µL of dimethyl sulfoxide (DMSO, Sigma–Aldrich) to solubilize the formazan crystals. The viability of surviving cells was assessed by measuring the optical density (OD) at 592 ηm, using a spectrophotometer. Cellular viability was determined according to the equation (T/C) x 100, where T corresponds the average optical density of the treated group and C represents the control group. In order to calculate the cytotoxic concentration of 50% ($CC_{50}$), the cells were treated with BthTX-I and BthTX-II in a two-fold serial dilution ranging from 0.003 to 25 µg/mL, and cell viability was measured after 72h. The $CC_{50}$ value was calculated using a non-linear regression at GraphPad Prism 8.0.

**2.4 Viral assays**

The JFH-1 HCVcc genotype 2a particles[43] were generated as described previously[44]. A suspension of 5 x $10^3$ Huh-7.5 cells/well were seeded to 96-well microplates incubated at 37 ºC with the atmosphere at 5% $CO_2$. After 24h, cells were infected with 100 focus-forming units (FFU)/well of JFH-1 HCVcc, and compounds were added at the highest non-cytotoxic concentration (cell viability ≥ 90%). The (-)-epigallocatechin gallate (EGCG, Sigma-Aldrich, USA) at 100 µM was used as a control of inhibition of HCV infection[45]. For all antiviral assays, cells were fixed 72 hours post-infection (h.p.i.) with 4% (v/v) paraformaldehyde (PFA), washed with 100 mM Glycine (Applichem, USA), and semi-permeabilized with 0.1% Triton X-100 (Vetec Labs, BR). The infectivity was marked by indirect immunofluorescence using a sheep anti-NS5A IgG[46] as the primary antibody and anti-sheep IgG, Alexa Fluor 594 conjugated, as a secondary antibody. Cells were analyzed in a fluorescence microscope (Axio Vert.A1 - Zeiss) in green light (500-565 ηm). Relative infectivity was expressed as focus-forming units per milliliter (FFU/mL).

**2.5 Evaluation of the effective concentration of 50% of active compounds activity HCV infectivity**

The active proteins were further assessed towards their inhibition profile on HCV infectivity. Briefly, cells were infected with JFH-1 HCVcc with BthTX-I or BthTX-II in a two-fold serial dilution ranging from 0.003 to 25 µg/mL for 72 h as described [42]. The supernatant was discarded, cells were rinsed with PBS, fixed, and the relative infectivity was measured as described above. The $EC_{50}$ was calculated using a non-linear regression at GraphPad Prism 8.0 and the values of $CC_{50}$ and $EC_{50}$ were used to calculate the selectivity index (SI = $CC_{50}/EC_{50}$).

### 2.6 BthTX-I and BthTX-II effects on HCV entry steps

For all entry assays, cells were infected with 100 PFU/well of JFH-1 HCVcc, and relative infectivity was measured 72 h.p.i. to access the inhibition rates as described in [42]. The molecules BthTX-I and BthTX-II were employed at 5 µg/mL, while EGCG at 100 µM (Sigma-Aldrich, USA) [47] was used as control of entry blockage.

To determine whether the compounds could confer cell protection against viral infection, a pre-treatment assay was carried out. For this, Huh-7.5 cells were exposed to each compound for 1h at 37°C before infection. Cells were rinsed and then infected with JFH-1 HCVcc for 4 h at 37 °C. After removing the inoculum, the inoculum was removed, additional PBS washes were performed to completely remove the non-endocytosed virus and fresh medium was added.

To evaluate the effect of each protein in HCV entry stage, JFH-1 HCVcc infectious supernatant to infect Huh-7.5 cells in the presence each protein for 4 h at 37 °C. The supernatant was removed, cells were washed extensively with PBS to the complete removal of inoculum and replaced with fresh complete medium. Further, a virucidal assay was conducted to assess the ability of the proteins to act directly on the viral particles. Infectious supernatant was prior to incubating with each compound for 1 h at 37 °C, and then used to infect cells for 4 h at 37 °C. The inoculum was removed, cells were washed extensively with PBS and replaced by fresh medium.

### 2.7 BthTX-I and BthTX-II activity on HCV replication

To identify if the proteins were able to inhibit the HCV replication, cells were infected with JFH-1 HCVcc (100 PFU/well) for 4h and thoroughly washed with PBS to eliminate any non-endocytosed virus. Then, cells were treated with each protein for 72 h, and relative infectivity was measured. Cyclosporine A at 1 µM (CsA, Sigma-Aldrich) was used as a positive control [48].

### 2.8 Combination of BthTX-I or BthTX-II with sofosbuvir

The antiviral activity of the proteins BthTX-I and BthTX-II were further identified towards the capacity of being employed jointly sofosbuvir (SOF), a direct-acting antiviral[49], and result in increased inhibition. The sofosbuvir was dissolved in DMSO (Sigma–Aldrich) and stored at -80°C. Cell viability and infectivity assays were performed with the compounds and sofosbuvir separately and with the combination of each protein plus SOF at 500 ηM. Due to the cytotoxicity profile of the association of the different molecules, BthTX-I and BthTX-II were tested at 0.38 µg/mL and 0.0036 µg/mL. respectively).

### 2.9 Molecular docking

In order to identify the mode of interaction between the E1-E2 glycoprotein from HCV (PDB: 7T6X) and BthTX-I (PDB: 3HZD) or BthTX-II (PDB: 2OQD), a blind protein-protein docking analysis was performed using HDOCK server[50] combining template-based and ab initio modeling. The resulting docking complexes were visualized in 3D using Chimera X[51] and analyzed in 2D with LigPlot+[52]. Prior to the analyses, the residues in PDBs files 3HZD and 2OQD were renumbered to correct discrepancies in their original amino acids sequence numbering[33].

### 2.10 Statistical analysis

Experiments were conducted in triplicate, and all assays were repeated at least twice to ensure result reproducibility. Mean differences were analyzed using one-way ANOVA, followed by Dunnett's test to compare results with the PBS control, utilizing GraphPad Prism 8.0 (GraphPad Software). Statistical significance was set at $P < 0.01$ (denoted by asterisks). $EC_{50}$ and $CC_{50}$ values were determined by transforming the data into Log(X), where X represents the concentration, and applying a non-linear regression with a four-parameter variable slope. All analyses were performed using GraphPad Prism 9.0.

## 3. Results

### 3.1 Isolation and purification of BthTX-I and BthTX-II

The purification of BthTX-I and BthTX-II were successfully carried out by the two chromatographic steps mentioned above. After the first chromatography step on Sephacryl S200 column, the fraction SPIV (**Figure 1A**), was identified by means of SDS PAGE containing phospholipase and myotoxins (**Figure 1A, insert**). The SPIV fraction was lyophilized and subsequently submitted to the CM-Sepharose column (Fig 1B). The BthTX-II was detected in the fraction CM- III, meanwhile BthTX-I was detected in the fraction CM-V. Both peaks were found to be highly pure when analyzed by SDS-PAGE (Fig.1B insert). A subsequent round of reverse-phase chromatography verified that the highly active fraction consisted of a single compound, which eluted as a single peak (**Figure 2**).

BthTX-I was identified through N-terminal amino acid sequencing using automated Edman degradation, revealing 20 residues (SLFELGKMILQETGKNPAKS) with 100% identity to the N-terminal sequence of BthTX-I, isolated from of *B. jararacussu* venom and complete amino acid sequence reported by Cintra et al. (1993) [53]. In addition, by same methods, BthTX-II also

presented 20 amino acid residues (DLWQWGQMILKETGKLPFPY) with 100% identity to the N-terminal sequence of the BthTX – II from Pereira et al. (1998).

### 3.2 Inhibitory effect of snake venom compounds on HCV infectivity

Prior to identifying the activity of the proteins on HCV replicative cycle, their cytotoxicity was assessed by treating cells with each molecule at four different concentrations performing MTT assay. The results demonstrated that BthTX-I, BthTX-II, and CM12 did not show any cytotoxicity at the highest concentration of 25 µg/mL (cell viability ≥ 90%), while PV, PIV, Bjara H, Bjara I, Bjara H2, and CM14 the highest non-cytotoxic concentration were ≥ 5 µg/mL, therefore, these concentrations were selected for the antiviral screening **(Supplementary Material 1)**.

To assess the inhibitory effect of these compounds on HCV replicative cycle, cells were infected with JFH-1 HCVcc and simultaneously treated with compounds at the highest non-cytotoxic concentrations. Infectivity levels were analysed 72 h.p.i by focus-forming units per milliliter of supernatant (FFU/mL). The results demonstrated that 8 compounds significantly inhibited HCV infectivity ($p < 0.001$) (**Figure 3**). Particularly, BthTX-I and BthTX-II, at a non-cytotoxic concentration, were able to block up to 99% of the virus infectivity ($p < 0.001$) (**Figure 3**), demonstrating the strong effect of these compounds on HCV life cycle.

### 3.3 BthTX-I and BthTX-II inhibit HCV with high selective indexes

Concentration-response curves were performed for BthTX-I and BthTX-II in order to determine the $CC_{50}$, $EC_{50}$, and SI values. To this, a two-fold serial dilution of BthTX-I or BthTX-II ranging from 0.003 to 25 µg/mL was used to treat the cells in the presence or absence of HCV. As a result, for BthTX-I the values of $CC_{50}$, $EC_{50}$ and SI were >25 µg/mL, 0.5 µg/mL, and > 50, in this order, while for BhtTX-II were 324.4 µg/mL, 0.02 µg/mL, and 16.220, respectively (**Figure 4**).

### 3.4 BthTX-I and BthTX-II compounds blocked HCV entry

In order to evaluate the ability of the venom compounds in blocking the virus entry into Huh-7.5 cells, the virus-containing inoculum and compounds were added simultaneously to Huh-7.5 cells and incubated for 4h. Relative infectivity was assessed using a focus forming unit assay. Results showed that the proteins BthTX-I and BthTX-II at 5 µg/mL inhibited 83.4% and 97.2% of HCV entry to the host cells, respectively ($p < 0.001$) (**Figure 5a**).

To further evaluate the compounds' inhibitory effects on viral entry, a virucidal assay was carried out. With that, a significant virucidal activity was observed for both compounds (BthTX-I and BthTX-II) which blocked 85% and 96% of virus entry, respectively ($p < 0.001$) (**Figure 5b**).

The protective effect of these compounds on host cells was also evaluated. For this, Huh-7.5 cells were pre-treated with the compounds for 1 hour at 37°C. After 4h of JFH-1 HCVcc infection, the supernatant was replaced by fresh medium and relative infectivity measured 72 h.p.i. The results showed that only BthTX-II reduced infectivity when cells were pre-treated with this compound, blocking 87% of virus entry (**Figure 3c**). In addition to the above data, these results suggest that BthTX-II acts on both the virus particle and the host cells (**Figure 5c**).

### 3.5 BthTX-I and BthTX-II do not affect post entry steps of HCV replicative cycle

These compounds were also evaluated for their ability to inhibit HCV replication, after the entry of viruses into the cells. Huh-7.5 cells were infected with JFH-1 HCVcc viral particles for 4h, the inoculum was then removed and washed extensively to completely remove the virus. Drug-containing medium was added and HCV infectivity was measured 72 h.p.i.. The results showed that non-cytotoxic concentrations of these compounds did not interfere with HCV replication (**Figure 6**).

### 3.6 The treatment with compounds BthTX-I and BthTX-II plus sofosbuvir did not significantly increase the antiviral activity

To verify if the antiviral activity of BthTX-I and BthTX-II is significantly increased with the addition of the sofosbuvir, the cell viability and infectivity assays were carried out with BthTX-I at 0.38 µg/mL, BthTX-II at 0.0036 µg/mL, SOF at 500 ηM and with the combination of BthTX-I plus SOF and BthTX-II plus SOF at the same concentrations above.

The results showed that the combined treatment did not decrease the cell viability in comparison with the monotherapies (**Figure 7a**), making it possible to compare the inhibition rates. Although the treatment with SOF and the combined treatments to be statistically different from non-treated control, there is no significant difference between the inhibition of SOF (61%) and SOF + BthTX-I (69%), neither SOF nor SOF + BthTX-II (52%) (**Figure 7b**).

### 3.7 In silico analyses suggest interactions between BthTX-I or BthTX-II and HCV E1E2 heterodimer

The protein-protein docking conducted between BthTX-I and II with the envelope glycoproteins E1 and E2 of HCV resulted in the interaction of phospholipases with both E proteins, albeit in slightly distinct positions (**Figures 8a and 8b**). BthTX-I exhibited fewer hydrogen bonds and hydrophobic interactions with glycoproteins E1 and E2 compared to BthTX-II (**Figures 8c and 8c and Table 1**). Furthermore, the interaction between BthTX-II appears to be stronger, as indicated by the greater number of interactions, with glycoprotein E2 rather than protein E1.

## 4. Discussion

HCV infection remains a significant global health concern and there is no vaccine to prevent this disease[54,55]. The infected patients are treated with direct-acting antivirals (DAAs) which are the currently available treatment[56], however, are expensive, can cause several side effects, and induce resistance variants[57,58]. Therefore, the development of new therapies is needed[59,60].

Snake venoms are a complex blend of compounds with diverse effects on both prey and human victims[61]. Many of these compounds are biologically active against parasites[62], bacteria[63], and viruses[30]. With this, herein, the antiviral activity of proteins isolated from snake venoms of the *Bothrops* genus species was investigate against HCV. The most effective proteins were the phospholipases $A_2$ BthTX-I and BthTX-II, with both impairing up to 99% of HCV infectivity, with a SI of > 50 and 16.220, respectively. Snake venom phospholipases (PLAs) catalyze the hydrolysis of glycerophospholipids at the sn-2 position, releasing free fatty acids and lysophospholipids. They play a crucial role in various biological processes, including lipid digestion, host defense, and the maintenance of cellular membrane homeostasis[64,65], but also with pathogen-inhibiting activity (bactericidal and antiviral activity)[66,67]. Interestingly, in accordance with our data, a similar PLA, the $PLA2_{CB}$ isolated from *Crotalus durissus terrificus* was found to be highly active in low $EC_{50}$ concentrations against the *Chikungunya virus* (CHIKV), an enveloped virus by disrupting viral membrane in CHIKV virions[68].

Further, Muller and coworkers evaluated the effect of PLAs, being of them the BthTX-I from *B. jararacussu*, against DENV and YFV. As a result, the BthTX-I, a $PLA_2$ without catalytic activity, showed virucidal activity with an SI of 15.4 for YFV and 22.6 for DENV-2, but when compared to $PLA2_{CB}$ and $PLA_2$-IC the obtained SI values were >135,000 and >35,000, in this order, for both viruses[31]. Their mechanism of action was described as disrupting viral membrane in virions, indicating that the phospholipases' enzymatic activity is crucial for their antiviral effect [30,31]. In this sense, these findings corroborate with our results since the

inhibition observed in the virucidal assay was 11% higher for BthTX-II (96% of inhibition), a phospholipase with low catalytic activity [69,70], in comparison with the BthTX-I (85%), without any catalytic activity; furthermore, the SI was also higher to BthTX-II (16.220) than to BthTX-I (>50). With these results, we can infer that catalytic activity plays a key role for the antiviral effect of phospholipases, being that further confirmed by Cassani and collaborators, which evaluated the same BthTX-I and BthTX-II against the ZIKV, another *Orthoflavivirus.* Their data revealed that the BthTX-II had an SI of $1.44 \times 10^5$ while BthTX-I had one of 149.4, being the increase on antiviral activity also related to the catalytic activity of this protein[33].

X-ray crystallography and spectroscopic analyses of BthTX-I have revealed that the monomer interface functions as a molecular hinge, leading to "open" and "closed" dimeric forms. It has been suggested that changes in the quaternary conformation of the membrane-bound protein may facilitate the insertion of the C-terminal loop region into the lipid bilayer, penetrating up to 13 Å. This insertion compromises membrane integrity and enhances permeability [71,72]. BthTX-II in turn, is an Asp49 $PLA_2$, that can act directly on membrane phospholipids, via glycerophospholipid cleavage and consequently membrane disrupting[73]. Therewith, we can suggest that the virucidal activity of PLA2s is due to their insertion into the viral envelope, with BthTX-I destabilizing the lipid bilayer and BthTX-II disintegrating such structure and, with that, both preventing the virus from entering the cell and continuing its cycle. In this sense, *in silico* analysis can propose the binding interactions of these toxins with the HCV envelope glycoprotein E1E2 complex, essential for HCV entry into the host cells. The viral surface glycoproteins regulate the entry process by undergoing conformational changes, transitioning from a less stable state to a more stable one[74]. In addition, these glycoproteins are synthesized as a complex with a second membrane glycoprotein (prM). A disintegration in this structure can cause conformational changes necessary for entry, effectively inhibiting the envelope from changing its conformation and consequently blocking the virus from infecting the cell.

Nowadays, the use of combined therapies with different antivirals has been used for treatment of diverse diseases, including against hepatitis C [75–77]. Here, we evaluated the effect of the combination treatment of BthTX-I or BthTX-II with sofosbuvir, a DAA currently used against HCV infections. The results showed that no synergistic effect occurs when comparing individual treatments with the combined ones, besides that, more studies can be carried out in order to discover any drug that could increase the antiviral effect of the compounds.

## 5. Conclusion

With this work, it was demonstrated that the proteins isolated from *Bothrops* snake venoms strongly inhibited the HCV infection *in vitro*, being the proteins BthTX-II and BthTX-I from *B. jararacussu* the ones with higher inhibition rates. Both proteins were able to block the viral infection in 96% and 84.7%, respectively, through virucidal mechanism of action due to their interaction with E1E2 complex. Furthermore, BthTX-II also showed protective effect on Huh-7.5 cells against HCV infection, inhibiting 86.7%. Thus, these proteins may contribute to the development of future Hepatitis C virus therapies functioning as a scaffold source of new antiviral molecules.

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**Captions to figures**

**Figure 1. Isolation of BthTX-I and BthTX-II from Bothrops jararacussu venom. (A)** Chromatographic profile of *B. jararacussu* crude venon (300 mg) on Sephacryl S 200 column under elution with 50mM ammonium bicarbonate, pH 8.0. Fraction of 3 mL were collected at a flow rate of 22.8mL/h, at room temperature. Inserted: 12% SDS- PAGE of SPIV fraction under reducing conditions (1) *B.jararacussu* venom (2) SPI, (3) SPII, (4)SPIII, (5)SPIV. Purification of BthTX– I and BthTX-II. **(B)** Chromatography of 20 mg of SPIV fraction on CM-Sepharose previously equilibrated with 50mM ammonium bicarbonate, pH 8.0, and then eluted on a concentration gradient of up to 500mM of the same buffer. Fraction of 4 mL were collected at a flow rate of 1,5mL/min, at room temperature. Inserted: 12% SDS- PAGE (1) CM I, (2) CIII (BthTX-II), (3) CM V (BthTX-I) fraction under reducing conditions (4); Molecular mass standards.

**Figure 2. HPLC reverse phase chromatography (column C18) analysis. (A)** BthTX-I (1 mg), was solubilized in 500 µL of 0.1% trifluoracetic acid (v/v) (TFA) and analyzed in a HPLC system equipped with a reverse phase C-18 column (2.0 x 250 mm) (Shimadzu). The column was equilibrated with the running solvents (solvent A – 0.1% TFA; solvent B – 70% acetonitrile) and eluted as follows: 10 min to 0% of B, concentration gradient from 0-100% of solvent B for 70 min, rising to 100% of B in 10 min, and maintaining for 10 min under a flow rate of 1 mL/min, totaling 90 min of running time at room temperature (25 ºC). The eluates were monitored at 280 nm by a detector coupled to the system. **(B)** BthTX-II (1 mg) was solubilized in 500 µL of 0.1% TFA (v/v) and analyzed by HPLC system using a reverse phase column to determine its purity, under the same conditions described above.

**Figure 3. Anti-HCV activity of snake venom compounds.** Huh-7.5 cells were treated with compounds for 72h and MTT assay was performed to determine cell viability (grey bars). To analyse the HCV infectivity under treatment of compounds, cells were infected with JFH-1 HCVcc and simultaneously treated with each compound at the highest non-cytotoxic concentration. Virus was titrated 72 h.p.i. by focus-forming units per milliliter of supernatant (FFU/mL) (black bars). PBS was used as untreated control and EGCG (100 µM) was used as control of inhibition of HCV infection. Mean values of two independent experiments, each conducted in triplicate, are presented along with standard deviation are shown. A p value of $< 0.05$ was considered statistically compared to the untreated control. (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

**Figure 4. Dose-response assay of BthTX-I and BthTX-II in HCV replication.** Huh-7.5 cells

were treated with BthTX-I and BthTX-II in a two-fold serial dilution ranging from 0.003 µg/mL to 25 µg/mL in the presence of absence of 100 pfu/well of JFH-1 HCVcc. After 72 h.p.i., MTT assay was performed to evaluate cell viability, and supernant was titered.

**Figure 5. Effect of the snake venom compounds on HCV entry.** (a) For entry assay, Huh-7.5 cells were infected with JFH-1 HCVcc and compounds BthTX-II (25 µg/mL) and BthTX-I (5 µg/mL) were immediately added. After 4h, the supernatant was replaced by fresh medium after repeated washes with PBS to remove the inoculum. (b) For virucidal assay, infectious supernatants were prior incubated with each compound for 1h at 37°C and then used to infect naive Huh-7.5 cells. The mixture was incubated with cells for 4h at 37°C. The inoculum was removed, cells were washed with PBS and replaced by fresh medium. (c) For pre-treatment assay, Huh-7.5 cells were treated with each compound for 1 hour at 37°C before infection. Following incubation, cells were rinsed with PBS and then infected with JFH-1 HCVcc for 4h. Infectious supernatant was removed, additional washes were performed, and fresh medium was added. PBS was used as untreated control and EGCG at 100 µM as control of inhibition for both assays. Virus was titrated 72 h.p.i. by focus-forming unit. Mean values of two independent experiments, each conducted in triplicate, are presented along with standard deviation are shown. $P < 0.05$ was considered statistically different from PBS. (***) $P < 0.001$.

**Figure 6. Snake venom compounds activity on HCV replication.** Huh-7.5 cells were infected with JFH-1 HCVcc for 4h, extensive washing was performed on cells to remove unbound virus and treatement with compounds BthTX-II at 25 µg/mL and BthTX-I at 5 µg/mL was conducted. PBS was used as untreated control and CsA at 1 µM as control of viral replication inhibition. Virus was titrated by focus-forming unit 72 h.p.i.. Mean values of two independent experiments, each conducted in triplicate, along with standard deviation are shown. $P < 0.001$ was considered statistically different from PBS. (***) $P < 0.001$.

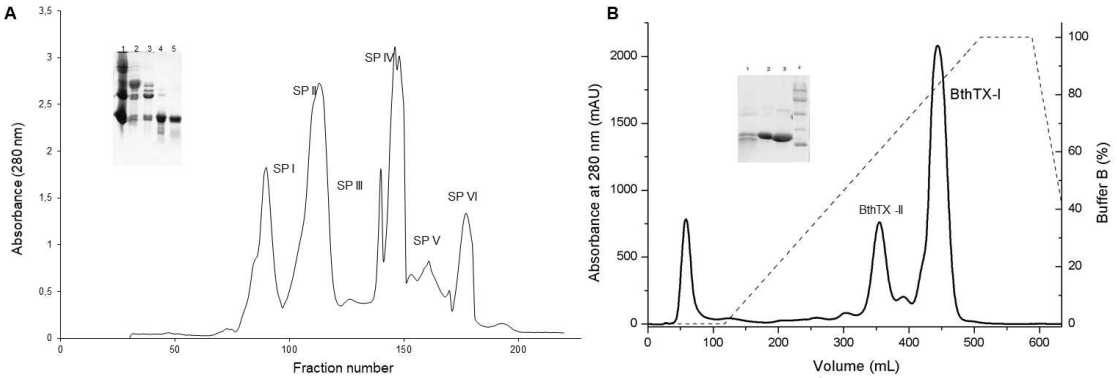
**Figure 7. Tridimensional structure of the complex between phospholipases BthTX-I (A) and II (B) with HCV glycoproteins E1 and E2 obtained by protein-protein docking.** E1E2 glycoprotein from HCV (PDB: 7T6X) and BthTX-I (PDB: 3HZD) or BthTX-II (PDB: 2OQD) a protein-protein blind docking analysis was performed using HDOCK server based on a hybrid algorithm of template and ab initio modeling. 3D images of the complexes were generated using Chimera X.

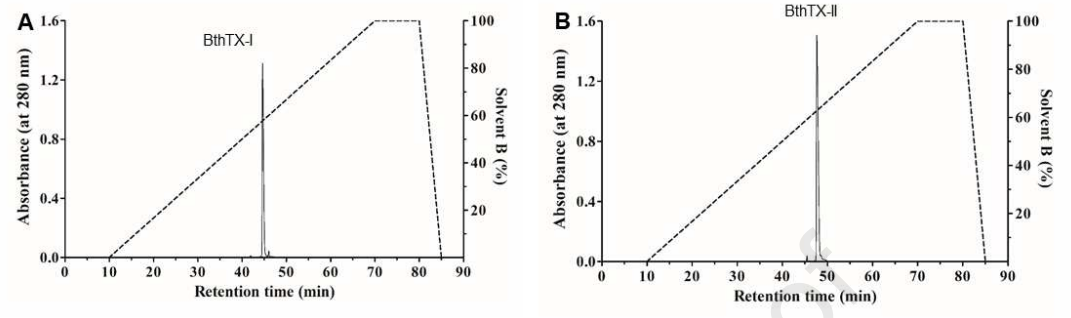
**Figure 8. 3D and 2D interaction diagram of the complex between phospholipases BthTX-I and II with HCV glycoproteins E1 and E2 obtained by protein-protein docking. (A and B)** A protein-protein blind docking analysis was performed using HDOCK server between the

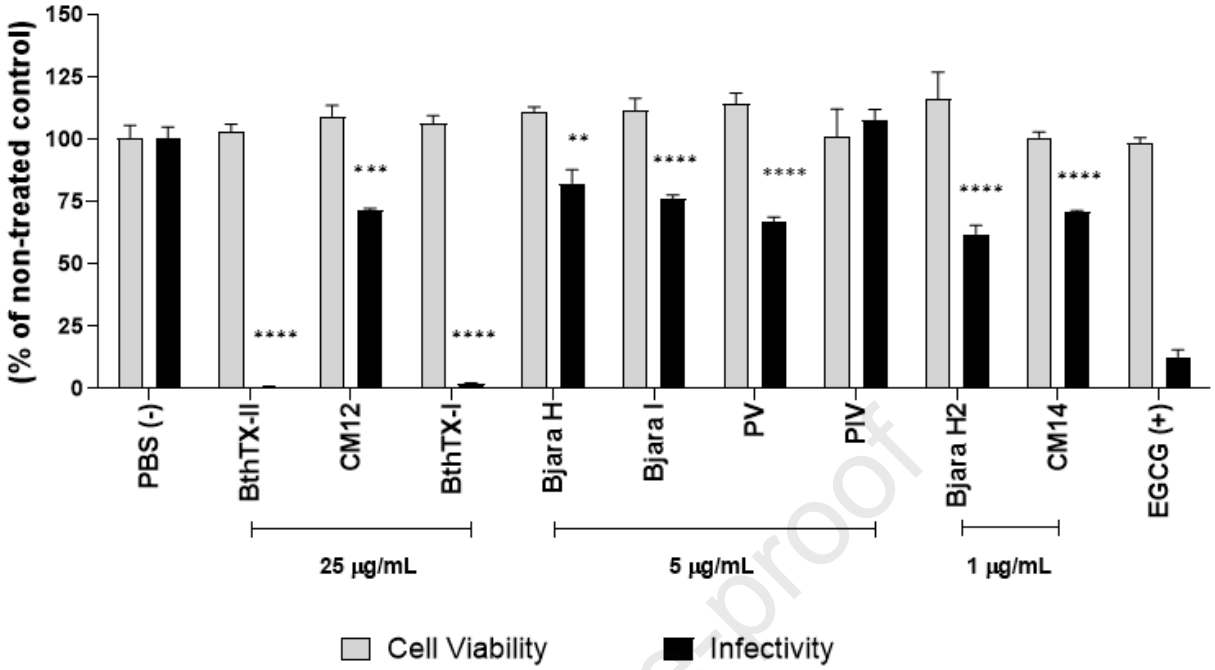
E1E2 glycoprotein from HCV (PDB: 7T6X) and BthTX-I (PDB: 3HZD) or BthTX-II (PDB: 2OQD). **(C and D)** 2D analysis of the interactions were generated using LigPlot+. Red arcs represent hydrophobic interactions from residues of E1 and E2 glycoproteins. Magenta arcs represent hydrophobic interactions from residues of BthTX $PLA_2$s. Green dashed lines represent hydrogen bonds.

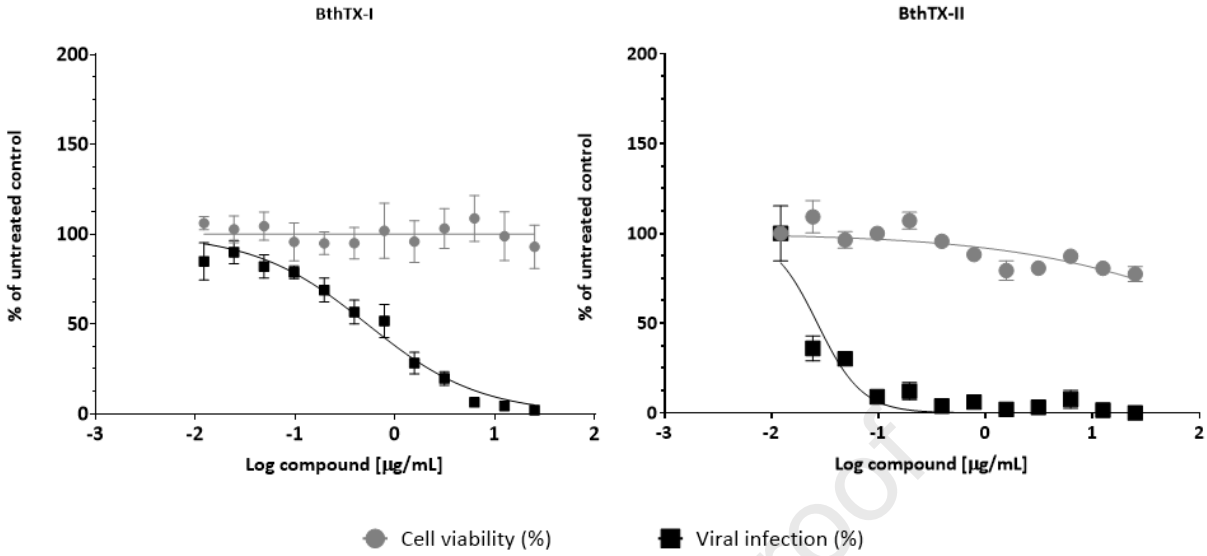
**Table 1. Number of residues with hydrogen bond and hydrophobic interactions observed for each toxin with HCV E1E2 glycoprotein.**

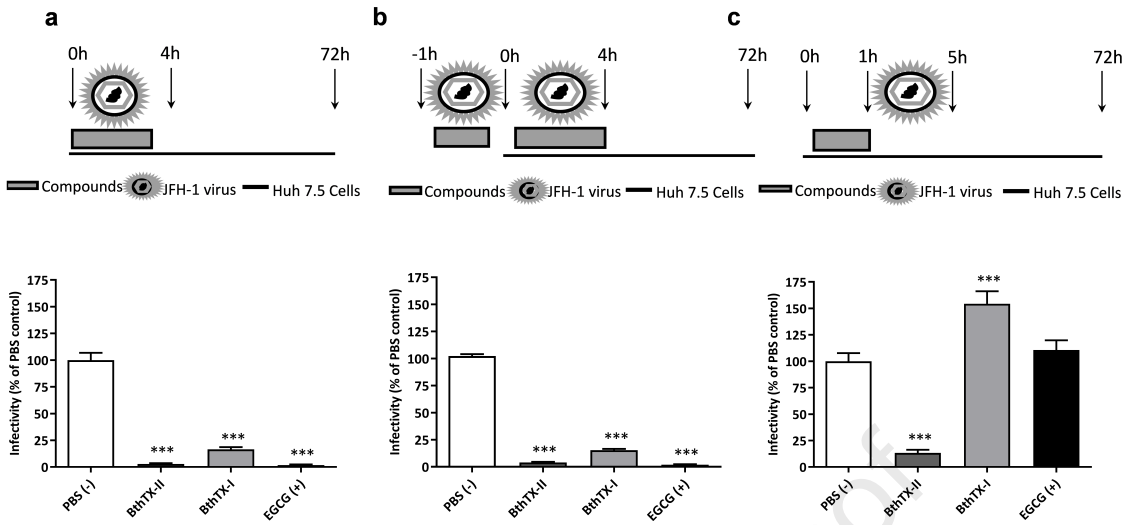
| | Hydrogen Bond | Hydrophobic Interaction |
|---|---|---|
| **BthTX-I** | 1 | 19 |
| **E1** | 1 | 9 |
| **E2** | 0 | 9 |
| **BthTX-II** | 3 | 22 |
| **E1** | 0 | 7 |
| **E2** | 3 | 11 |

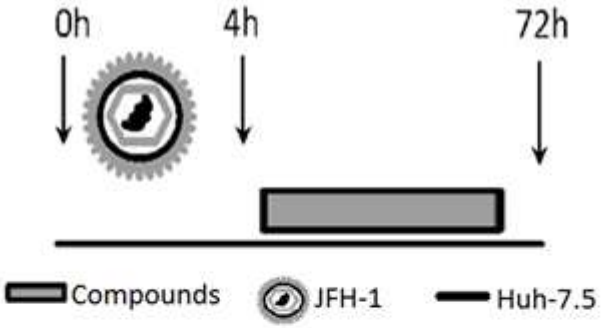


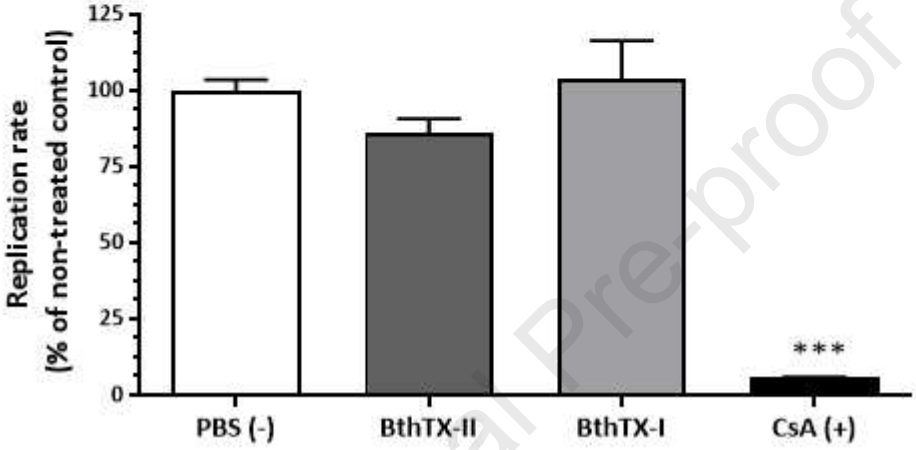


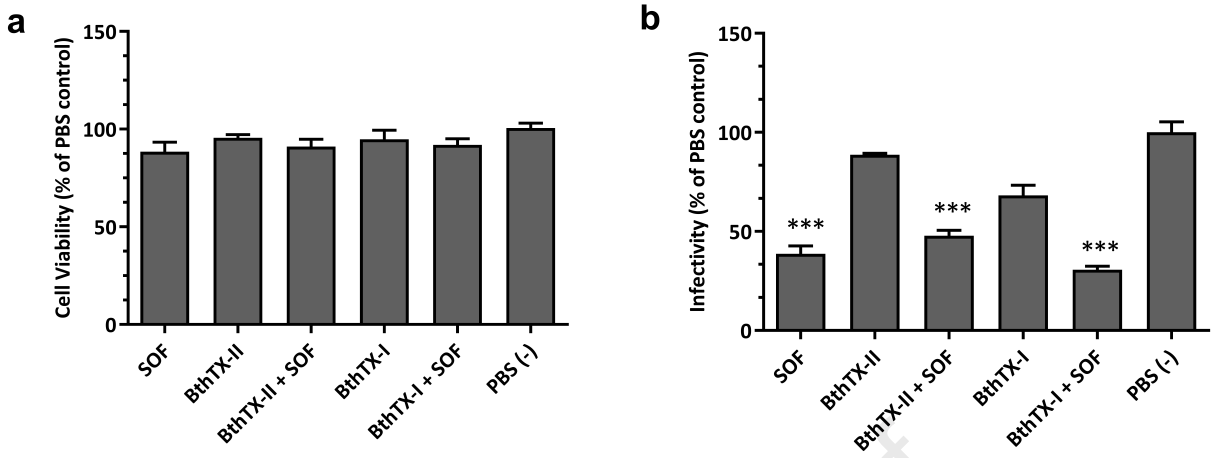


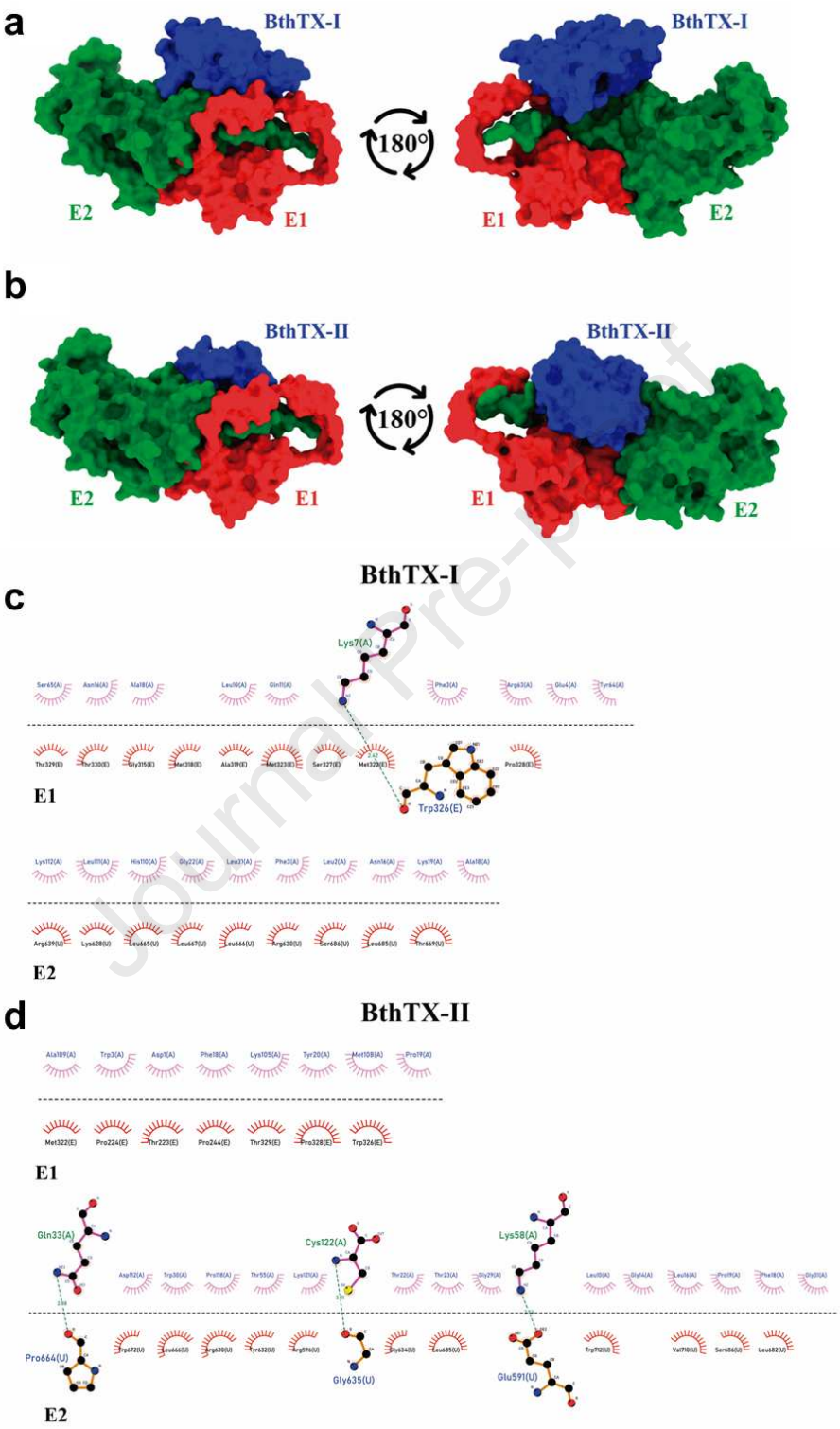










**HIGHLIGHTS**

- 8 toxins isolated from snakes venom of *Bothrops* genus inhibited up to 99% of HCV infection;
- Bothropstoxins I and II (BthTX-I/II) strongly inhibits HCV *in vitro*;
- BthTX-I/II impair the early stages of HCV infection;
- BthTX-I/II can protect cells from HCV infection;
- In silico binding interactions were observed between BthTX-I/II and HCV E1-E2 glycoprotein.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: