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Campus de São José do Rio Preto

Carina Machado Pereira

Avaliação da atividade antiviral de compostos naturais e sintéticos no ciclo replicativo do HCV *in vitro*

São José do Rio Preto
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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Microbiologia, junto ao programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Financiadora: CAPES

Orientadora: Profª Drª Ana Carolina Gomes Jardim
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"Nas grandes batalhas da vida, o primeiro passo para a vitória é o desejo de vencer."

Mahatma Gandhi

RESUMO

A hepatite C é uma doença causada pelo vírus da Hepatite C (HCV) que acomete aproximadamente 115 milhões de pessoas. O tratamento atual para os pacientes infectados consiste na combinação de antivirais de ação direta (DAAs), que apesar de apresentar resposta virológica sustentada satisfatória já apresentam variantes resistentes descritas. Estes medicamentos também representam significativos gastos para o sistema público de saúde, além de causarem efeitos colaterais. Desta forma, o desenvolvimento de novos agentes terapêuticos se faz necessário. Compostos naturais e seus derivados sintéticos têm sido descritos pela atividade antiviral contra diversos vírus, como os vírus da Dengue (DENV), Febre amarela (YFV), Herpes (HSV), Imunodeficiência Humana (HIV) e HCV. Nesse estudo, a atividade antiviral de 10 compostos isolados de venenos de serpentes do gênero *Bothrops* (TOXs) e de 16 acridonas sintéticas (Facs) foi avaliada, utilizando linhagem celular Huh-7.5 e partículas virais JFH-1 HCVcc genótipo 2a. Oito TOXs e dois Facs inibiram significativamente a infecção pelo HCV. Os compostos com maior inibição, TOX1 (SI = 1736), TOX3 (SI = 13,5), Fac15 (SI = 1,72) e Fac17 (SI = 1,65), demonstraram reduzir a infecção em 96%, 84,7%, 58% e 48%, respectivamente, agindo diretamente nas partículas virais. TOX1 e Fac15 também reduziram a infectividade viral em 87% e 39% nas células hospedeiras por ação protetora, após o pré-tratamento das células. Com este trabalho, concluímos que tanto os compostos isolados de venenos de serpentes do gênero *Bothrops* quanto as acridonas sintéticas podem ser eficientes na inibição do HCV.

Palavras-chave: Hepatite C. HCV. Antiviral. Veneno de serpente. Acridonas sintéticas.

ABSTRACT

Hepatitis C is a disease caused by Hepatitis C virus (HCV) that affects approximately 115 million people. The current treatment for infected patients consists of the combination of direct acting antivirals (DAAs), which despite presenting satisfactory sustained virological response already presents resistant variants described. These drugs also represent significant costs to the public health system and cause side effects. Therefore, the development of new therapeutic agents is needed. Natural compounds and their synthetic derivatives have been described as antivirals against several viruses as Dengue (DENV), Yellow fever (YFV), Herpes (HSV), Human immunodeficiency virus (HIV) and HCV. In this study, the antiviral activity of 10 compounds isolated from Bothrops *snake venoms (TOXs) and 16 synthetic acridones (Facs) was evaluated, using Huh-7.5 cell line and JFH-1 HCVcc genotype 2a virus particles. Eight TOXs and two Facs significantly inhibited the HCV infection. The compounds with the highest inhibition, TOX1 (SI = 1736), TOX3 (SI = 13.5), Fac15 (SI = 1.72) and Fac17 (SI = 1.65), demonstrated to reduce the infection in 96%, 84.7%, 58% and 48%, respectively, by acting directly on the viral particles. TOX1 and Fac15 also reduced 87% and 39% of viral infectivity in host cells by protective action after pre-treatment of cells. With this work, we conclude that either compounds isolated from* Bothrops *snake venoms and synthetic acridones may be efficient in inhibiting HCV.*

Keywords: Hepatitis C. HCV. Antiviral. Snake venom. Synthetic acridones.

Sumário

CAPÍTULO I

Considerações gerais

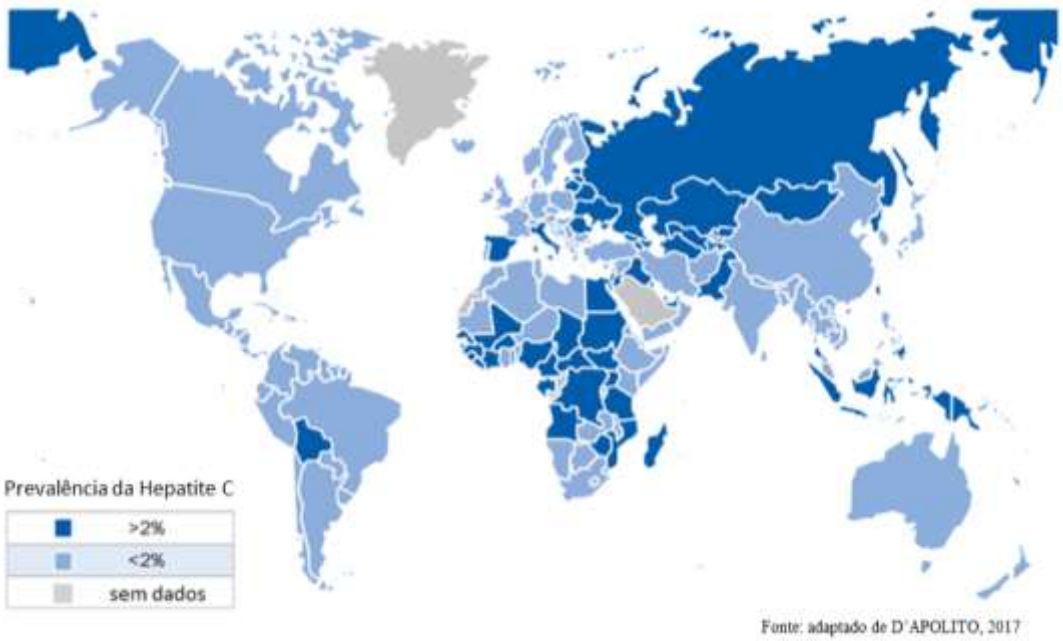
1. Introdução

1.1. A Hepatite C

Com o desenvolvimento de diagnósticos específicos para os vírus da hepatite A (VHA) e da hepatite B (VHB) na década de 1970, ficou claro que a maioria dos casos de hepatite decorrentes de transfusão de sangue não era causada por estes ou outros agentes virais conhecidos (CHOO et al., 1989). Passaram então a denominar esse tipo de doença como hepatite não-A e não-B, que ainda na década de 1970 recebeu a denominação de hepatite C, quando Choo e colaboradores identificaram o vírus da hepatite C (HCV) como causador da doença (CHOO et al., 1989). A prevalência global da infecção pelo HCV tem sido estimada em aproximadamente 115 milhões de pessoas em todo o planeta (LANINI et al., 2016) (Figura 1). Entre 71 e 79 milhões desses pacientes apresentam infecção crônica, sendo maior a prevalência no Mediterrâneo Oriental (2,3%), seguido pela Região Europeia (1,5%) (GOWER et al., 2014; LANINI et al., 2016; WHO, 2017).

De 1999 a 2016, mais de 182.389 pacientes confirmados com HCV foram identificados pelo sistema de vigilância brasileiro (BRASIL, 2017). Além disso, estudos demonstraram uma prevalência do vírus de 0,62% na região Norte, 0,55% no Nordeste, 0,28% no Centro-Oeste, 0,43% no Sudeste e 0,46% no Sul (PALTANIN; REICHE, 2002; BRASIL, 2017).

Figura 1. Prevalência global da hepatite C.



Fonte: adaptado de D'APOLITO, 2017

A Hepatite C se caracteriza em duas fases, aguda e crônica (MAASOUMY; WEDEMEYER, 2012). A fase aguda sintomática ocorre em cerca de 15% dos pacientes que estão infectados com o HCV, apresentando sintomas como febre, falta de apetite, vômito, dor abdominal e icterícia, sendo por esta razão muitas vezes não diagnosticada corretamente (MAHESHWARI et al., 2008; WHO, 2016). A taxa de diagnóstico estimado na Inglaterra, por exemplo, é de 35%, o que sugere que 65% da população total HCV-positiva permanece sem diagnóstico (MILLER; DILLON, 2015). Cerca de 50-80% dos pacientes com hepatite C aguda progridem para hepatite C crônica e de 20 a 50% dos pacientes apresentam cura espontânea sem tratamento (CHO et al., 2014).

A hepatite C crônica é uma das principais causas de doença hepática e transplante de fígado em todo o mundo, pois pode causar danos contínuos nesse órgão e resultar em cirrose e carcinoma hepatocelular (CASTRO et al., 2015; WANDELER et al., 2015). Pacientes com esse tipo de hepatite podem reportar sintomas como desconforto abdominal direito, náusea, fadiga, dor muscular, dor articular e perda de peso, no entanto, esses sinais clínicos não são comuns e na maioria dos casos são restritos a cirrose hepática avançada (MAASOUMY; WEDEMEYER, 2012). Tratamentos antivirais reduzem a progressão da fibrose hepática e podem reverter a cirrose, porém, mesmo em países desenvolvidos, a mortalidade por hepatite C está aumentando devido ao diagnóstico incorreto e ao tratamento inadequado (PRECIADO et al., 2014).

A transmissão do HCV ocorre principalmente pelo contato com sangue contaminado, pela transfusão de sangue e compartilhamento de agulhas e seringas não esterilizadas (LAI et al., 2003; SHEN et al., 2014). O uso de drogas injetáveis e injeções não seguras resultou em um grande número de infecções por HCV durante o século 20 (ARMSTRONG, 2003).

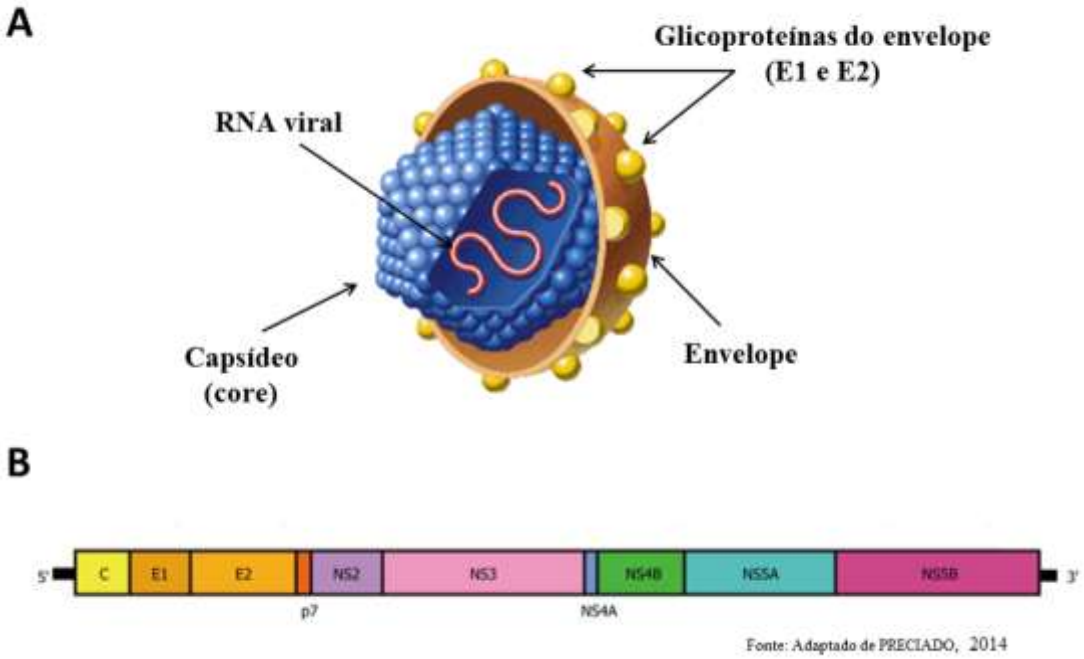
O HCV também pode ser transmitido verticalmente em aproximadamente 5% dos casos e sexualmente em 0,07% ao ano entre casais heterossexuais monogâmicos, porém esses modos de transmissão são muito menos comuns (TERRAULT et al., 2013; WHO, 2016; EINBERG et al., 2017).

1.2. O vírus da hepatite C

O HCV é um vírus envelopado pertencente à família *Flaviviridae* e ao gênero *Hepacivirus*, que possui molécula de RNA fita simples de polaridade positiva com cerca de 10.000 nucleotídeos (CHOO et al., 1991; HOUGHTON, 2002). O envelope, constituído de bicamada lipídica com as glicoproteínas E1 e E2 ancoradas, envolve o nucleocapsídeo,

composto por múltiplas cópias da proteína core (capsídeo) e pelo genoma de RNA (DRUMMER et al., 2003; BARTENSCHLAGER et al., 2013) (Figura 2).

Figura 2. Esquema tridimensional (A) e genoma (B) do HCV.



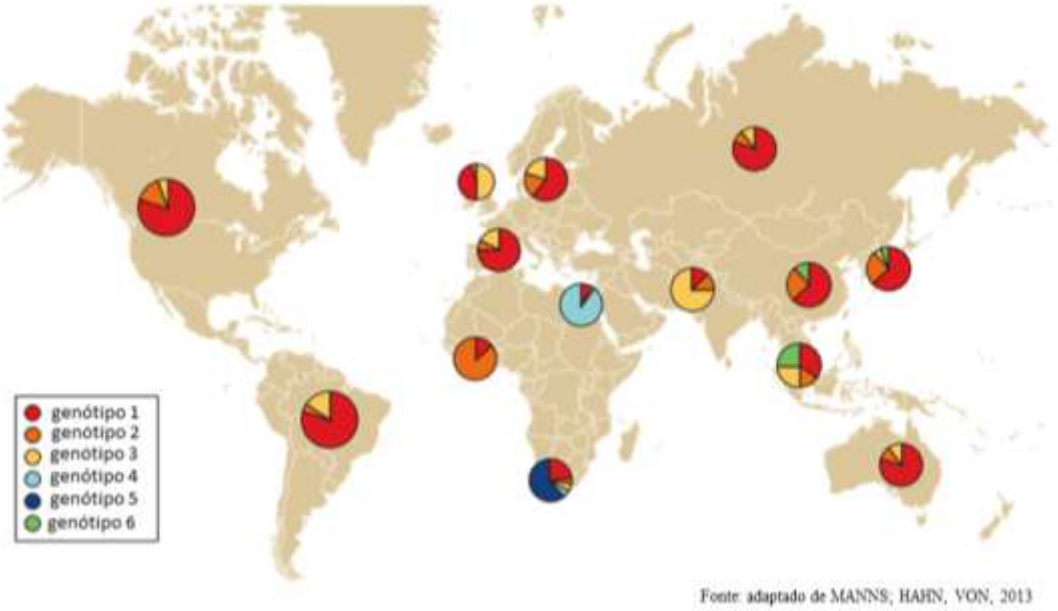
O genoma viral é traduzido em uma poliproteína precursora, que processada resulta nas proteínas estruturais core, E1 e E2, na pequena proteína de canal iônico p7 e nas seis proteínas não estruturais NS2, NS3, NS4A, NS4B, NS5A e NS5B (DUGUM; O'SHEA, 2014). Várias das proteínas não estruturais são enzimas e são, portanto, um atraente alvo de drogas antivirais. Estas incluem as duas proteases virais NS2/3 e NS3/4A, a RNA helicase NS3/NTPase, a RNA polimerase dependente de RNA NS5B, e a proteína multifuncional NS5A, envolvida em diferentes estágios do ciclo de vida do HCV, incluindo a replicação do RNA e montagem e liberação do vírion (MANNS; HAHN, VON, 2013; GAETANO, 2014; SUN et al., 2015).

Até o momento, foram identificados sete genótipos do HCV (MURPHY et al., 2014). Os genótipos 1, 2 e 3 tem distribuição geográfica ampla, enquanto os genótipos 4, 5, 6 e 7 são geralmente limitados a regiões geográficas específicas (HAJARIZADEH et al., 2013; MURPHY et al., 2014) (Figura 3).

O genótipo 1 do HCV é o genótipo mais prevalente no mundo, com 83,4 milhões de casos (46,2% de todos os casos de HCV), seguido pelo genótipo 3, com 54,3 milhões de

casos (30,1%). Os genótipos 2, 4 e 6 representam 22,8% dos casos de HCV e o genótipo 5 contribui com menos de 1%. Para o genótipo 7, identificado pela primeira vez em 2014 na África Central, ainda não existem dados consistentes sobre a sua prevalência e distribuição global (MURPHY et al., 2014). A frequência do genótipo 4 é mais alta da África Central ao Oriente Médio, o genótipo 5 prevalece apenas no sul da África e o genótipo 6 ocorre com maior frequência no Leste e Sudeste Asiático (MESSINA et al., 2015). Os últimos dados do Brasil revelam que o genótipo 1 é o mais prevalente, com 78,4% da população infectada, seguido pelo genótipo 3 (17,9%) (PERONE et al., 2008).

Figura 3. Distribuição genotípica global do HCV.



Fonte: adaptado de MANNS, HAHN, VON, 2013

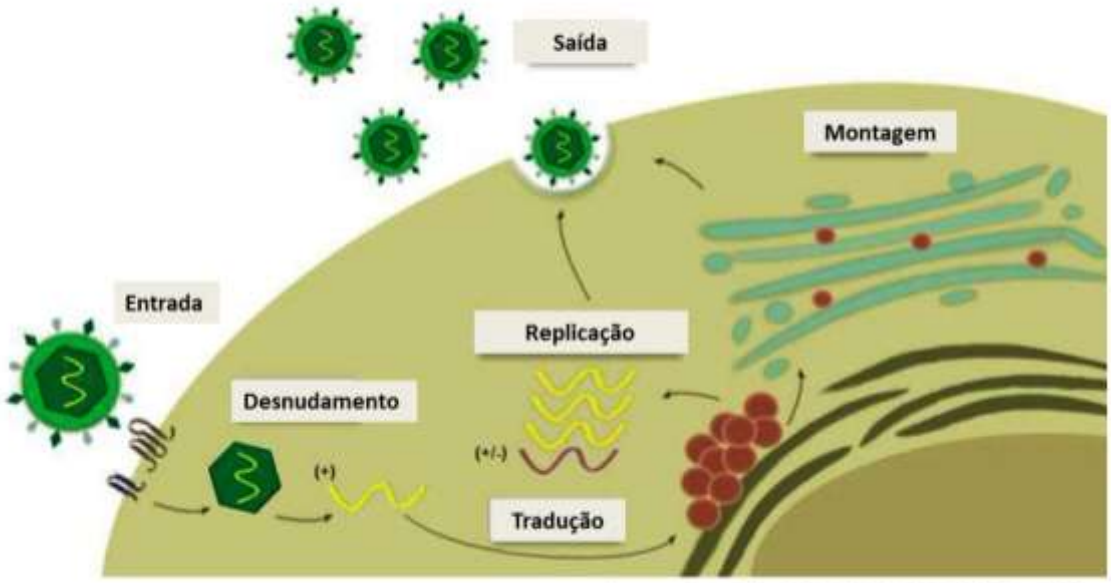
Os genomas completos dos diferentes genótipos diferem em sítios nucleotídicos em pelo menos 30%. Os genótipos 1, 2, 3, 4 e 6 são ainda subdivididos em subtipos, que diferem de 20 a 25% dentro de cada genótipo (SIMMONDS et al., 2005). Existem também variantes genéticas do vírus denominadas quasispécies, que estão altamente relacionadas entre si, diferindo em menos de 10% do genoma viral (ZHOU et al., 2007).

1.3. Ciclo replicativo do HCV

O HCV entra na célula através de endocitose mediada por receptor, que ocorre por uma série de interações entre as glicoproteínas do envelope viral (E1 e E2) com moléculas de superfície celular como glicosaminoglicanos (GAG), grupo de diferenciação 81 (CD81), ocludina (OCLN), claudina-1 (CLDN1), receptores de lipoproteínas de baixa densidade

(LDLR) e receptor de eliminação de classe B tipo I (SR-BI) (BARTENSCHLAGER et al., 2011; BHLER; BARTENSCHLAGER, 2012). Após a entrada ocorre o desnudamento, quando o genoma de RNA de sentido positivo é liberado no citoplasma; neste local ocorre a tradução do RNA do HCV, produzindo uma poliproteína grande (CHOI J., CORDER N. L B., 2015). A poliproteína é então cortada pela protease sinal do hospedeiro e as proteases virais NS2 e NS3/4A geram produtos proteicos individuais, incluindo NS5B, a replicase viral (CHOI J., CORDER N. L B., 2015). Juntos, NS3/4A, NS4B, NS5A e NS5B constituem as proteínas virais da máquina de replicação, que replica o genoma de RNA de sentido positivo através de um intermediário de cadeia negativa, complementar ao genoma (LOHMANN, 2013). Os RNAs de polaridade positiva produzidos podem ser usados para tradução, replicação, ou então ser empacotados para constituir novos vírus (BARTENSCHLAGER et al., 2013; DUBUISSON; COSSET, 2014). As proteínas estruturais se associam (core) ou se integram (E1, E2 e p7) com a membrana do retículo endoplasmático (RE) e formam oligômeros funcionais que promovem a montagem da nova partícula viral. O envelope viral é adquirido por brotamento na membrana do RE e as novas partículas virais são liberadas por meio de via secretora (COUNIHAN et al., 2011; COLLER et al., 2012). A figura 4 representa o ciclo replicativo do HCV.

Figura 4. Ciclo replicativo do HCV.



Fonte: adaptado de CHOI J., CORDER N. L. B., 2015

1.4. Tratamento

O interferon-α foi aprovado pela *Food and Drug Administration* (FDA) dos Estados Unidos para o tratamento de infecções com HCV em 1991, a partir de então, todas as terapias licenciadas no período passaram a incluir alguma forma de interferon-α. As respostas melhoraram em 1998 com a combinação do interferon-α com ribavirina oral, e em 2001 pela associação da molécula de interferon-α com polietileno glicol, produzindo o peginterferon-α (THOMAS, 2013). Para a infecção com o HCV genótipo 1, de maior incidência, um avanço adicional veio em 2011, com a aprovação dos antivirais de ação direta (DAAs) que atuam como inibidores de protease viral, como o telaprevir e o boceprevir, que impedem a protease viral NS3 de clivar as enzimas responsáveis pela replicação viral (KISER et al., 2013; SERRANTI et al., 2014).

A eficiência do tratamento contra o HCV é medido principalmente pela taxa de Resposta Virológica Sustentada (*Sustained Virological Response*, SVR), definida como ausência de RNA do vírus no sangue dos pacientes ao final do tratamento, e seis meses após o seu término (THOMAS, 2013). No tratamento com peginterferon-α em combinação com ribavirina cerca de 50% dos pacientes com o genótipo 1 do vírus desenvolveram SVR, e com a adição de telaprevir/boceprevir na terapia convencional pegIFN/ribavirina houve um aumento de 20 - 30% (PAN et al., 2012). Apesar de ser clara a eficácia da terapia tripla com telaprevir ou boceprevir, a baixa barreira à resistência continuava a ser uma limitação importante, além dos efeitos colaterais (SERRANTI at al., 2014).

Nos tratamentos baseados em interferon-α e ribavirina são observados efeitos colaterais semelhantes aos sintomas de gripe, anemia e redução do número de plaquetas e leucócitos. Além disso, 19% dos pacientes apresentam efeitos adversos graves como impotência, distúrbios da tireoide, sangramento intestinal, hemólise e anemia. (WOHLFARTH; EFFERTH, 2009). Os efeitos mais graves do telaprevir são erupção cutânea e anemia, e do boceprevir são anemia, perda de paladar, diminuição do número de neutrófilos e desconforto anal (PAN et al., 2012).

Em 2013 foram aprovados dois novos DAAs, o sofosbuvir, um inibidor da polimerase NS5B, e o simeprevir, um inibidor de protease de segunda geração. Esses DAAs são administrados oralmente, em combinação ou não com interferon-α e/ou ribavirina, dependendo do genótipo do vírus (DUGUM; O'SHEA, 2014). Mais recentemente, foram aprovados novos tratamentos com combinações de diferentes DAAs, livres de interferon (HOLMES; THOMPSON, 2015). A combinação de sofosbuvir e daclastavir, inibidor da

NS5A, por exemplo, foi aprovada em 2015 na Europa para os genótipos 1, 2 e 3, e nos EUA para o genótipo 3 (HOLMES; THOMPSON, 2015). Esses tratamentos apresentaram SVR acima de 90% para a maioria dos genótipos e dos pacientes (HOLMES; THOMPSON, 2015).

A bradicardia foi relatada como um efeito colateral após a injestão de sofosbuvir e daclatasvir por pacientes que receberam amiodarona, um medicamento cardíaco, indicando que pacientes tratados com esse medicamento devem ser monitorados (RENET et al., 2015), e um caso de convulsões foi apresentado como possível efeito colateral da terapia antiviral com sofosbuvir e simeprevir (SYAL et al., 2014).

No Brasil, a Comissão Nacional de Incorporação das Tecnologias no SUS (CONITEC) optou por descontinuar o uso dos DAAs de primeira geração (boceprevir e telaprevir) em julho de 2015, e o sofosbuvir, o simeprevir e o daclatasvir foram adicionados ao protocolo terapêutico do SUS (CONITEC, 2015). Em março de 2017, foi adicionado o tratamento com a associação dos fármacos ombitasvir (inibidor de NS5A), dasabuvir (inibidor da polimerase NS5B), veruprevir (inibidor de protease NS3/4A) e ritonavir (potencializador farmacocinético) (CONITEC, 2017).

Terapias baseadas em DAAs custam aproximadamente 84.000 dólares por 12 semanas de tratamento, sendo esses regimes inacessíveis em muitos países (PEARLMAN, 2012; ZHANG et al., 2015). Além disso, estudos tem demonstrado que mutações específicas podem conferir resistência antiviral a esses tratamentos de maneira muito rápida (THOMPSON et al., 2011; TAO et al., 2017).

Os efeitos colaterais, somado ao custo elevado e ao surgimento de variantes virais resistentes aos novos tratamentos, demonstram a necessidade da busca constante por novas terapias anti-HCV.

1.5. Compostos naturais e sintéticos com potencial antiviral

Compostos naturais têm demonstrado considerável potencial no desenvolvimento de produtos terapêuticos, e muitos trabalhos reportaram o uso de compostos extraídos de fontes animais e vegetais como possíveis fármacos, inclusive com atividade antiviral contra o HCV (JIN et al., 2008; YAN et al., 2011; DABBOUSEH; JENSEN, 2013; MATSUMOTO et al., 2013; SHIMIZU, J. F. et al., 2017). Além disso, compostos naturais apresentam características como elevada diversidade química, baixo custo de produção e efeitos colaterais mais leves ou inexistentes (KITAZATO et al., 2007).

Venenos de escorpiões, por exemplo, foram descritos com atividade anti-HCV, reduzindo a infectividade por ação direta nas partículas virais (YAN et al., 2011; EL-BITAR et al., 2015); e toxinas isoladas de serpentes foram efetivas na inibição de diversos vírus como o da Febre amarela, Dengue, Oropouche, Mayaro, Rocio Vírus e Herpes (HUBBARD et al., 2012; MULLER et al., 2012, 2014). Além disso, recentemente Shimizu e colaboradores demonstraram o efeito anti-HCV de toxinas isoladas da serpente *Crotalus durissus terrificus*, atuando na entrada, replicação ou liberação da partícula viral (SHIMIZU, J. et al., 2017).

O uso de compostos sintéticos com base nas estruturas de compostos naturais também tem sido estudado devido às vantagens apresentadas pela síntese como a produção em larga escala, a possibilidade de modificações químicas para potencializar suas atividades biológicas e a patenteabilidade (STANKIEWICZ-DROGOŃ et al., 2010; FERNÁNDEZ-CALIENES VALDÉS, 2011).

As acridonas são compostos amplamente encontrados em plantas da família Rutaceae e são caracterizadas pela presença de um grupo carbonil na posição 9 (DEMEUNYNCK et al., 2001). São conhecidas por suas atividades farmacológicas já descritas, dentre elas a ação antiviral de amplo espectro, agindo contra vírus de DNA e RNA (YAMAMOTO et al., 1989; TABARRINI et al., 2006; MAZZUCCO et al., 2015). Acridonas sintéticas são descritas por diversos autores com atividade anti-HCV, demonstrando um grande potencial antiviral desses compostos contra o vírus a ser estudado neste trabalho (STANKIEWICZ-DROGON et al., 2008; MANFRONI et al., 2009; CAMPOS et al., 2017).

2. Objetivos Gerais

O objetivo geral do trabalho foi avaliar a atividade antiviral de compostos naturais e sintéticos contra o vírus da hepatite C (HCV) *in vitro*.

2.1. Objetivos Específicos

1. Analisar a viabilidade celular de 10 compostos isolados de venenos de serpentes do gênero *Bothrops* e de 16 acridonas sintéticas;
2. Avaliar a atividade antiviral dos compostos por ensaio de infectividade viral;
3. Investigar o efeito dos compostos com atividade antiviral nas etapas de entrada e replicação.

3. Referências

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CAPÍTULO II

“Hepatitis C virus infection is inhibited *in vitro* by *Bothrops* snake venoms compounds”

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Hepatitis C virus infection is inhibited *in vitro* by *Bothrops* snake venoms compounds

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Abstract

Hepatitis C is a liver disease caused by the Hepatitis C virus (HCV) and about 115 million people are estimated to be currently infected worldwide. The available therapy to treat infected patients is based mostly on direct-acting antivirals (DAAs), however, this treatment is expensive, presents several side effects and antiviral resistance has been documented, demonstrating the need for new therapeutic approaches. In this context, natural compounds have demonstrated medical interest due to their biological activities. Compounds isolated from snake venoms have shown antiviral activity against a range of viruses such as Dengue (DENV), Yellow fever (YFV), Herpes virus (HSV) and HCV. In this study, the activity of 10 compounds isolated from venoms of different snake species of the *Bothrops* genus were evaluated against HCV replicative cycle *in vitro*. The cell viability Huh-7.5 cell line under treatment was evaluated by MTT assay and the antiviral activity was analyzed using the full length JFH-1 system; virus was titrated by focus formation units assay. Eight compounds inhibited up to 99% of HCV infection, being the most potent inhibitory rates observed to TOX1 and TOX3. The CC_{50} and EC_{50} values were determined by MTT and infectivity assays for TOX1 (EC_{50} of 0.0036 µg/mL and SI = 1736) and TOX3 (EC_{50} of 0.37 µg/mL and SI = 13.51). These compounds demonstrated to block 96% and 84.7% of HCV infectivity, respectively, by directly acting on the virus particles. TOX1 also demonstrated a protective effect in cells treated prior to HCV infection of approximately 86.7%. In conclusion, these compounds showed to inhibit the HCV infectivity by either acting on the virus particles or protecting the cells against infection.

1. Introduction

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

HCV-related liver disease may gradually advances from chronic hepatitis to liver cirrhosis and hepatocellular carcinoma (HCC), and presents considerable morbidity and mortality rates [4–6]. The global prevalence of HCV was estimated to be 115 million people and approximately 71 million people are living with chronic HCV infection worldwide [7–9].

Since 2011, the treatment of HCV infection is mostly based on direct-acting antivirals (DAAs), that presents Sustained Virological Response (SVR) rates higher than 90% (MILLMAN et al., 2017; WHO, 2017). However, the high cost of DAA-based treatment (around US$ 7,000 weekly for each DAA) and documented resistance-associated variants to these treatment demonstrate the need of searching for new therapeutic approaches [2,12,13].

Several pharmacologically active components from animal venoms have been described in the literature. Bee venom from the honey bee *Apis Melifera* L. presents various biological properties including antibacterial, antiparasitic and antiviral. These compounds inhibited the replication of enveloped viruses such as Influenza A virus, Vesicular stomatitis virus, Respiratory syncytial virus and HSV, and also inhibited the replication of non-enveloped viruses such as Human papiloma virus, Enterovirus-71 and Coxsackie virus [14,15]. Compounds isolated from the *Scorpio maurus palmatus* [16] and *Heterometrus petersii* [17] scorpions venoms inhibited HCV *in vitro* through virucidal activity, by directly interacting with the viral membrane and decreasing the virus infectivity.

Toxins isolated from snake venoms inhibited several virus like DENV, YFV, Rocio virus, Oropouche virus, Mayaro virus and HSV [18–21]. Shimizu and collaborators showed that the treatment with toxins isolated from the venom of *Crotalus durissus terrificus* inhibited HCV entry, replication and release [22].

In this study, we investigated the activity of 10 compounds isolated from *Bothrops jararacussu*, *B. moojeni*, *B. alternatus* and *B. jararaca* venoms on 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 serpentarium "Centro de Extração de Toxinas Animal", registered at the Ministry of the Environment, nº 3002678. Isolation and purification of the phospholipase TOX1 and myotoxins TOX3 and TOX10 from *B. jararacussu*; the phospholipases TOX2 and TOX9 from *B. moojeni*; the peptide pools TOX6 and TOX7 from *B. alternatus* and the peptide pools TOX4, TOX5 and TOX8 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 [21,23].

Lyophilized compounds were dissolved in PBS (Phosphate-Buffered Saline), filtered and stored at -80°C. The compounds were diluted in 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 [24] was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) and supplemented with 10% fetal bovine serum (Cutilab, 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) [25]. A suspension of 5 x 10^3 Huh-7.5 cells/well was transferred to 96-well microplates the day prior to the assay and incubated at 37 ºC with atmosphere at 5% CO_2. Supernatant was replaced by fresh DMEM containing drugs at specific concentrations and maintained for 72h. After treatment, DMEM containing MTT at the final concentration of 1 mg/mL was added to cells, incubated for 30 min and replaced with 100 µL of dimethyl sulfoxide (DMSO, Sigma–Aldrich) to solubilize the formazan crystals. Surviving cells were measured by optical density (OD) of each well at 592 ηm, using a spectrophotometer. Cellular viability was calculated

according to the equation (T/C) x 100, where T represents the mean optical density of the treated group and C represent control group. In order to calculate the cytotoxic concentration of 50% (CC_{50}), cells were treated with increasing doses of the compounds and cell viability was measured. The CC_{50} value was calculated using Prism (Graph Pad).

2.4. Viral assays

The virus assays was performed with the compounds at non-cytotoxic concentration (minimum of 90% of cell viability). The JFH-1 HCVcc genotype 2a particles [26] were generated as described previously [27]. A suspension of 5 x 10^3 Huh-7.5 cells/well was seeded to 96-well microplates the day prior to the assay and incubated at 37 ºC with the atmosphere at 5% CO_2. Then, cells were infected with JFH-1 HCVcc, approximately 100 focus-forming units (FFU)/well, and compounds were added at the highest non-cytotoxic concentration at different time points, depending on the stage of HCV life cycle to be evaluated. Cells were fixed 72 hour post-infection (h.p.i.) with 4% (vol/vol) paraformaldehyde (PFA), washed with 100 mM Glycine (Applichem, USA) and semi-permeabilized with 0.1% Triton X-100 (Vetec Labs, BR). Intracellular virus was marked by indirect immunofluorescence using a sheep anti-NS5A IgG [28] as primary antibody and anti-sheep IgG, Alexa Fluor 594 conjugated, as secondary antibody. Cells were analyzed in fluorescence microscope (Axio Vert.A1 - Zeiss) in green light (500-565 ηm). Infectivity was expressed as focus-forming units per milliliter (FFU/mL).

2.4.1. Evaluation of compounds activity on HCV infectivity

The HCV infectivity assay was carried out to determine whether the venoms compounds exhibited anti-HCV activity in any part of the virus life cycle. Cells were infected with JFH-1 HCVcc in the presence of the compounds for 72 hours. The supernatant was removed, cells were washed with PBS, fixed and intracellular virus was titrated as described in virus assays. The (-)-epigallocatechin gallate (EGCG, Sigma-Aldrich, USA) at 100 µM was used as a control of inhibition of HCV infection [29]. For effective concentration of 50% (EC_{50}), cells were infected in the presence of increasing doses of compounds and infectivity was measured. The EC_{50} was calculated using Prism (Graph Pad) and the values of CC_{50} and EC_{50} were used to calculate the selectivity index ($SI = CC_{50}/EC_{50}$).

2.4.2. Antiviral activity on entry steps

The activity on virus entry steps was evaluated by using JFH-1 HCVcc infectious supernatant to infect naive Huh-7.5 cells in the presence of compounds for 4h at 37°C. The

supernatant was removed, cells were washed extensively to the complete removal of inoculum and replaced with fresh complete media.

The virucidal assay was performed to evaluate the ability of the compounds to act directly on the viral particles. Infectious supernatant was prior incubated with each compound for 1h at 37°C, and then used to infect Huh-7.5 cells for 4h at 37°C. The inoculum was removed, cells were washed extensively and replaced by fresh media.

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

For all entry assays, virus was titrated 72 h.p.i. to access the inhibition rates. EGCG at 100 µM (Sigma-Aldrich, USA) [29] was used as control of entry blockage.

2.4.3. Effect against HCV replication

To verify if the compounds are able to inhibit the HCV replication, cells were infected with JFH-1 HCVcc for 4h and washed extensively with PBS to remove non-endocytosed virus particles. Then, cells were treated with compounds for 72h and intracellular virus was titrated. Cyclosporine A at 1 µM (CsA, Sigma-Aldrich) was used as control of inhibition of replication [30].

2.5. Statistical analysis

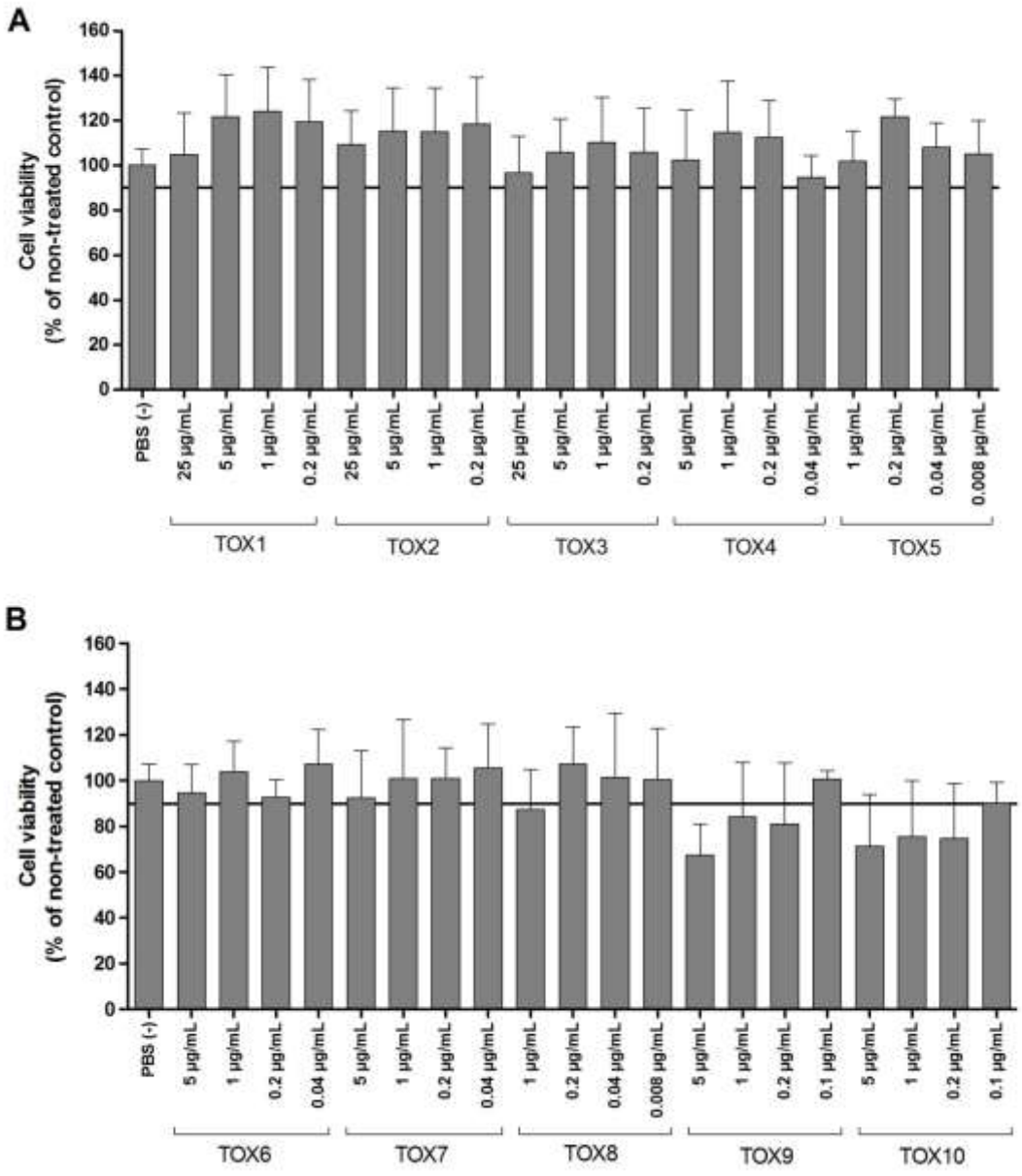
Individual experiments were performed in triplicate and all assays were performed a minimum of two times to confirm the reproducibility of the results. Mean differences of readings were compared using analysis of variance (one-way ANOVA) and Dunnett test to compare the results with the PBS control, using GraphPad Prism 5.0 software (GraphPad Software). P values of less than 0.001 (indicated by asterisks) were considered to be statistically significant.

3. Results

3.1. Inhibitory effect of snake venom compounds on HCV infectivity

To determine the potential activity of snake venom compounds on HCV replicative cycle, we first screened for their cytotoxicity by treating cells with each compound at four different

concentrations performing MTT assay. The results demonstrated that TOX1 and TOX2 did not show any cytotoxicity at the highest concentration (25 µg/mL), and for the other compounds, the highest non-cytotoxic concentration (with a minimum of 90% of cell viability) was selected for further analysis (Supplementary Material 1).



Supplementary material 1. Cell viability of snake venom compounds. Naïve Huh-7.5 cells were treated with the compounds at four different concentrations for 72 hours, when cell viability was measured by the MTT assay. Cell viability of compounds TOX1 – TOX5 are represented in A and TOX6 – TOX10 are represented in B.

We next assessed the inhibitory effect of these compounds on HCV replicative cycle. For this, cells were infected with JFH-1 HCVcc and simultaneously treated with compounds at the highest non-cytotoxic concentrations. Infectivity levels were analyzed 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 1). Particularly, TOX1 and TOX3 at nontoxic concentration were able to block up to 99% of the virus infectivity ($p < 0.001$) (Figure 1), demonstrating the strong effect of these compounds on HCV life cycle.

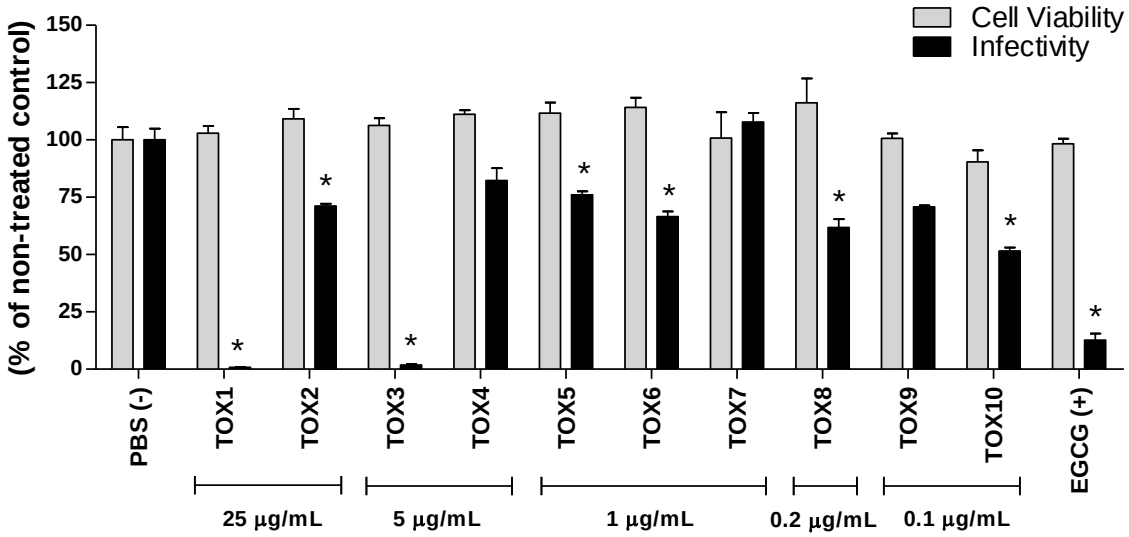


Figure 1. Anti-HCV activity of snake venom compounds. Huh-7.5 cells were treated with compounds for 72h and MTT assay was performed to determine cell viability (grey bars). To analyze the HCV infectivity under treatment of compounds, cells were infected with JFH-1 HCVcc and simultaneously treated with 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 measured in triplicate including the standard deviation are shown. $p < 0.001$ was considered statistically different of untreated control (*).

Concentration-response curves were performed for these compounds in order to determine the CC_{50}, EC_{50} and SI values. The concentrations used for TOX1 were of 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.09, 0.04, 0.02, 0.01, 0.006 and 0.003 µg/mL and for TOX3 were 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.020, 0.010, 0.005 and 0.002 µg/mL. Considering that both compounds did not show cytotoxicity for Huh-7.5 cells in the maximum concentration tested (6.25 for TOX1 and 5 µg/mL for TOX3), this concentrations were considered as the CC_{50} to calculate the SI ($SI = CC_{50}/EC_{50}$). The EC_{50} and SI were of 0.0036 µg/mL and 1736 for TOX1 and 0.37 µg/mL and 13.51 for TOX3, respectively (Figure 2).

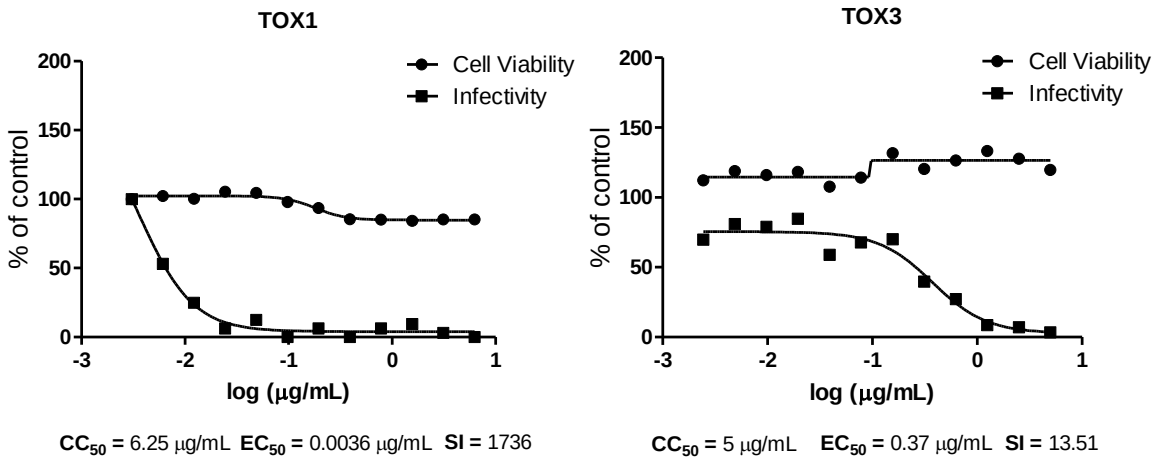


Figure 2. Cytotoxic Concentration of 50% (CC_{50}), Effective Concentration of 50% (EC_{50}) and Selectivity Index (SI) of TOX1 and TOX3. Huh-7.5 cells were treated with TOX1 at 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.09, 0.04, 0.02, 0.01, 0.006 and 0.003 µg/mL and TOX3 at 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.020, 0.010, 0.005 and 0.002 µg/mL (for MTT assay), and simultaneously infected with JFH-1 HCVcc (for infectivity assay) for 72h, at 37°C.

3.2. Snake venom compounds blocked HCV entry, but did not interfere with replication

In order to evaluate the ability of the venom compounds in blocking the virus entry into Huh-7.5 cells, virus-containing inoculum and compounds were simultaneously added to Huh-7.5 cells and incubated for 4h. Cells were extensively washed with PBS to remove any remaining virus or compounds, replaced with fresh medium and incubated for 72 h. The intracellular virus was titrated by using a focus forming unit assay. The results showed that compounds TOX1 at 25 µg/mL and TOX3 at 5 µg/mL significantly inhibited 97.2% and 83.4% of HCV entry to the host cells, respectively ($p < 0.001$) (Figure 3a).

To further investigate the inhibitory activity of compounds on entry blockage the virucidal assay was carried out. Supernatant containing JFH-1 HCVcc was incubated with compounds for 1 hour at 37°C prior to the infection of Huh-7.5 cells. The inoculum of virus and compounds were transferred to the naïve cells and incubated for 4 hours. Cells were washed for the complete removal of the inoculum, replaced with fresh media and incubated for 72 hours, when virus was titrated. A significant virucidal activity was observed for both compounds (TOX1 and TOX3) which blocked 96% and 85% of virus entry, respectively ($p < 0.001$) (Figure 3b).

We also investigated whether the antiviral activity of these compounds was related to their effects on host cells. To this end, Huh-7.5 cells were pre-treated with the compounds for 1 hour at 37°C, followed by washes with PBS to remove any trace of compounds, prior to infection with JFH-1 HCVcc virus for 4 hours. Supernatant was replaced by fresh media after further PBS washes and intracellular virus was titrated 72 h.p.i. The results showed that only TOX1 significantly inhibited infectivity when cells were previously treated with this compound, blocking 87% of virus entry (Figure 3c). In addition to the above data, these results suggest that TOX1 act on both the virus particle and the host cells. By the other hand, the compound TOX3 significantly increased the viral infectivity in 54.4% (Figure 3c).

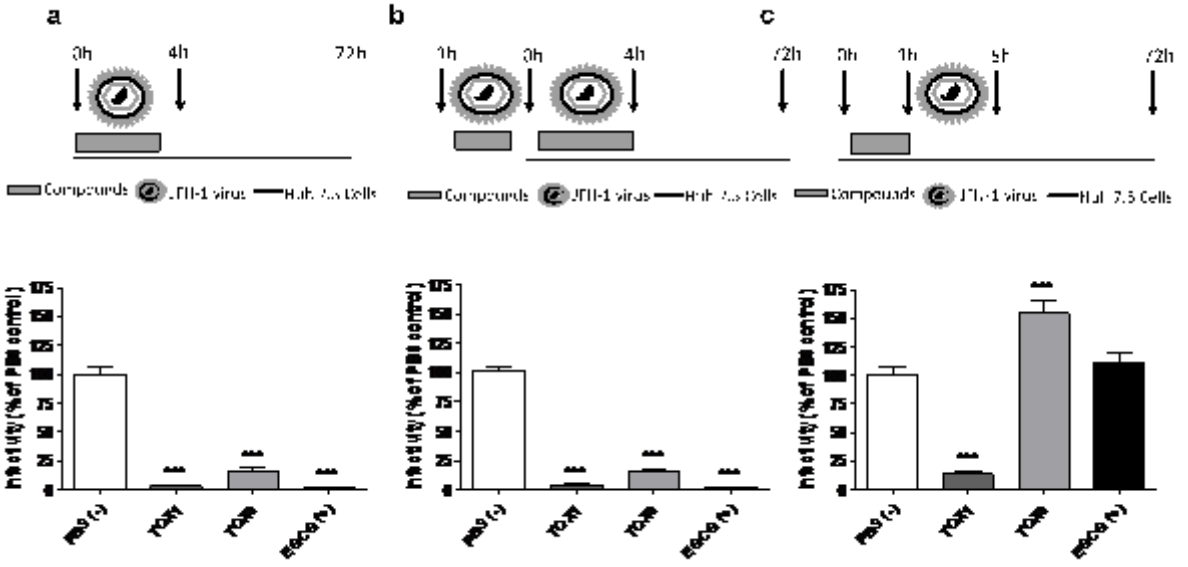


Figure 3. Effect of the snake venom compounds on HCV entry. (a) For entry assay, Huh-7.5 cells were infected with JFH-1 HCVcc and compounds TOX1 (25 µg/mL) and TOX3 (5 µg/mL) were immediately added. After 4h, the supernatant was replaced by fresh medium after repeated washes with PBS to remove the inoculum. (b) For virucidal assay, infectious supernatant was 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 media. (c) For pre-treatment assay, Huh-7.5 cells were treated with each compound for 1 hour at 37°C prior to infection. After incubation, cells were washed with PBS and infected with JFH-1 HCVcc for 4h. Infectious supernatant was removed, additional washes were performed and fresh media 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 measured in triplicate including the standard deviation are shown. $p < 0.001$ was considered statistically different from PBS (*).

These compounds were also evaluated for their ability to inhibit the 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. Drugs-containing media 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 4).

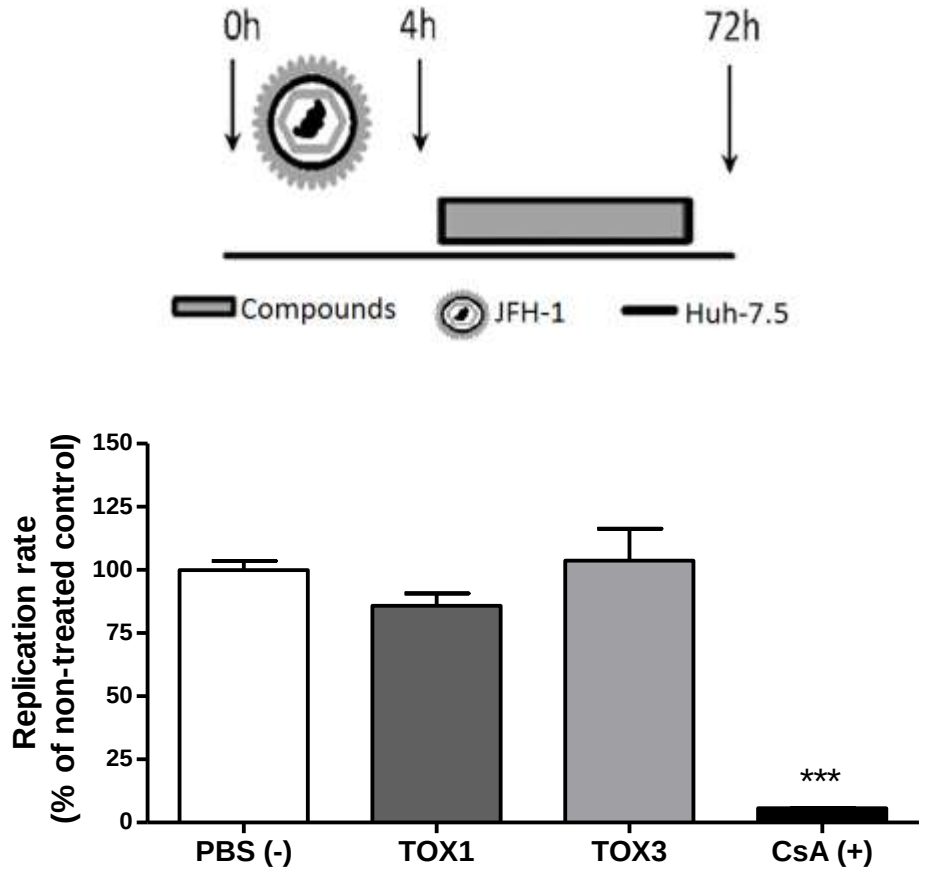


Figure 4. Snake venom compounds activity on HCV replication. Huh-7.5 cells were infected with JFH-1 HCVcc for 4h, cells were washed extensively to remove virus and treated with compounds TOX1 at 25 µg/mL and TOX3 at 5 µg/mL. PBS was used as untreated control and CsA at 1 µM as control of viral replication inhibition. Virus was titrated by focus-forming unit 72 h.p.i. Mean values of two independent experiments each measured in triplicate including the standard deviation are shown. $p < 0.001$ was considered statistically different from PBS (*).

4. Discussion

Hepatitis C virus (HCV) infection is a major worldwide health problem and there is no vaccine to prevent this disease [31,32]. Moreover, the currently available treatments are expensive, present several side effects and resistance variants have been described [12,13]. Therefore, the development of new therapies is need [33,34].

Snake venoms are a complex mixture of compounds, which have a diverse array of actions both on prey and human victims [35], many of these compounds are biologically active against parasites [18], bacteria [36] and viruses [20].

Here, we investigated the antiviral activity against HCV of compounds isolated from snake venoms of the *Bothrops* genus species and we observed that 8 compounds had antiviral activity. The most effective compounds were the phospholipase TOX1 and the myotoxin TOX3, with up to 99% of infectivity inhibition.

We observed that phospholipase TOX1 inhibited the HCV, acting on viral particles (96% of inhibition) and by a protective effect on cells against the virus infection (87%), being an antiviral of multiple effects. The myotoxin TOX3 also showed strong activity against viral particles, inhibiting 85% on virucidal assay, however, the pre-treatment of the cells with this compound provoked more than 50% of increase on viral infectivity.

The phospholipases of snake venoms catalyze the hydrolysis of the glycerophospholipids at the sn-2 position, releasing free fatty acids and lysophospholipids. Therefore, they play a key role in various biological activities, such as lipid digestion, host defense and homeostasis of cellular membranes [37,38]. Phospholipase A_2 (PLA_2) from snakes have been described with neurotoxic, myotoxic, hemolytic and bactericidal activity [39,40]. Muller et al. has found that phospholipases at non-cytotoxic concentrations showed high antiviral activity against the enveloped virus as DENV, YFV, Rocio virus, Oropouche virus and Mayaro virus. They act on virus particles, suggesting that the enzymatic activity of the phospholipases is important for the antiviral effect [20,21]. To test this hypothesis, they analyzed the effect of TOX3 from *B. jararacussu*, a PLA_2 without catalytic activity, against YFV and DENV, and although TOX3 showed virucidal activity, the SI obtained for this toxin was lower (15.4 for YFV and 22.6 for DENV-2) when compared to the obtained for PLA_2-CB (>135,000) and PLA_2-IC (>35,000) for both viruses [21]. This findings corroborate with our results since the inhibition observed in the virucidal assay was 11% higher for TOX1 (96% of inhibition), a phospholipase with low catalytic activity [41,42], in comparison with the myotoxin TOX3 (85%), without any catalytic activity; furthermore, the SI was also higher to TOX1 (1736) than to TOX3 (13.5). With these results, we can infer that catalytic activity play an important role for the antiviral effect of phospholipases.

The toxin TOX3 contain a surface that binds to the target membrane, appropriately called the interfacial binding surface (IBS), which is composed of a surface cleft and is lined with highly conserved hydrophobic residues that bind the acyl-chains of the phospholipid substrate, together with the surrounding ring of charged and polar residues [43]. Bortoleto-Bugs 2007 shown that amphiphilic molecules accumulate at the IBS of both monomers in the TOX3 dimer, and upon accumulation a quantity of molecules equal to the aggregation

number, it becomes energetically favorable for the bound molecules to dissociate from the protein in the form of a micelle. The accumulation and release of amphiphilic molecules by the IBS has been called the "Micellar Nucleation Model" (MNM) and predicts that the TOX3 requires only a transient association with the exterior leaflet of the phospholipid bilayer to permeabilize the membrane [44].

X-ray crystallography and spectroscopic studies of TOX3 have shown that the interface of monomers acts as a molecular hinge resulting in "open" and "closed" forms of the dimer, and it has been proposed that alterations in the quaternary conformation of the membrane bound protein may result in the insertion of the C-terminal loop region in the lipid bilayer of up to a depth of 13 Å, causing the loss of the integrity of the membrane [45,46]. Therewith, we can suggest that the virucidal activity of TOX3 is due to its insertion into the viral envelope, disintegrating such structure and preventing the virus from entering the cell and continue its cycle.

5. Conclusion

With this work, we demonstrated that the compounds from *Bothrops* snake venoms analyzed strongly inhibited the HCV infection *in vitro*. Toxins TOX1 and TOX3 from *B. jararacussu* presented the highest inhibition rates, demonstrating to block the viral infection in 96% and 84.7%, respectively, through virucidal mechanism of action. Furthermore, TOX1 also showed protective effect on Huh-7.5 cells against HCV infection, inhibiting 86.7%. Thus, these toxins may contribute for the development of future Hepatitis C virus therapies.

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CAPÍTULO III

“Synthetic acridones inhibit Hepatitis C virus entry *in vitro*”

*Este artigo está formatado como manuscrito de acordo com as normas do periódico *Journal of General Virology* (com algumas alterações estruturais para melhor se adequar ao formato da dissertação).

Synthetic acridones inhibit Hepatitis C virus entry *in vitro*

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Abstract

Hepatitis C is a severe disease that chronically affects 1% of the world population, more than 70 million individuals. This disease is caused by the Hepatitis C virus (HCV), a member of *Flaviviridae* family. The current treatment for the infected patients is based on direct-acting antivirals (DAAs), in monotherapy or combined with interferon and ribavirin, and treated patients can achieve approximately 90% of Sustained Virological Response (SVR). However, this treatment is expensive, there are several side effects and resistance-associated variants (RAVs) are described. Therefore, new treatments have been continuously searched. In this context, acridones have been described to possess antiviral activity against several viruses like Dengue virus (DENV), Herpes simplex virus (HSV), Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV). In this study we evaluated the anti-HCV activity of 16 acridones synthesized based on natural chemical scaffolds. The non-cytotoxic concentration of each compound was determined by cell viability assay (MTT) in Huh-7.5 cell line, and later, the acridones were screened by their anti-HCV activity by performing infectivity assay based on the JFH-1 HCVcc system. The acridones Fac15 and Fac17 demonstrated to possess antiviral potential inhibiting HCV infectivity up to 55.7% and 51.7% at non-toxic concentration, respectively. The CC_{50} and EC_{50} values were determined by MTT and HCV infectivity assays for Fac15 (EC_{50} of 5.6 µM and SI = 1.72) and Fac17 (EC_{50} of 12.7 µM and SI = 1.65). No inhibitory effect was observed against HCV replication for these compounds, however, they showed a reduction of HCV infectivity of 58% and 48%, respectively, acting directly on viral particles. Additionally, Fac15 also demonstrated protective activity to cells against the infection of 39%.

1. Introduction

The global prevalence of chronic Hepatitis C virus (HCV) infection is estimated to be 71 million individuals (1% of the world population) [1, 2]. The HCV is an enveloped virus with a single-stranded RNA of approximately 10,000 nucleotides grouped into the *Flaviviridae* family [3, 4].

Currently, seven genotypes of HCV with several subtypes are described [5, 6]. The genotypes differ in approximately 30 – 35% of nucleotide sites, with 20 – 25% nucleotide variability among subtypes [7]. The high genetic variation among HCV variants contributes to the inexistence of a vaccine, and to the resistance to the therapies available against HCV infection [8, 9].

The treatment of HCV infection was based on the combination of peginterferon alfa (PEG-IFNα) and ribavirin (RBV) for years [10–12]. Since 2011, the outcomes have improved due to the approval of direct-acting antivirals (DAAs) that inhibit non-structural proteins involved in the HCV replicative cycle [13–15]. However, this treatment is expensive, there are side effects and resistance-associated variants (RAVs) are described [16–19]. Therefore, researches for new therapies to treat patients infected with HCV are necessary [8, 20].

Alkaloids are a central class of natural products which have been extensively used as modern drugs or drug prototypes [21]. Acridones, mainly found in vascular plants from Rutaceae family, are alkaloids characterized by the presence of a carbonyl group at the 9-position [22, 23]. Several pharmacological activities have been described for acridones and include insecticidal [24], anticancer [22], antibacterial [25], antimalarial [26] and antiviral activities [27, 28]. Acridones extracted from *Glycosmis parva* (Rutaceae) [29] and citrus plant (Rutaceae) [28] showed antiviral activity against HSV type 1 and 2. Citrusinine-I, the same acridone isolated from citrus plant, inhibited cytomegalovirus (HCMV) with EC_{50} of 1.5 µg/mL [27].

The use of synthetic compounds based on the natural compounds scaffolds has been studied due to advantages presented by synthesis as the production in large scale, the possibility of chemical modifications to have their biological activities potentiated, and the patentability [26, 30]. Synthesized acridone derivatives demonstrated to be effective against viruses like DENV [31], HIV-1 [32], Human adenovirus (HADV) [33] and Junin virus (JUNV) [34]. The antiviral activity of synthetic acridones is also described for HCV [30, 35–37]. These data indicate that synthetic acridones could be potential antiviral against HCV.

In this work, we investigated the antiviral effect of 16 synthetic acridones against HCV replicative cycle. The data obtained showed that two of these acridones can reduce HCV infectivity.

2. Material and Methods

2.1. Acridones

The acridones were synthesized under the supervision of Prof. Dr. Luis Octavio Regasini at Laboratory of Green and Medicinal Chemistry, Department of Chemistry and Environmental Sciences, of the Institute of Biosciences, Letters and Exact Sciences, Unesp - São José do Rio Preto - São Paulo – Brazil, using the protocol previously described [23]. The compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich), and stored at -80°C. Compounds were diluted in complete medium immediately prior to the experiments to reach a maximum final concentration of 0.1% DMSO. For all the assays performed, control cells were treated with medium added with DMSO at the final concentration of 0.1%.

2.2. Cell culture

The human hepatoma cell line Huh-7.5 [38] was cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich) supplemented with 10% fetal calf serum (Cutilab, 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) at 37 ºC in a humidified 5% CO_2 incubator.

2.3. Cell viability assay

The cell viability of acridones was determined by MTT [3-(4,5-dimeth- ylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Sigma-Aldrich, St. Louis, MO, USA) [39]**.** Huh-7.5 cells were seeded at the density of 5 x 10^3 to 96-well microplates the day prior to the assay and incubated at 37 ºC with atmosphere at 5% CO_2 overnight. The culture medium was removed and medium containing the acridones at specific concentrations was added to the cell culture and maintained for 72h at 37°C in a humidified 5% CO_2 incubator. DMEM containing MTT at the final concentration of 1 mg/mL was then added to each well, incubated for 30 min at 37ºC and replaced with 100 µL of DMSO to solubilize the formazan crystals. The absorbance was measured at 592 ηm on a plate reader (FLUOstar Omega/BMG LABTECH, Offenburg, BW, DE). Cell viability was calculated according to the equation (T/C) × 100, which T and C represented the mean optical density of the treated and control groups,

respectively. In order to calculate the cytotoxic concentration of 50% (CC_{50}), cells were treated with increasing doses of Fac15 or Fac17 and cell viability was measured. The CC_{50} value was calculated using Prism (Graph Pad).

2.4. Viral assays

For viral assays JFH-1 HCVcc genotype 2a particles [40] were generated as previously described [41]. Naive Huh-7.5 cells were infected with JFH-1 HCVcc particles, approximately 100 focus-forming units (FFU)/well, and compounds were added at different time points, depending on the stage of HCV life cycle to be evaluated. Cells were fixed 72 hours post-infection (h.p.i.) with 4% (vol/vol) paraformaldehyde (PFA), washed with 100 mM Glycine (Applichem, USA) and semi-permeabilized with 0.1% Triton X-100 (Vetec Labs, BR). Intracellular virus was detected by indirect immunofluorescence using a sheep anti-NS5A IgG [42] as primary antibody and anti-sheep IgG, Alexa Fluor 594 conjugated, as secondary antibody. Cells were washed, maintained in PBS and analyzed onto fluorescence microscope (Axio Vert.A1 - Zeiss) in under 500-565 ηm. The infectivity was expressed as focus-forming units per milliliter of supernatant (FFU/mL).

2.4.1. Effect of acridones on HCV infectivity

In order to evaluate the effects of acridones on HCV infectivity, Huh-7.5 cells were infected with JFH-1 HCVcc particles and simultaneously treated with compounds at the highest non-cytotoxic concentration (above 80% of cell viability) for 72h. The infectivity was analyzed by focus-forming units per milliliter of supernatant (FFU/mL). For effective concentration of 50% (EC_{50}), cells were infected in the presence of increasing doses of compounds and infectivity was measured. The EC_{50} was calculated using Prism (Graph Pad) and the values of CC_{50} and EC_{50} were used to calculate the selectivity index ($SI = CC_{50}/EC_{50}$), which suggests the potential antiviral activity of the compounds.

2.4.2. Antiviral activity of acridones on HCV entry steps

To verify the activity of acridones on HCV entry step, Huh-7.5 cells were infected with viral particles and simultaneously treated with the acridones Fac15 at 5 µM and Fac17 at 10 µM for 4h. The supernatant was removed, cells were extensively washed with PBS to the complete removal of inoculum, and replaced with fresh complete media.

The virucidal assay was performed in order to evaluate if the compounds act directly on the viral particles. Infectious supernatant was prior incubated with each compound for 1h at

37°C, and then this mixture was used to infect naive Huh-7.5 cells for 4h at 37°C. The solution was removed, cells were washed extensively and replaced by fresh media.

Pre-treatment assay was carried out to determine whether any compound could confer cell protection against viral infection. Huh-7.5 cells were treated with each compound for 1h at 37°C prior to infection. Cells were washed and infected with JFH-1 HCVcc for 4h at 37°C. The inoculum was then removed, additional washes were performed to completely removal of non-endocytosed virus and fresh media was added.

For these assays, virus was titrated 72 h.p.i. by focus-forming units per milliliter of supernatant (FFU/mL). DMSO and (-)-epigallocatechin gallate at 100 µM (EGCG, Sigma-Aldrich, USA) [43] were used as non-treated and positive controls, respectively.

2.4.3. Effect of acridones on HCV replication

In order to analyze the effect of acridones on HCV replication, naive Huh-7.5 cells were first infected with viral particles for 4h. The cells were extensively washed to remove non-endocytosed virus particles, and the acridones Fac15 at 5 µM and Fac17 at 10 µM were added. Intracellular virus was titrated 72 h.p.i. by focus-forming units per milliliter of supernatant (FFU/mL). DMSO was used as untreated control and cyclosporine A at 1 µM (CsA, Sigma-Aldrich) as control of inhibition of replication [44].

2.5. Statistical analysis

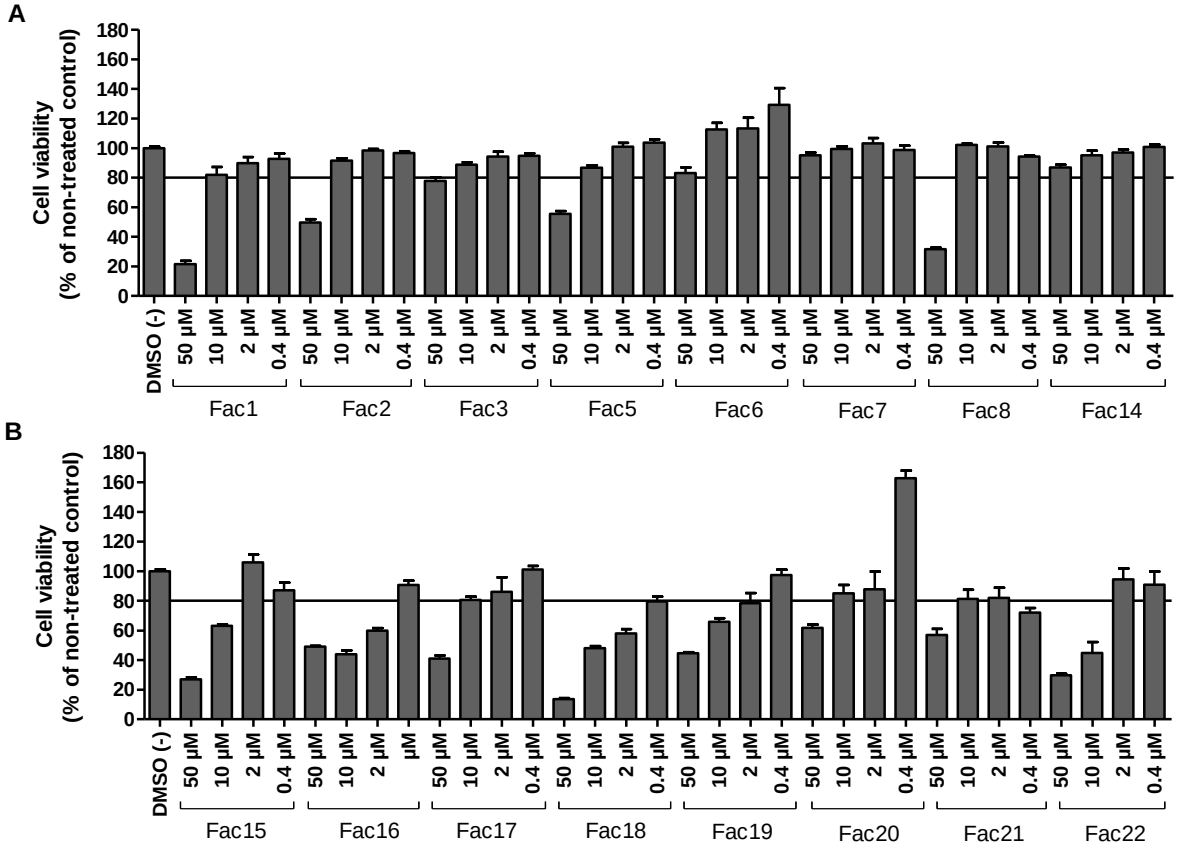
Individual experiments were performed in triplicate and all assays were performed a minimum of two times to confirm the reproducibility of the results. Mean differences of readings were compared using analysis of variance (one-way ANOVA) and Dunnett test using GraphPad Prism 5.0 software (GraphPad Software). P values of less than 0.001 (indicated by asterisks) were considered to be statistically different of DMSO control.

3. Results

3.1. Cell viability assay

The highest non-toxic concentrations of each compound was determined by MTT assay. Naïve Huh 7.5 cells were treated with the compounds at 50 µM, 10 µM, 2 µM and 0.4 µM for 72 hours and cell viability was measured by the MTT assay (Supplementary Material 1). The highest non-cytotoxic concentration was 50 µM for Fac6, Fac7 and Fac14; 10 µM for Fac1, Fac2, Fac3, Fac5, Fac8, Fac17, Fac20 and Fac21; 2 µM for Fac15 and Fac22 and at 0.4 µM

for Fac16, Fac18 and Fac19. All subsequent assays were performed under the highest non-toxic concentrations.



Supplementary material 1. Cell viability of acridones. Naïve Huh-7.5 cells were treated with the compounds at 50, 10, 2 and 0.4 µM for 72 hours, and cell viability was measured by the MTT assay. Cell viability of acridones Fac1 – Fac14 are represented in A and Fac15 – Fac22 are represented in B.

3.2. Acridones Fac15 and Fac17 inhibit HCV infectivity

Huh-7.5 cells were infected with JFH-1 HCVcc and simultaneously treated with each compound at the highest non-cytotoxic concentration. Infectivity levels were assessed 72 h.p.i. by focus-forming units per milliliter of supernatant (FFU/mL). The results demonstrated that Fac15 and Fac17 significantly inhibited HCV infectivity up to 55.7% and 51.7%, respectively ($p < 0.001$) (Figure 1).

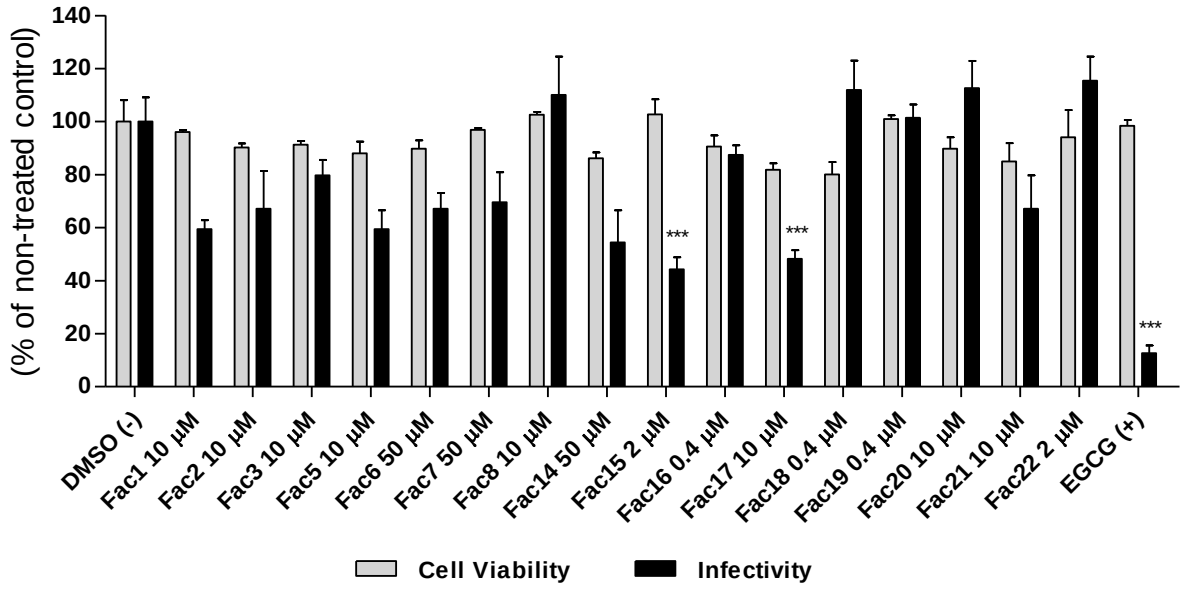


Figure 1. Anti-HCV activity of synthetic acridones. Huh-7.5 cells were treated with acridones for 72h and MTT assay was performed to determine cell viability (grey bars). To analyze the HCV infectivity under treatment of compounds, cells were infected with JFH-1 HCVcc and simultaneously treated with each acridone 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). DMSO was used as non-treated control. The assays were performed in triplicates and in two independent events. $p < 0.001$ was considered statistically different of DMSO control (*).

Concentration-response curves were performed for these compounds in order to determine the CC_{50}, EC_{50} and SI values. The values obtained were CC_{50} of 9.7 µM and EC_{50} of 5.6 µM for Fac15, and CC_{50} of 21 µM and EC_{50} of 12.7 µM for Fac17. The compounds showed SI values of 1.72 and 1.65, respectively, demonstrating that both acridones have similar antiviral potential (Figure 2).

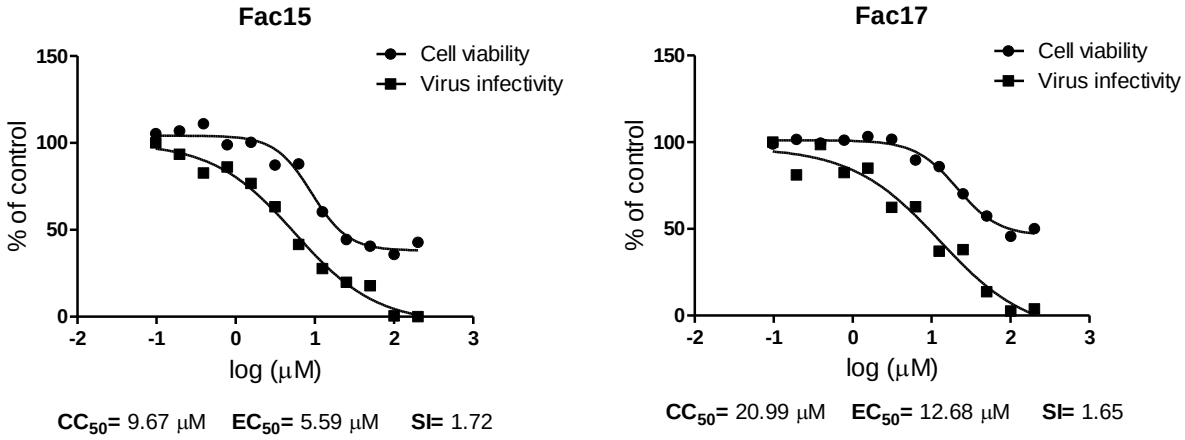


Figure 2. Cytotoxic Concentration of 50% (CC_{50}), Effective Concentration of 50% (EC_{50}) and Selectivity Index (SI) of Fac15 and Fac17. Huh-7.5 cells were treated with Fac15 and Fac17 at 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 and 0.09 μM (for MTT assay), and simultaneously infected with JFH-1 HCVcc (for infectivity assay) for 72h, at 37°C.

3.3. Antiviral activity of acridones on HCV entry

To evaluate the ability of the acridones in blocking the virus entry into Huh-7.5 cells, virus-containing inoculum and compounds (Fac15 at 5 μM or Fac17 at 10 μM) were simultaneously added to Huh-7.5 cells and incubated for 4h. Cells were extensively washed with PBS to completely remove remaining virus or compounds and replaced with fresh medium for 72 hours, when intracellular virus was titrated by focus formation unit assay. The results demonstrated that Fac15 and Fac17 were able to block HCV entry up to 58% and 50%, respectively (Figure 3a).

In order to verify if the compounds show a direct effect on viral particles, the virucidal assay was carried out. For this, JFH-1 HCVcc virus was incubated with the compounds at 37°C for 1h and this solution was used to infect Huh-7.5 cells for 4h. Cells were washed and incubated with fresh medium for 72 hours, when virus was titrated. A significant virucidal activity was observed for the compounds Fac15 and Fac17, which inhibited the virus ability to infect the cell in 58% and 48%, respectively ($p < 0.001$) (Figure 3b). These data suggest that an anti-HCV mechanism of action for these compounds might be related to a direct action on the virus particle structure.

To investigate whether the antiviral activity of these compounds is related to their effects on host cells the pre-treatment assay was carried out. Huh-7.5 cells were pre-treated with the compounds for 1 hour at 37°C, followed by washes with PBS to remove any trace of

compounds, prior to infection with JFH-1 HCVcc virus for 4 h. The analysis showed that only Fac15 significantly inhibited infectivity when cells were previously treated with this compound, inhibiting 39% of virus entry (Figure 3c). Thus, these results suggest that Fac15 act on both the virus particle and the host cells.

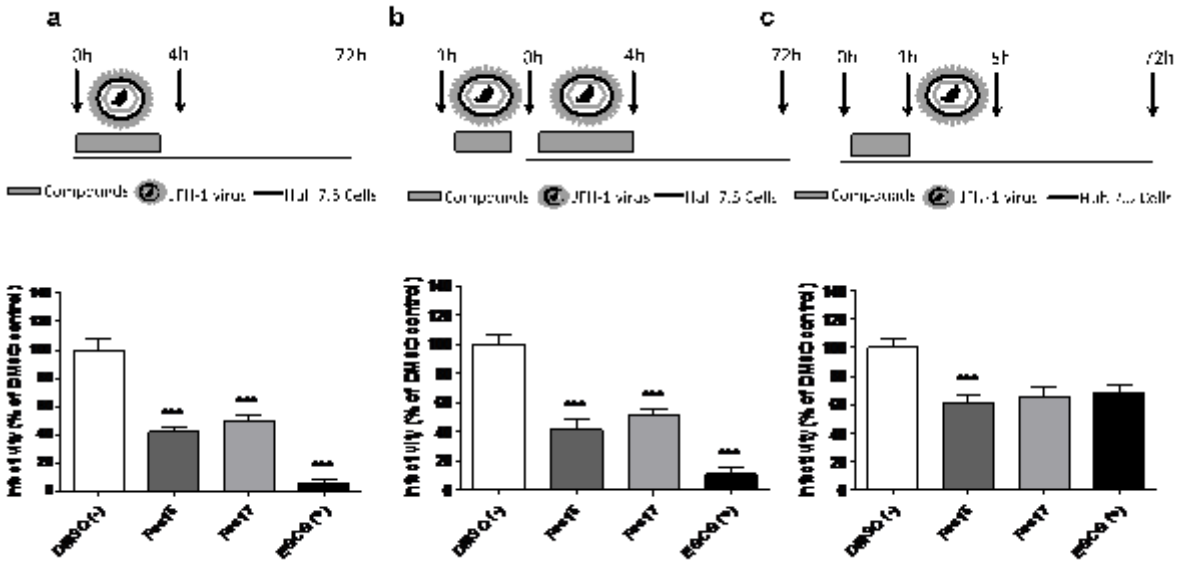


Figure 3. Antiviral effect of the acridones on HCV entry. (a) For entry assay, Huh-7.5 cells were infected with JFH-1 HCVcc and compounds Fac15 at 5 µM and Fac17 at 10 µM were immediately added. After 4h, the supernatant was replaced by fresh medium after repeated washes with PBS to remove the inoculum. (b) For virucidal assay, infectious supernatant was 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 media. (c) For pre-treatment assay, Huh-7.5 cells were treated with each compound for 1 hour at 37°C prior to infection. After incubation, cells were washed with PBS and infected with JFH-1 HCVcc for 4h. Infectious supernatant was removed, additional washes were performed and fresh media was added. DMSO 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 measured in triplicate including the standard deviation are shown. $p < 0.001$ was considered statistically different from DMSO (*).

3.4. The acridones did not reduce HCV replication

These acridones were also evaluated for their ability to inhibit the HCV replication. For this, Huh-7.5 cells were infected with JFH-1 HCVcc viral particles for 4h, the supernatant was removed and cells were washed extensively to complete removal of viral. Drugs-containing media was added and HCV infectivity was measured 72 h.p.i. by focus-forming units per milliliter of supernatant (FFU/mL). The results showed that non-cytotoxic concentrations of these compounds had no effect on HCV replication (Figure 4).

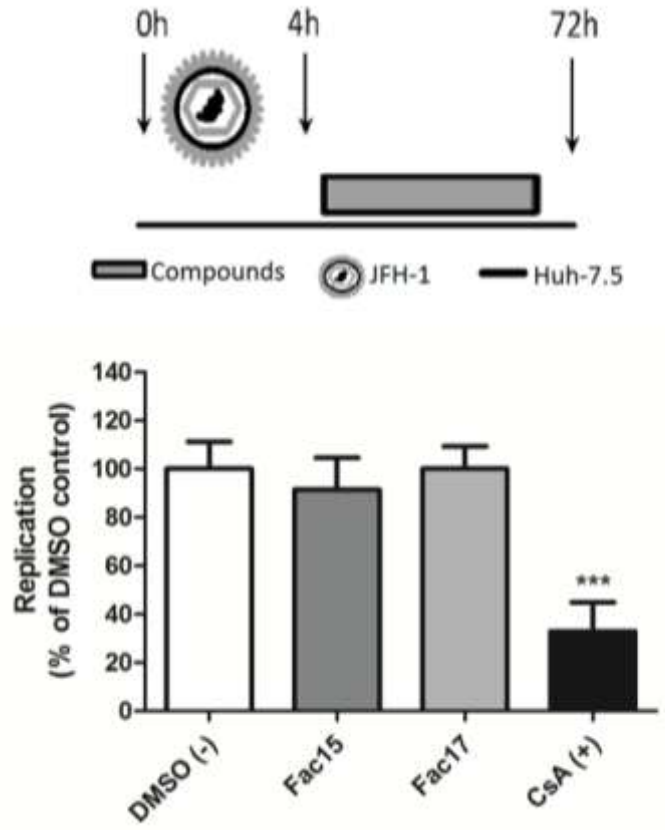


Figure 4. Evaluation of acridones activity on HCV replication. Huh-7.5 cells were infected with JFH-1 HCVcc for 4h, cells were washed extensively to remove virus, and drugs-containing media Fac15 (5 µM) and Fac17 (10 µM) was added. The cells were maintained for 72h at 37°C, then the intracellular virus was titrated by focus-forming units per milliliter of supernatant (FFU/mL). DMSO was used as untreated control and CsA at 1 µM as control of inhibition of viral replication. The assays were performed in triplicate and in two independent events. $p < 0.001$ was considered statistically different of DMSO control (*).

4. Discussion

The HCV is a global health problem that affects chronically approximately 70 million individuals worldwide [2]. The current treatment for these patients is expensive, presents several side effects, and viral resistance has been described [18, 19]. Therefore, the search for new therapeutical approaches is need.

Natural and synthetic acridones have demonstrated to possess a variety of biological activity. They have been described as antitumor agents due to their DNA or RNA binding ability and capacity of inhibiting telomerase and topoisomerase, process that result in the disorder of the biological functions in living cells [45, 46]. Some acridones from Rutaceae plants have presented strong antiviral activity against viruses with DNA genomes like HSV-1

and HSV-2 [28], and Epstein-Barr virus [47]. For RNA viruses, acridones showed activity against HIV-1 [32], Bovine viral diarrhea virus (BVDV) [48], DENV [49] and HCV [37].

Here, we showed that the synthetics acridones Fac15 and Fac17 inhibit HCV infectivity in the JFH-1 HCVcc full length system. Manfroni and collaborators showed the antiviral activity of the acridone derivative thiazolpiperazinyl on HCV replication, by inhibiting the NS3 helicase activity (IC_{50} of 110 ± 12 μM) in a Huh-7 derived cell line harbouring genotype 1b HCV replicon [35]. Similarly, Stankiewicz-Drogon showed that the acridone-4-carboxamides derivative acts as inhibitor of NS3 helicase activity with IC_{50} of 3.82 μM [36]. The same author described the acridone-4-carboxamide derivative as inhibitor of HCV replication, acting on helicases of genotypes 1a and 3a (IC_{50} of 8.4 μM and 7.3 μM, respectively) [30]. This author concludes that in the replicon system used, this compound can act via inhibition of both NS3 and NS5B activities, efficiently inhibiting HCV replication with SI value of 40.5 [36]. Another work demonstrated that the synthetic acridone Fac4 inhibited 90% of HCV replication in genotype 2a subgenomic replicon and approximately 70% in the JFH-1 HCV full length virus. Fac4 also inhibited up to 80% of the viral release from Huh-7.5 cells, and authors suggested that this inhibition could be correlated to the intercalation of this compound with the dsRNA, producing during the viral replication process [37]. Contrarily with these works, our results showed the HCV inhibition by other mechanisms, by acting on viral particles inhibiting 58% (Fac15) and 48% (Fac17), and also by protecting of host cells against the infection in 39% (Fac15).

The methanol extract of the stem barks of *Morus mesozygia* Stapf. (Moraceae) was submitted to several liquid chromatographies for Nicolle and co-authors in 2009, and Fac17 was one of the pure compounds identified by means of spectrometric methods [50]. In the same study, the compounds were tested as inhibitor of breast cancer in HEK-293 human cells, and Fac17 showed IC_{50} of 7.9 μM [50]. Although Fac17 previously demonstrated to possess biological activities, our results are the first description of the antiviral activity of Fac17.

The chemical structure and associated activities of Fac15 is still unpublished in the literature, being this work the first description of a biological activity of this compound. Therefore, our results demonstrate the importance of acridones as antivirals, acting both on the viral particles or protecting the cells against the infection.

5. Conclusion

In summary, acridones can be effective against HCV infection. The active compounds were Fac15 and Fac17, with 58% and 48% of infectivity inhibition, respectively, acting directly on viral particles. Furthermore, Fac15 also demonstrated protective activity to the cells against the infection of 39%. Thus, more studies are necessary to better understand the action mechanisms of these compounds.

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CAPÍTULO IV

Conclusão

1. Conclusão

A realização do presente trabalho foi importante para demonstrar novos componentes com atividade antiviral contra o HCV. Compostos isolados de veneno de serpentes do gênero *Bothrops*, bem como acridonas sintéticas, se mostraram com potencial anti-HCV, uma vez que reduziram a infectividade viral.

Os quatro compostos que apresentaram maior inibição TOX1, TOX3, Fac15 e Fac17 apresentaram atividade diretamente nas partículas virais. Adicionalmente, o composto isolado de serpente TOX1 e a acridona Fac15 também apresentaram efeito protetor para as células contra a infecção viral.

Dessa maneira, os compostos com atividade antiviral mais efetiva (TOX1 e TOX3) apresentam potencial para se tornar um medicamento contra a infecção pelo HCV, além da possibilidade de modificação das estruturas químicas das acridonas sintéticas para um possível aumento de seu potencial antiviral.

CAPÍTULO V

Artigos Publicados

PLOS ONE

RESEARCH ARTICLE

Multiple effects of toxins isolated from *Crotalus durissus terrificus* on the hepatitis C virus life cycle

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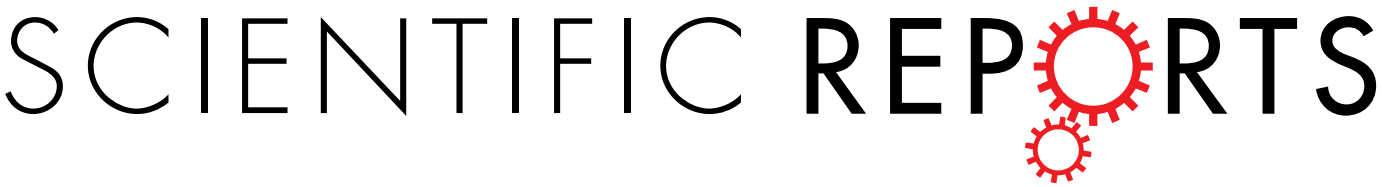
Abstract

Hepatitis C virus (HCV) is one of the main causes of liver disease and transplantation worldwide. Current therapy is expensive, presents additional side effects and viral resistance has been described. Therefore, studies for developing more efficient antivirals against HCV are needed. Compounds isolated from animal venoms have shown antiviral activity against some viruses such as *Dengue virus*, *Yellow fever virus* and *Measles virus*. In this study, we evaluated the effect of the complex crotoxin (CX) and its subunits crotapotin (CP) and phospholipase A_2 (PLA_2-CB) isolated from the venom of *Crotalus durissus terrificus* on HCV life cycle. Huh 7.5 cells were infected with HCVcc JFH-1 strain in the presence or absence of these toxins and virus was titrated by focus formation units assay or by qPCR. Toxins were added to the cells at different time points depending on the stage of virus life cycle to be evaluated. The results showed that treatment with PLA_2-CB inhibited HCV entry and replication but no effect on HCV release was observed. CX reduced virus entry and release but not replication. By treating cells with CP, an antiviral effect was observed on HCV release, the only stage inhibited by this compound. Our data demonstrated the multiple antiviral effects of toxins from animal venoms on HCV life cycle.

Introduction

Hepatitis C is a disease caused by Hepatitis C virus (HCV) infection, essentially characterized by liver inflammation. Chronic infection may progress to cirrhosis or hepatocellular carcinoma and represents one of the major causes of liver diseases and transplants [1]. Approximately 130–150 million people are chronically infected worldwide [2].



OPEN

Flavonoids from *Pterogyne nitens* Inhibit Hepatitis C Virus Entry

Jacqueline Farinha Shimizu[1,2], Caroline Sprengel Lima[3], Carina Machado Pereira[1], Cintia Bittar[1], Mariana Nogueira Batista[1], Ana Carolina Nazaré[3], Carlos Roberto Polaquini[3], Carsten Zothner[4], Mark Harris[4], Paula Rahal[1], Luis Octávio Regasini[3] & Ana Carolina Gomes Jardim[1,2]

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Hepatitis C virus (HCV) is one of the leading causes of liver diseases and transplantation worldwide. The current available therapy for HCV infection is based on interferon-α, ribavirin and the new direct-acting antivirals (DAAs), such as NS3 protease and NS5B polymerase inhibitors. However, the high costs of drug design, severe side effects and HCV resistance presented by the existing treatments demonstrate the need for developing more efficient anti-HCV agents. This study aimed to evaluate the antiviral effects of sorbifolin (1) and pedalitin (2), two flavonoids from *Pterogyne nitens* on the HCV replication cycle. These compounds were investigated for their anti-HCV activities using genotype 2a JFH-1 subgenomic replicons and infectious virus systems. Flavonoids 1 and 2 inhibited virus entry up to 45.0% and 78.7% respectively at non-cytotoxic concentrations. The mechanism of the flavonoid 2 block to virus entry was demonstrated to be by both the direct action on virus particles and the interference on the host cells. Alternatively, the flavonoid 1 activity was restricted to its virucidal effect. Additionally, no inhibitory effects on HCV replication and release were observed by treating cells with these flavonoids. These data are the first description of 1 and 2 possessing *in vitro* anti-HCV activity.

Hepatitis C virus (HCV) was identified in 1989 as the causative agent of hepatitis C[1]. It infects millions of people worldwide and is the major cause of liver disease and transplantation. According to World Health Organization (WHO), more than 350,000 people die currently from liver disease related to HCV infection[2].

HCV is an enveloped, single stranded positive-sense RNA virus, which belongs to the Flaviviridae family, genus *Hepacivirus*[3]. There is no effective vaccine for prevention of the HCV infection and, until recently, the only treatment for HCV infected patients was based on pegylated interferon and ribavirin association (PEG-IFN + RBV)[4]. The availability of new direct acting antivirals (DAAs) such as simeprevir, daclatasvir and sofosbuvir have increased rates of sustained virological response (SVR) with treatment efficacies as high as 90% for most common HCV genotypes[5–8]. However, the current treatments present several side effects, high costs[9] and resistant variants were described even for the recent therapies approved by Food and Drugs Administration (FDA)[10,11]. Therefore, despite the introduction of interferon-free regimens, the therapy regime in many countries is still based on PEG-IFN + RBV.

The high costs and potential for developing viral resistance presented by the existing treatments demonstrate the need for improving therapeutic options against HCV. In this context, natural sources have demonstrated to provide a wide source of compounds, which can be evaluated for their antiviral properties[12].

Flavonoids represent an important class of compounds, which are produced by plants as a response to microbial infections[13]. Several flavonoids have been described to possess antiviral activities. Epigallocatechin from green tea was demonstrated to inhibit replication of Enterovirus 71[14], Chikungunya virus[15] and HCV[16]. Naringenin from grapefruit showed antiviral effects against Herpes simplex virus type 1 (HSV-1)[17] and HCV[18]. Silibin and ladanein have also demonstrated anti-HCV activities by inhibiting the viral entry step[19,20]. In this context, the high structural similarity between ladanein and flavonoids from *Pterogyne nitens* Tul. (Leguminosae) and other active flavonoids encouraged us to search for their potential anti-HCV activity by inhibition of viral entry.

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