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Campus de São José do Rio Preto

Jacqueline Farinha Shimizu

Atividade antiviral de compostos naturais no ciclo replicativo do HCV

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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.

Orientador: Profª. Drª. Ana Carolina Gomes Jardim

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Comissão Examinadora

Profª. Drª. Ana Carolina Gomes Jardim
UFU – Uberlândia
Orientador

Profª. Drª. Monika Aparecida Coronado
UNESP – São José do Rio Preto

Prof. Dr. Aripuanã Sakurada Aranha Watanabe
FAMERP – São José do Rio Preto

São José do Rio Preto
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“*As nuvens mudam sempre de posição, mas são sempre nuvens no céu. Assim devemos ser todo dia, mutantes, porém leais com o que pensamos e sonhamos; lembre-se, tudo se desmancha no ar, menos os pensamentos”.* (Paulo Beleki)

LISTA DE SIGLAS E ABREVIATURAS

3' NTR – "Non-translated 3' region", Região 3' não traduzida

5' NTR - "Non-translated 3' region", Região 5' não traduzida

bp – "base pairs", pares de bases

C - Proteína do Core/ capsídeo

CP - Crotapotina

CX - Crotoxina

Da - Daltons

DAAs – "Direct-acting antivirals", Agentes antivirais de ação direta

E1 - Proteína do Envelope 1

E2 - Proteína do Envelope 2

FDA – "*Food and Drug Administration"* Agência reguladora de medicamentos

HCV – "Hepatitis C Virus", Vírus da Hepatite C

IRES – "Internal ribosome entry site", Sítio interno de entrada ribosomal

kb – "Kilobases"

LD – " Lipids Droplets", Gotículas lipídicas

LDLs – "Low-density lipoproteins", Lipoproteínas de baixa

VLDLs – "Very-low density lipoproteins", Lipoproteínas de muito baixa densidade

NS2 – "Non-structural 2 protein", Proteína Não-estrutural 2

NS3 - "Non-structural 3 protein", Proteína Não-estrutural 3

NS4A – "Non-structural 4A protein", Proteína Não-estrutural 4A

NS4B - "Non-structural 4B protein", Proteína Não-estrutural 4B

NS5A - "Non-structural 5A protein", Proteína Não-estrutural 5A

NS5B - "Non-structural 5B protein", Proteína Não-estrutural 5B

PEG-IFN – "Pegylated interferon"- Interferon peguilado

PLA_2-CB – "Phospholipase A_2 – basic chair", Fosfolipase A_2 – Cadeia básica

RE - Retículo Endoplasmático

RNA – "Ribonucleic acid", Ácido ribonucléico

RVS - Resposta virológica sustentada

RESUMO

A Hepatite C é uma doença causada pelo vírus da Hepatite C (HCV), que afeta milhares de pessoas em todo o mundo. Representa um problema de saúde pública, sendo uma das principais causas de doenças e transplantes relacionados ao fígado. Não há uma vacina contra o HCV e os tratamentos atuais não são eficazes para todos os pacientes tratados, apresentando muitos efeitos colaterais e alto custo de desenvolvimento. Desta forma, fica evidente a necessidade do desenvolvimento de novas abordagens terapêuticas que produzam uma melhor resposta virológica sustentada, efeitos colaterais mais brandos e menor custo de produção. Neste contexto, compostos naturais podem fornecer uma fonte alternativa para a identificação de produtos com potencial terapêutico. O presente trabalho teve como objetivo avaliar os efeitos de compostos naturais, isolados do veneno da serpente *Crotalus durissus terrificus* (complexo heterodimérico crotoxina e suas subunidades crotapotina e Fosfolipase A_2), e do extrato das folhas de *Pterogyne nitens* (sorbifolina e pedalitina), no ciclo replicativo do HCV *in vitro*. Estes compostos foram testados quanto às suas atividades antivirais por meio de infecção e tratamento de células Huh-7.5, e realização de ensaios de luciferase, western-blotting e imunofluorescência. Os dados obtidos demonstraram que tanto os compostos isolados de *C. durissus terrificus* quanto de *P. nitens* possuem efeito anti-HCV, sendo que alguns compostos inibiram mais de uma etapa do ciclo replicativo viral. Portanto, os múltiplos efeitos anti-HCV apresentados pelo tratamento com esses compostos demonstraram o potencial terapêutico de fontes naturais no tratamento da Hepatite C.

Palavras-chave: vírus da Hepatite C, antivirais, compostos naturais

ABSTRACT

Hepatitis C is a disease caused by Hepatitis C virus (HCV) that affects thousands of people worldwide. Represents a public health problem, being one of the main causes of liver disease and transplantation. There is no vaccine for HCV and the current therapy is not effective for all treated patients, presents many side effects and high cost of development. Thus, there is an evident need to develop new therapeutic approaches which result in a better sustained virologic response, milder side effects and lower production cost. In this context, natural compounds can provide an alternative source for the identification of products with therapeutic potential. This study aimed to evaluate the effects of natural compounds, isolated from Crotalus durissus terrificus venom (*heterodimeric complex crotoxin and its subunits crotapotin and phospholipase* A_2*), and from leaves extract of Pterogyne nitens (sorbifolin e pedalitin), on HCV life cycle in vitro. These compounds were screened for their antiviral activities by infecting and treating Huh-7.5 cells, and performing luciferase, western blotting and immunofluorescence assays. The data obtained demonstrated that both compounds isolated from Crotalus durissus terrificus and from P. nitens possess anti-, and some compounds inhibited more than one step of the virus life cycle. Therefore, the multiple anti-HCV effects presented by the treatment with these compounds demonstrated the therapeutic potential of natural sources in the treatment of Hepatitis C.*

Keywords: Hepatitis C Virus, antivirals, natural compounds

Sumário

CAPÍTULO I:

FUNDAMENTAÇÃO TEÓRICA

1. INTRODUÇÃO

1.1. Histórico e Patologia

A Hepatite C é uma doença causada pelo vírus da Hepatite C (HCV), e constitui uma das principais causas de doenças hepáticas, assim como de transplantes de fígado no mundo (BARTENSCHLAGER; LOHMANN; PENIN, 2013). Segundo dados recentes aproximadamente 130 a 150 milhões de pessoas estão infectadas cronicamente. A estimativa é de que 3 a 4 milhões de pessoas são infectadas com o HCV anualmente, e mais de 500.000 pessoas morrem por doenças no fígado relacionadas à Hepatite C (W.H.O, 2015).

A infecção é caracterizada principalmente pela inflamação do fígado, apresentando uma fase aguda que pode desenvolver-se em um estado crônico. Na fase aguda, aproximadamente 15 % dos pacientes conseguem eliminar a infecção espontaneamente, sendo que os 85 % restante dos infectados desenvolvem o estado crônico da doença. Em 20 % dos casos de hepatite C crônica pode haver o desenvolvimento de cirrose após 25 a 30 anos da infecção, e destes, 1 a 5 % podem progredir para falência hepática devido à cirrose ou ao carcinoma hepatocelular (ASHFAQ et al., 2011; MORENO-OTERO, 2005; WHO, 2015). A maioria dos pacientes pode permanecer assintomática em ambas as fases, aguda e crônica, por anos e só serem diagnosticados durantes exames de saúde (RAIMONDI et al., 2009).

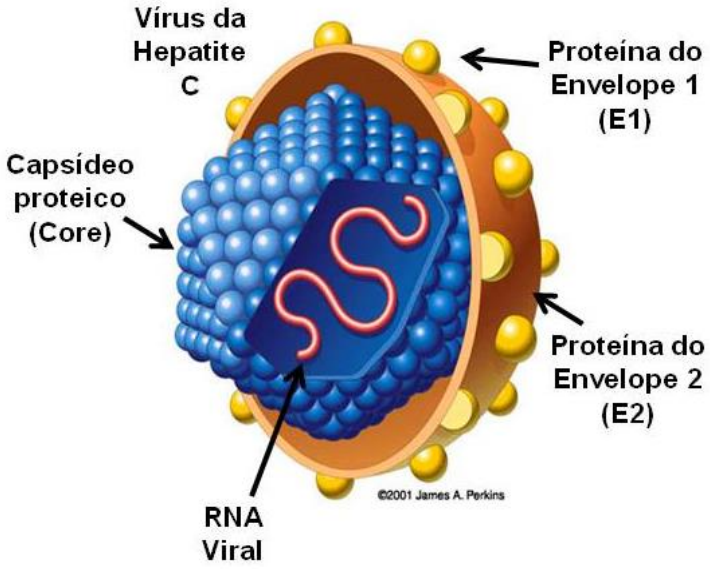
1.2. O Vírus da Hepatite C

O HCV foi identificado pela primeira vez como agente causador da Hepatite C em 1989 por Choo e colaboradores (CHOO et al., 1989), o qual era até então conhecido como vírus da Hepatite não-A e não-B.

Pertencente ao gênero *Hepacivirus* da família Flaviviridae, o HCV possui como material genômico uma fita simples de RNA de polaridade positiva de aproximadamente 9600 nucleotídeos (DUBUISSON; COSSET, 2014; SIMMONDS et al., 2005). A partícula viral mede aproximadamente 50 nm, e é formada por um envelope viral derivado das membranas do hospedeiro, no qual estão inseridas as glicoproteínas E1 e E2 do vírus, um capsídeo proteico formado pelas proteínas do Core, e o genoma viral (Figura 1A). O RNA viral é traduzido em uma poliproteína precursora de 3000 aminoácidos, que é clivada por proteases virais e do hospedeiro em 10 proteínas virais estruturais e não estruturais. Entre as proteínas estruturais estão as glicoproteínas do envelope E1 e E2, as do capsídeo e a proteína p7, enquanto entre as não estruturais estão as proteínas NS2, NS3, NS4A, NS4B, NS5A e NS5B (BARTENSCHLAGER; LOHMANN; PENIN, 2013; KAITO et al., 1994; TALWANI et al., 2012) (Figura 1B).

Figura 1: Partícula viral esquematizada, demonstrando as proteínas do core, do envelope (E1 e E2) e RNA viral – Adaptado de JAMES, 2001 (A). poliproteína viral codificada pelo HCV, demonstrando as proteínas estruturais e não estruturais do vírus – Adaptado de BARTENSCHLAGER et al., 2013 (B).

(A)



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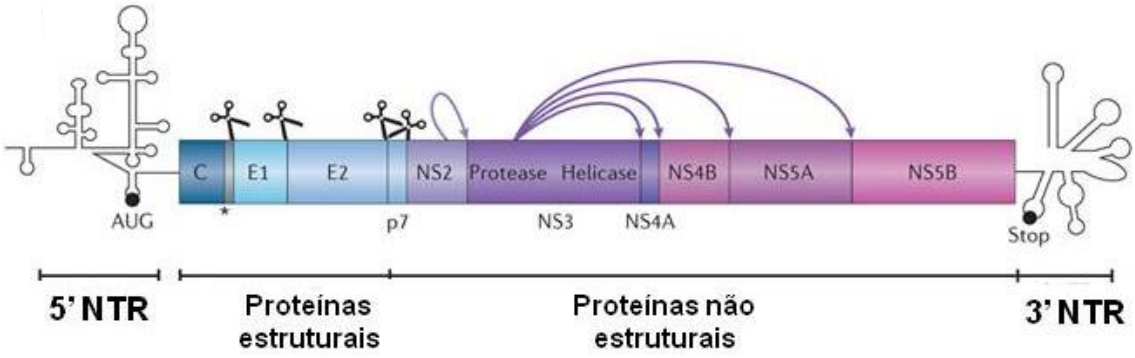


A proteína do capsídeo (C) encontra-se na região mais conservada do genoma viral e está relacionada com a composição do nucleocapsídeo viral. Outras funções atribuídas à proteína do Core inclui a modulação da transcrição de genes, supressão da resposta imune e proliferação celular (PENIN et al., 2004). Além disso, há evidências que o core pode se associar a gotículas lipídicas, sendo essencial para recrutar outros componentes virais importantes na montagem de novas partículas (DUBUISSON; COSSET, 2014).

As proteínas transmembrânicas E1 e E2 são as mais variáveis dentre as proteínas do HCV. São os principais alvos dos anticorpos neutralizantes e estão relacionadas com o reconhecimento das células hepáticas, pelas quais o vírus apresenta tropismo (SIMMONDS, 2013).

A viroporina p7 é uma proteína intrínseca de membrana, apresenta atividade cátion seletiva, sendo essencial para a liberação dos vírions das células hospedeiras. Juntamente às proteínas do core, E1, E2 e NS2, formam o módulo de montagem das partículas virais (BARTENSCHLAGER; LOHMANN; PENIN, 2013).

As proteínas não estruturais apresentam diferentes funções, estando associadas principalmente ao processamento da poliproteína precursora viral, desenrolamento da dupla fita de RNA intermediária, e patogênese viral. Estas proteínas também formam o complexo de replicação viral (COUNIHAN; RAWLINSON; LINDENBACH, 2011).

A primeira proteína não estrutural NS2 está relacionada à maturação de outras proteínas virais, e juntamente com a NS3 formam uma cisteino-protease responsável pela clivagem da região NS2/NS3 (DUBUISSON; COSSET, 2014; LINDENBACH; RICE, 2005).

A NS3 é uma proteína multifuncional, a qual apresenta um domínio N-terminal serino protease e um domínio C-terminal RNA helicase/NTPase. Não apresenta um domínio transmembrânico, mas há uma associação

desta proteína com a NS4A, que funciona como um cofator. Quando isto ocorre, a NS3 é encontrada associada ao retículo endoplasmático rugoso (RE), atuando de maneira importante para formar o complexo replicativo viral (MUKHERJEE et al., 2012; ROMANO et al., 2012).

A proteína NS4A apresenta 54 aminoácidos e, além de atuar como cofator da NS3, interage com outras proteínas, contribuindo na replicação do RNA e produção de novas partículas virais (PHAN et al., 2011). Já a região NS4B possui atividade de GTPase e ATPase, estando também relacionada com a hiperfosforilação da NS5A e induzindo o RE na formação de uma estrutura denominada rede membranosa, que facilita a síntese da fita de RNA positiva (PHAN et al., 2011).

Com aproximadamente 450 aminoácidos, a proteína NS5A é composta por uma α-hélice anfipática N-terminal e mais 3 domínios (MACDONALD et al., 2004). Esta proteína é de fundamental importância no ciclo replicativo viral, regulando várias etapas do processo replicativo, auxiliando na replicação e montagem, e interagindo tanto com as proteínas do hospedeiro como com outras proteínas virais (FRIDELL et al., 2011; LIM et al., 2012). Com isso, esta proteína é um dos principais alvos na busca de novos tratamentos contra o HCV (NGUYEN et al., 2011; VERDEGEM et al., 2011).

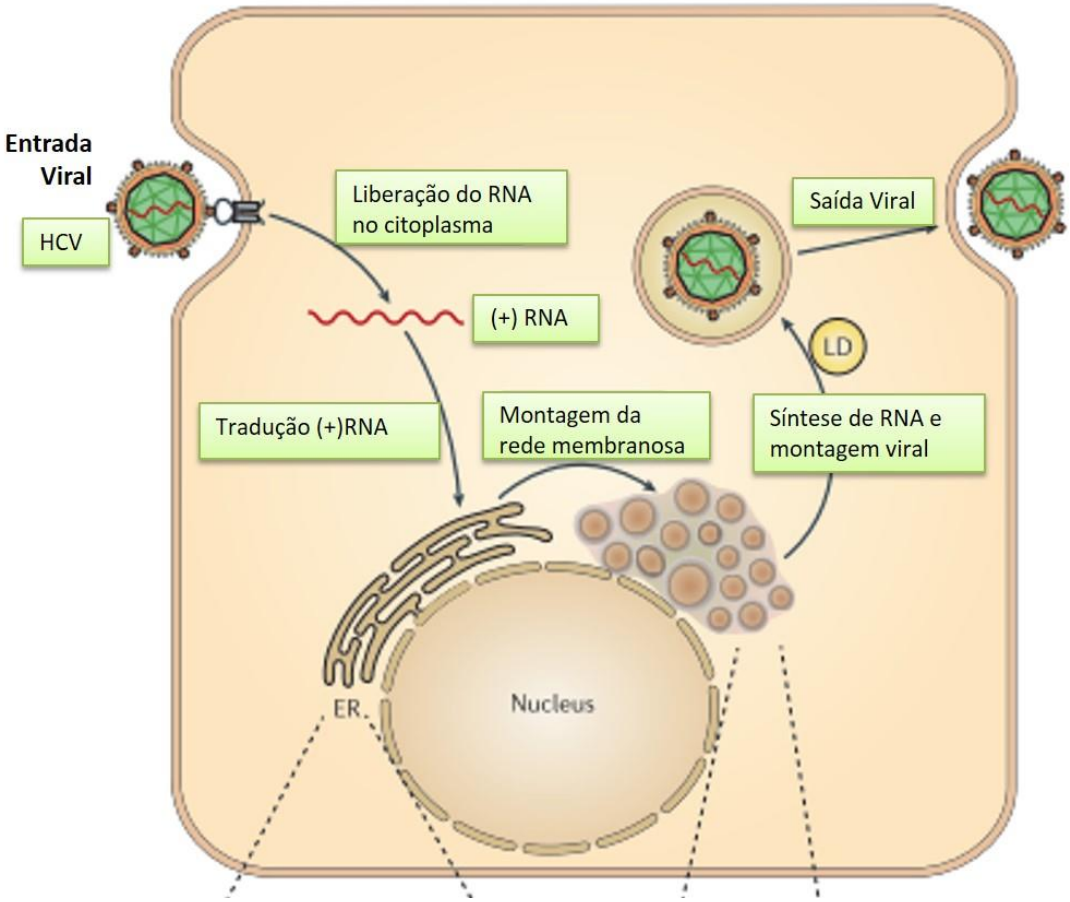
A proteína NS5B atua como uma RNA polimerase dependente de RNA, e está relacionada diretamente com a replicação do material genético viral que dará origem a novos genomas virais, e estes constituirão juntamente às proteínas estruturais, novos vírions (CHEN et al., 2012; QIU et al., 2011).

1.2.1 Ciclo replicativo do HCV

As partículas virais do HCV estão associadas a lipoproteínas de baixa e muito baixa densidade (low-density – LDLs e very-low-density

lipoproteins – VLDLs), e esta associação está relacionada à infectividade, indicando a contribuição dos componentes lipoproteicos para a entrada eficiente do HCV nas células hospedeiras (ANDRÉ et al., 2002; BARTENSCHLAGER et al., 2011; LINDENBACH; RICE, 2013). O primeiro estágio da infecção pelo HCV consiste da ligação do vírus à superfície da célula hospedeira, pela interação específica entre as glicoproteínas do envelope viral e receptores celular como CD81, SRB1, CLDN1 e OCLN, seguido da endocitose mediada por clatrina (ROUILLE et al., 2006; ZEISEL; FELMLEE; BAUMERT, 2013). Após a entrada do vírus e liberação do material genético, o sítio interno de entrada ribossomal (Internal ribosome entry site - IRES) promove a iniciação da tradução da poliproteína, que é seguido pelo processamento das proteínas virais. As proteínas não estruturais se associam do lado citoplasmático da membrana do RE onde interagem entre si e com as proteínas hospedeiras para formar a maquinaria de replicação viral. Essa maquinaria usa seu próprio genoma como molde para transcrição de fita complementar negativa de RNA. A fita negativa ou dupla fita, por sua vez, serve como uma molécula replicativa intermediária na síntese de novas moléculas de RNA de polaridade positiva que podem ser usadas para tradução, replicação ou então serem empacotadas para constituir novos vírions (BARTENSCHLAGER; LOHMANN; PENIN, 2013; DUBUISSON; COSSET, 2014). As proteínas estruturais se associam (core) ou se integram com a membrana do retículo endoplasmático (RE) (E1, E2 e p7) e formam oligômeros funcionais que promoverão a montagem das novas partículas virais. O envelope viral é adquirido por brotamento na membrana do RE, processo que parece estar associado à maquinaria de VLDL, e as novas partículas virais são liberadas (BERGER et al., 2012; COUNIHAN; RAWLINSON; LINDENBACH, 2011; HUANG et al., 2007). O esquema do ciclo replicativo do HCV é representado na figura 2.

Figura 2: Ciclo de replicativo do HCV. A fita de RNA positivo é liberada no citoplasma, interagindo com o reticulo endoplasmático (RE) para a tradução da fita e formação dos complexos replicativos, estes complexos se associam com membranas derivadas do RE formando a rede membranosa. Para a formação de novos vírions o (+)RNA é direcionados para gotículas lipídicas (LD) para serem empacotados e depois liberados. Adaptado de Li et al., 2015



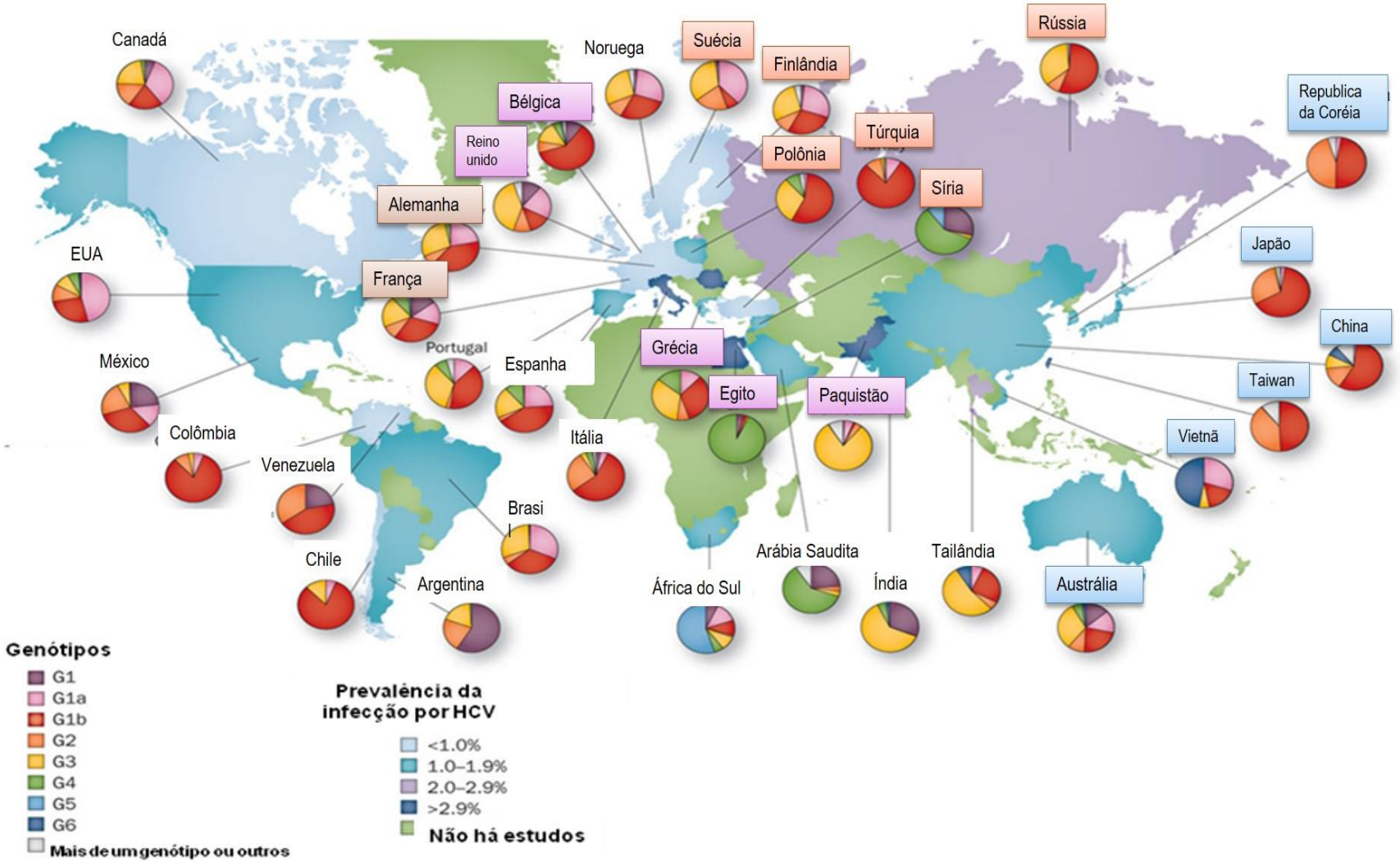
1.3. Epidemiologia e transmissão

A distribuição do HCV é bem ampla. Sua maior prevalência está localizada na África e no Oriente Médio, podendo chegar em até 10%. Além disso, a região asiática em números absolutos apresenta aproximadamente 50% da população mundial de infectados pelo HCV. (HAJARIZADEH; GREBELY; DORE, 2013) (Figura 3).

A principal via de transmissão do HCV é por exposição percutânea de sangue e derivados do plasma, sendo a utilização de agulhas e seringas contaminadas uma das principais fontes de transmissão, principalmente em usuários de drogas (WHO, 2015). Até o final da década de 80, quando o HCV ainda não havia sido identificado, a principal via de transmissão ocorria por transfusões sanguíneas. Em países desenvolvidos, o aumento da incidência do HCV está relacionado há um aumento do número de usuários de drogas (HAJARIZADEH; GREBELY; DORE, 2013; WANDELER et al., 2015).

A transmissão por meios de procedimentos como a colocação piercings, tatuagens, agulhas de acupuntura e técnicas utilizadas por manicure e pedicure também foram documentadas como fator de risco para infecção por HCV (CONITEC, 2015; LEMOS et al., 2014). Há também relatos de transmissão perinatal do HCV entre crianças recém-nascidas e mães infectadas (ATTALLAH et al., 2015; GARCIA-TEJEDOR et al., 2015; OHTO et al., 1994). Existem poucas evidências de que o semêm, saliva, lágrimas e urina estejam relacionados com a transmissão do vírus, embora o RNA viral tenha sido encontrado nesses fluídos (SIMMONDS, 2013).

Figura 3. Prevalência da infecção e distribuição mundial dos genótipos do HCV. Adaptado de John Wiley & Negro (2011); Hajarizadeh (2013)



1.4. Diversidade Genética do HCV

O HCV apresenta alta variabilidade genética, sendo possível diferenciar 7 genótipos, com aproximadamente 30% de diferenças entre as sequências nucleotídicas (MURPHY et al., 2007). Segundo um estudo realizado por SIMMONDS (2013), os genótipos 1, 2 e 3 apresentam uma distribuição mais ampla, enquanto os demais estão relacionados a regiões geográficas específicas. O genótipo 1 é o mais abrangente geograficamente, sendo o mais comum na América do Norte, no Oeste e Norte da Europa, América do Sul, Ásia e Austrália (HAJARIZADEH; GREBELY; DORE, 2013). O genótipo 2 é mais comum no oeste da África, o genótipo 3 no sul e sudeste asiático, o genótipo 4 na África central e Oriente Médio, o genótipo 5 é quase exclusivamente encontrado no sul da África e o genótipo 6 no sudeste asiático. O genótipo 7 foi descoberto recentemente (MURPHY et al., 1996; SIMMONDS, 2004, 2013) (Figura 3).

Estes 7 genótipos estão divididos em 70 subtipos que apresentam uma variação de 20 a 25% nas sequências nucleotídicas, sendo os subtipos mais frequentes são 1a, 1b, 2a, 2b, 3a, 4a e 6a (SIMMONDS, 2013). Existem também variantes genéticas do vírus denominadas quasiespécies, que estão altamente relacionadas entre si, diferindo em menos de 10% do genoma viral (DOMINGO et al., 2006; MARTELL et al., 1992; ZHOU et al., 2007).

A variação nas quasispecies representa um grande problema para os indivíduos infectados, devido às implicações do potencial adaptativo do HCV (GALE; FOY, 2005). Tal variabilidade genética, juntamente a outros fatores virais e do hospedeiro, pode estar relacionada aos diferentes níveis de virulência, uma vez que alguns genótipos parecem estar associados a patologias mais graves, e também a maiores taxas de escape, podendo levar à resistência ao tratamento (THIMME; BINDER; BARTENSCHLAGER, 2012).

1.5. Tratamento

Não existe atualmente uma vacina contra o HCV, os estudos para o desenvolvimento de vacinas e outras abordagens como o uso de RNAs de interferência, apesar dos grandes avanços, ainda se encontra em fases de estudo, não sendo aprovada a utilização em humanos (JAHAN et al., 2011; KHALIQ et al., 2010; SWADLING; KLENERMAN; BARNES, 2013).

Os métodos utilizados no tratamento não apresentam resultados eficazes para todos os pacientes tratados (POVEDA et al., 2014). O Interferon-α (IFN-α) foi o primeiro medicamento utilizado no tratamento de pacientes com hepatite C crônica, mas foi efetivo apenas em uma parcela dos indivíduos (JAECKEL et al., 2001). Com a introdução da ribavirina, o tratamento combinado apresentou uma taxa de resposta virológica sustentada (RVS) de 35 a 40 %, sendo de 29 % para pacientes infectados com o genótipo 1 após 48 semanas de terapia, e 70 % para os genótipos 2 e 3 após 24 semanas de tratamento (DEUTSCH; HADZIYANNIS, 2008; JAECKEL et al., 2001; POYNARD et al., 1998, 2003).

Posteriormente, sugiram tratamentos à base de Interferons modificados pela adição de uma molécula de polietileno glicol (PEG-IFN). A adição dessa molécula retarda a eliminação do IFN possibilitando que esse se mantenha em uma concentração estável no sangue, e juntamente com a ribavirina, apresentaram melhores resultados. Em estudos com pacientes cronicamente infectados, a taxa de RVS foi de 76 a 84 % em pacientes com infecção pelo genótipo 2 ou 3, e de 42 a 52% em pacientes infectados com HCV do genótipo 1 (KLENERMAN; FLEMING; BARNES, 2009; ZEUZEM, 2008).

Na busca por tratamentos mais eficientes contra o HCV, o uso de agentes antivirais de ação direta (DAAs), que tem como alvo as proteínas virais como a NS5A e NS3-4A, demonstraram um potencial na inibição da replicação viral (WOHLFARTH; EFFERTH, 2009). Em 2011, a agência reguladora de medicamentos dos Estados Unidos (FDA - Food and Drug

Administration) aprovou o uso dos inibidores de protease NS3-4A, Boceprevir e o Telaprevir, associados à terapia com interferon e ribavirina no tratamento de pacientes do genótipo 1, elevando para 90% a taxa de RVS (NAGGIE, 2012).

No Brasil, o governo aprovou recentemente a utilização dos DAAs de segunda geração Simeprevir e Daclastavir para o tratamento da hepatite C. Estes DAAs estão sendo distribuídos desde outubro de 2015 para o tratamento no Sistema Único de Saúde (SUS) (CONITEC, 2015). A segunda geração de DAAs apresenta efeitos colaterais mais brandos e um menor tempo de tratamento (WYLES, 2013). Entretanto, a combinação desses antivirais empregados no tratamento dependerá dos genótipos dos pacientes e, em alguns casos, poderão ser adicionais à terapia com interferon e ribavirina (CONITEC, 2015).

Apesar dos tratamentos mais recentes baseados em DAAs apresentarem melhores taxas de RSV e uma duração menor de tratamento (aproximadamente 12 semanas), alguns estudos já demonstraram que mutações específicas podem conferir resistência viral a estes tratamentos (BARTH, 2015; POVEDA et al., 2014; THOMPSON; LOCARNINI; BEARD, 2011). Além disso, apresentam custos de produção muito elevados, mesmo com a diminuição do tratamento para 12 semanas, limitando o uso em países subdesenvolvidos. Estes países ainda utilizam terapias como o Interferon convencional combinado à ribavirina para tratar pacientes infectados com genótipos menos agressivos, sendo os tratamentos mais recentes aplicados apenas para o genótipo 1. Adicionalmente, os tratamentos existentes também demonstram uma série de efeitos colaterais para os pacientes tratados. Tais fatos tornam necessária a busca por novos métodos de intervenção para o tratamento da Hepatite C.

1.6. Compostos naturais com potencial antiviral

Apesar dos recentes avanços no tratamento para Hepatite C, ainda existem muitos desafios a serem vencidos. É inerente a necessidade do desenvolvimento de novas terapêuticas, e neste contexto, compostos naturais podem servir como alternativas para o desenvolvimento de novas abordagens anti-HCV. Muitos trabalhos reportaram o uso de compostos extraídos de fontes naturais animais e vegetais como possíveis agentes terapêuticos, inclusive com atividade antiviral contra o HCV (JIN et al., 2008; MATSUMOTO et al., 2013; YAN et al., 2011); DABBOUSEH; JENSEN, 2013).

1.6.1. Toxinas animais

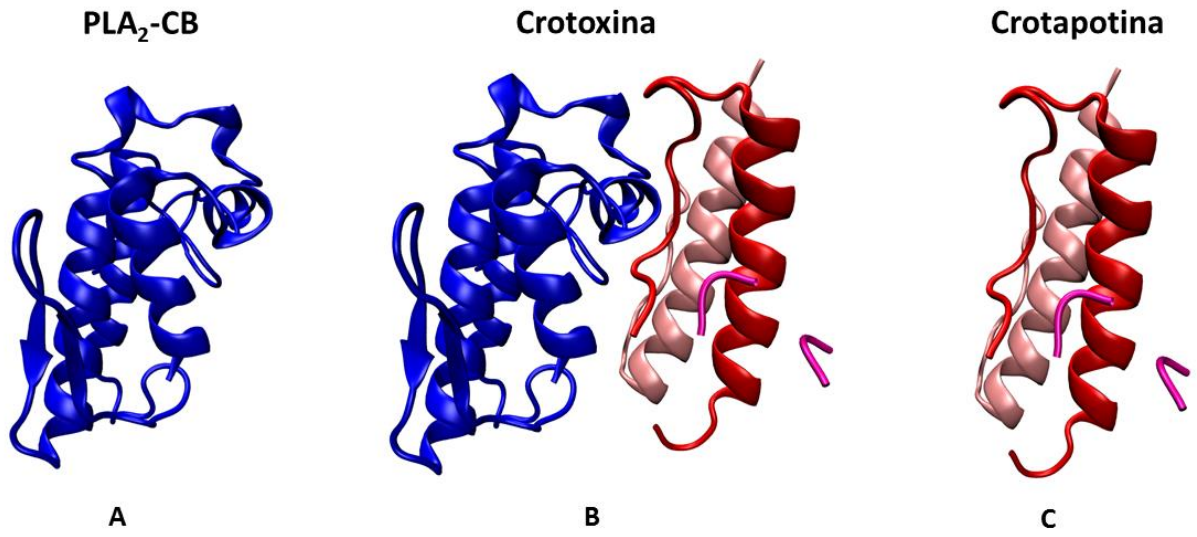
Toxinas isoladas de animais como serpentes peçonhentas vêm sendo amplamente estudadas com relação a suas aplicações (BISWAS et al., 2012; BORDON et al., 2012; KOH; KINI, 2012; MEENAKSHISUNDARAM; SWENI; THIRUMALAIKOLUNDUSUBRAMANIAN, 2009; MULLER et al., 2014; SANT'ANA et al., 2008). Venenos de serpente contém uma mistura de compostos bioativos como proteínas e polipeptídios que apresentam uma alta atividade metabólica. Estes compostos podem existir na forma de monônеros ou formar complexos (FAURE; SAUL, 2012; LEE, 1972).

O primeiro componente purificado e cristalografado de um veneno animal foi a crotoxina, um componente do veneno de *Crotalus durissus terrificus* (Slotta K, 1938). Esta proteína é formada por um complexo heterodimérico que apresenta atividades imunomoduladora, anti-inflamatoria, antitumoral, antimicrobiana e analgésica documentadas (Yan, 2006; Zhu, et al., 2008; Sampaio, 2006; Sampaio, 2010). Outros complexos isolados de venenos de serpente também vêm sendo estudados quanto a suas atividades (Doley, 2009). Alguns destes, inclusive a crotoxina, vêm

sendo investigados quanto a sua atividade antiviral (CECILIO et al., 2013; MARCUSSI et al., 2011; MEENAKSHISUNDARAM; SWENI; THIRUMALAIKOLUNDUSUBRAMANIAN, 2009).

Dennis et al. (2011) demonstraram que a crotoxina (CX) é um heterodímero composto por subunidades, denominadas de crotapotina (CP) e fosfolipase A_2 (PLA_2-CB), ligadas não covalentemente (Figura 4). Podem ser encontradas 4 isoformas de cada subunidade, que diferem na sequência dos aminoácidos apenas em 8 resíduos, sendo então a CX uma mistura de variantes formadas pela combinação das diferentes isoformas. A subunidade PLA_2-CB apresenta um caráter básico e peso molecular de aproximadamente 16.400 Da. Dentre os vários tipos de fosfolipases, a PLA_2-CB trata-se de uma fosfolipase secretada ($sPLA_2$), do grupo GIIA, pertencente a super família das fosfolipases A_2 (FAURE et al., 1993; KINI, 2003; DENNIS et al., 2011). Já a subunidade CP apresenta caráter ácido, com peso molecular de aproximadamente 9.000 Da. É formada por três cadeias polipeptídicas (α, β, γ) unidas por ligações dissulfeto, originadas da clivagem de seu precursor, a pró-crotapotina. A CP age com uma chaperona impedindo a subunidade PLA_2-CB de se ligar a sítios não específicos, ao mesmo tempo em que a direciona para os sítios alvo (DOLEY; KINI, 2009; HENDON; FRAENKEL-CONRAT, 1971; MULLER et al., 2012). Estudos com o complexo CX e suas subunidades, realizados por Radvanyi et al., em 1985, concluíram que a subunidade PLA_2-CB só foi tóxica quando combinada com outra subunidade PLA_2-CB, formando um dímero PLA_2-CB-PLA_2-CB.

Figura 4. Estrutura em cristal do complexo crotoxina do veneno de *Crotalus durissus terrificus*. A subunidade básica (PLA_2-CB) é mostrada em azul (A). O complexo crotoxina com suas duas subunidades (B). A subunidade ácida (crotapotina) é mostrada em vermelho, rosa e rosa claro (PDB, 3R0L).



Dentre a diversidade de aplicações para compostos extraídos de animais, foi identificada atividade inibitória no ciclo replicativo de diferentes vírus, apresentando uma taxa considerável de inibição da replicação de vírus da família Flaviviridae (MULLER et al., 2012; PARIDA et al., 2002; YAN et al., 2011). O complexo CX demonstrou efeito inibitório contra vírus da febre amarela e o da dengue (MULLER et al., 2012). Alguns compostos encontrados em venenos animais como a fosfolipase A2 (PLA-2) (FENARD et al., 1999) e a Mucroporin-M1 também demonstraram atividade inibitória contra HCV, vírus da Imunodeficiência Humana (HIV), coronavírus associado à síndrome respiratória aguda grave (SARS-CoV), vírus da influenza aviária (H5N1) e vírus do sarampo (LI et al., 2011; PETRICEVICH; MENDONÇA, 2003; XING et al., 2012; YAN et al., 2011).

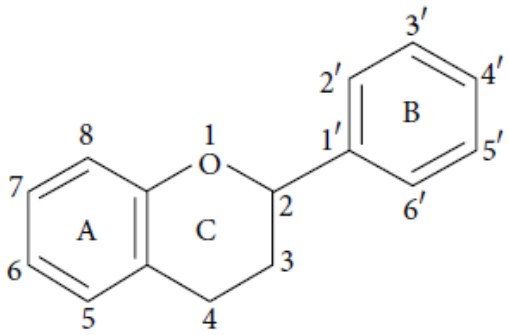
Com isso, a utilização de toxinas extraídas de serpentes peçonhentas como alternativa para o desenvolvimento de novos antivirais vem sendo estudadas para outros vírus. Porém, pouco se sabe sobre a ação destas toxinas no ciclo replicativo do HCV.

1.6.2. Flavonoides

Os flavonoides são uma classe importante de compostos naturais que apresentam vários benefícios à saúde, sendo utilizados na medicina. Muitos são descritos na literatura com atividade antioxidante (LEOPOLDINI et al., 2006), anti-inflamatória (SERAFINI; PELUSO; RAGUZZINI, 2010), hepatoprotetora (ZHU et al., 2012), anticancerígena (YI et al., 2005), e atividade antiviral, como a silibina (BLAISING et al., 2013), a epigalo-catequina-3-galato (CALLAND et al., 2012a), e a naringenina (GOLDWASSER et al., 2011; LYU; RHIM; PARK, 2005; NAHMIAS et al., 2008).

Quimicamente os flavonoides são compostos por um esqueleto de 15 carbonos, consistindo de 2 anéis benzênicos (A e B) ligados via anel pirano heterocíclico (C) (Figura 5). As várias classes de flavonoides diferem no nível de oxidação e no padrão de substituição do anel C (MIDDLETON, 1998).

Figura 5. Estrutura básica de um flavonoide. Fonte: KUMAR; PANDEY, 2013

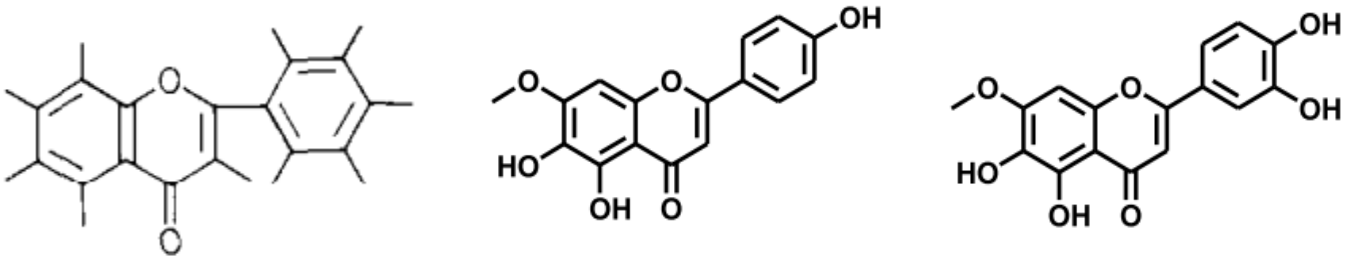


Dentro da classe dos flavonoides existe a subclasse das flavonas (Figura 6A), a qual demonstrou apresentar atividades antioxidante (FERNANDES et al., 2008), antibacteriana contra *Helicobacter pylori* (ISOBE et al., 2006), proteção contra danos ao DNA e inibição da

peroxidação lipídica (LIU et al., 2006). E em estudos computacionais apresentou atividade antiviral contra integrasse de HIV-1 (LAMEIRA et al., 2006) e anti-Picornavirus (SOUZA et al., 2004).

As flavonas pedalitina (Figura 6B) e sorbifolina (Figura 6C) são metabólitos secundários que pertencem à classe dos flavonoides, já isoladas em algumas espécies de plantas, como *Ruellia tuberosa* (LIN et al., 2006), *Mentha pulegium* e *Mentha suaveolens* (ZAIDI et al., 1998b). São descritas na literatura com atividades antibacteriana (ISOBE; NAGATA, 2010), antioxidante (FERNANDES et al., 2008; MASUOKA; ISOBE; KUBO, 2006) e anticancerígena (NITODA; ISOBE; KUBO, 2008), demonstrando um grande potencial de atividades biológicas. Porém, ainda não são descritas na literatura atividades antivirais para estes compostos.

Figura 6. Estrutura básica de uma flavona (A). Estrutura da Pedalitina (B) e Sorbifolina (C). Adaptado de HAVSTEEN, 1983; REGASINI,2015.



A utilização de medicamentos à base de plantas é uma prática antiga, sendo que muitos compostos naturais e seus derivados vêm sendo utilizados como medicamentos (BALUNAS; KINGHORN, 2005). O ácido acetil-salicilíco e a penicilina são exemplos clássicos desse uso (BUTLER, 2004).

Muitos compostos naturais extraídos de plantas são descritos com enorme potencial terapêutico, apresentando diversas atividades como anticâncer (COSTA-LOTUFO et al., 2010), antimalárica (VAN AGTMAEL; EGGELTE; VAN BOXTEL, 1999) e antidepressiva (CÍCERO BEZERRA FELIPE et al., 2007). Também são descritos na literatura compostos com uma taxa considerável de inibição da replicação de vírus da família Flaviviridae, incluindo a atividade anti-HCV (BALUNAS; KINGHORN, 2005; PARIDA et al., 2002). Jardim *et al.,* identificou uma potente atividade antiviral em compostos extraídos de plantas brasileiras sobre a replicação do HCV (JARDIM et al., 2015), corroborando com outros trabalhos, e demonstrando que a utilização de flavonoides extraídos de plantas como alternativa na terapia para o tratamento do HCV vem sendo amplamente estudada (CALLAND et al., 2012b).

Cerca de 40 novos medicamentos de origem natural foram lançados no mercado entre os anos de 2000 e 2010 (BRAHMACHARI, 2012). Neste contexto, a utilização de compostos naturais de fontes animais ou vegetais já demonstrou diversas atividades descritas na literatura, dentre elas a atividade antiviral, podendo ser considerados fontes promissoras para descobertas de futuros antivirais e resultar na melhoria do tratamento da Hepatite C.

2. OBJETIVOS

Avaliar a atividade antiviral de compostos naturais derivados de origem animal e vegetal no ciclo replicativo do HCV *in vitro*.

2.1. Objetivos específicos

- Avaliar a atividade anti-HCV do complexo CX, e das subunidades PLA_2-CB e CP, isolados do veneno da serpente *Crotalus durissus terrificus*;
- Analisar a atividade dos compostos sorbifolina (PN2) e pedalitina (F18), isolados da planta *Pterogyne nitens* no ciclo replicativo do HCV*;*
- Investigar quais etapas do ciclo replicativo do HCV estão sendo inibidas pelo tratamento com tais compostos naturais, e o possível mecanismo de ação desses compostos.

CAPÍTULO II:

MULTIPLE EFFECTS OF TOXINS ISOLATED FROM *CROTALUS DURISSUS TERRIFICUS* ON THE HEPATITIS C VIRUS LIFE CYCLE

*Este capítulo está formatado como manuscrito, com algumas alterações estruturais para melhor se adequar ao formato da dissertação. Este trabalho foi submetido à revista "Journal of Virology".

MULTIPLE EFFECTS OF TOXINS ISOLATED FROM CROTALUS DURISSUS TERRIFICUS ON THE HEPATITIS C VIRUS LIFE CYCLE

Shimizu, J.F.*[1]; Batista, M. N.[1]; Campos, G. R.F[1]; Bittar, C. [1]; Cintra, A.C.O.[2]; Sampaio, S.V.[2]; Aquino, V.H.[3]; Rahal, P.[1]; Jardim, A.C.G.[4].

Contact details:

Jacqueline Farinha Shimizu[1]
Email: jacquelineshimizu@gmail.com

Mariana Nogueira Batista[1]
Email: batista_m.n@hotmail.com

Guilherme Rodrigues Fernandes Campos[1]
Email: guilhermecampos07@gmail.com

Cintia Bittar[1]
Email: cibittar@gmail.com

Adélia Cristina Oliveira Cintra[2]
Email: acocintra@hotmail.com

Suely Vilela Sampaio[2]
Email: suvilela@usp.br

Victor Hugo Aquino[3]
Email: vhugo@fcfrp.usp.br

Paula Rahal[1]
Email: rahalp@yahoo.com.br

Ana Carolina Gomes Jardim*[1,4]
Email: caroljardim@gmail.com

[1]Genomics Study Laboratory, São Paulo State University, IBILCE, S. José do Rio Preto, SP, Brazil

[2]Laboratory of Virology, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, SP, Brazil

[3]Laboratory of Toxinology, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, SP, Brazil

[4]Laboratory of Virology, Institute of Biomedical Science, ICBIM, Federal University of Uberlândia, Uberlândia, MG, Brazil

*Corresponding author. Tel.: +55 (34) 3225-8679

Address: Avenida Amazonas, Bloco 4C – sala 216. Umuarama. Uberlândia, MG, Brazil, CEP: 38405-302

E-mail address: acgjardim@icbim.ufu.br

ABSTRACT

Hepatitis C virus (HCV) is one of the main causes of liver disease and transplantation worldwide. The new direct-acting antivirals (DAAs), such as the NS3 protease inhibitors, have improved therapeutic options. However, DAAs therapy is expensive, presents additional side effects and viral resistance has been described. Therefore, studies for developing more efficient antivirals against HCV are needed. In this context, natural sources can provide an alternate approach for the identification of products with therapeutic potential. 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 in 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. These toxins could be used as components of new anti-HCV therapies by their potent and broadly activity, or as a template to the development of new antivirals.

Keywords: Hepatitis C Virus, *Crotalus durissus terrificus*, antiviral, natural compounds.

1. 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 (WANDELER; BRUGGMANN; RAUCH, 2015). Approximately 130 -150 million people are chronically infected worldwide (WHO, 2015).

HCV is grouped into the genus *Hepacivirus* within the family *Flaviviridae.* Virions are enveloped and present and a single stranded positive-sense RNA genome surrounded by a proteic capsid (BARTENSCHLAGER; LOHMANN; PENIN, 2013a). There is no vaccine for preventing HCV infection and the current interferon-based treatment is not effective for all treated patients (HOUGHTON, 2011).

Recently, the addition of the direct-acting antiviral agents (DAAs) which target viral proteins such as NS5A and NS3-4A to the standard therapy increased the sustained virological response (SVR) rates (WOHLFARTH; EFFERTH, 2009). However, the triple therapy based on pegylated-Interferon-alpha plus ribavirin and DAAs is expensive, limited by several side effects, and not effective for all treated patients (DORE, 2012; LIANG, 2013; PEARLMAN, 2012). Additionally, studies have demonstrated that specific mutations may confer viral resistance to its treatment (THOMPSON; LOCARNINI; BEARD, 2011). Therefore, the search for new therapeutics for the treatment of HCV infection is of great interest and could provide a substantial benefit to the global public health (TALWANI et al., 2012).

In this context, compounds extracted from natural sources have shown therapeutic potential for treating chronic hepatitis C (DABBOUSEH; JENSEN, 2013; JIN et al., 2008; YAN et al., 2011). Toxins isolated from animals as the poisonous snakes have been widely studied with respect to their applications, including antiviral properties (LI et al., 2011;

MEENAKSHISUNDARAM; SWENI; THIRUMALAIKOLUNDUSUBRAMANIAN, 2009b; MULLER et al., 2014; PETRICEVICH; MENDONÇA, 2003). Snake venoms are a mixture of bioactive compounds that possess numerous metabolic activities (BAILEY; WILCE, 2001). These compounds previously demonstrated to inhibit the life cycle of a range of viruses, including the *Flaviviridae* family. Components of snake venoms have shown antiviral activity against *Dengue virus* (DENV), *Yellow Fever virus* (YFV), *Oropouche virus* (OROV), *Mayaro virus* (MAYV) (MULLER et al., 2014, 2012), *Measles virus* (MeV) (PETRICEVICH; MENDONÇA, 2003) and *Human immunodeficiency virus* (HIV) (FENARD et al., 1999b; MEENAKSHISUNDARAM; SWENI; THIRUMALAIKOLUNDUSUBRAMANIAN, 2009a). Therefore, toxins isolated from venomous snakes may provide an alternative approach for the development of new antivirals.

In this study, we investigated the antiviral effects of the complex crotoxin and its subunits crotapotin and phospolipase A_2 isolated from the venom of *Crotalus durissus terrificus* (HENDON; FRAENKEL-CONRAT, 1971; MULLER et al., 2012) on HCV life cycle. The data obtained showed that these toxins can inhibit different stages of the viral replicative cycle.

2. MATERIAL AND METHODS

2.1.Toxins

Isolation and purification of the crotoxin complex (CX), and its subunits phospholipase A2 (PLA_2-CB) and crotapotin (CP) **(Figure 1)** were carried out as previously described (HENDON; FRAENKEL-CONRAT, 1971; MULLER et al., 2012).

Lyophilized toxins were dissolved in PBS (Phosphate-Buffered Saline), filtered and stored at -80°C. Compounds were diluted in complete medium immediately prior to the experiments.

2.2 Cell Culture

The human hepatoma cell line Huh-7.5 was grown in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, USA) supplemented with 100 U/mL penicillin (Gibco Life Technologies, USA), 100 mg/mL streptomycin (Gibco Life Technologies, USA), 1% non-essential amino acids (Gibco Life Technologies, USA), 1 % HEPES (Gibco Life Technologies, USA) and 10% fetal bovine serum (FBS; Cultilab, BR) at 37 ºC in a humidified 5% CO_2 incubator. The subgenomic replicon (SGR) cell line harboring genotype 2a SGR-Feo-JFH-1 (WYLES et al., 2009) was maintained in DMEM supplemented with 500 µg/mL G418 (Sigma-Aldrich, USA).

2.3 Cytotoxicity assay

Cytotoxicity of toxins was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich, USA) method (MOSMANN, 1983). Huh-7.5 or SGR-harboring cells were cultured in DMEM medium in a 96-well plate at a density of 5 x 10^3 per well and incubated at 37 ºC in a humidified 5% CO_2 incubator overnight. Two fold serial dilutions of toxins (100 to 1.56 µg/mL) were added to the cell culture. Cells treated with PBS were used as untreated control. After 48 h incubation at 37 ºC, DMEM containing MTT at the final concentration of 1 mg/mL was added to each well, incubated for 1 hour and replaced with 100 µl of DMSO to solubilize the formazan crystals. Surviving cells were measured by optical density (OD) at 562 nm, using a spectrophotometer. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration required to reduce the cell number by 50% compared to that for the untreated control. All experiments were performed in triplicates and repeated a minimum of three times.

2.4. Luciferase-based replication assay

Huh-7.5 cells stably harboring the SGR-Feo-JFH-1 were seeded into 96-well plates at a density of 5 x 10^3 per well and toxins at specific concentrations were added. After 48 h, cells were harvested by lysis with Passive Lysis Buffer (Promega, USA) and HCV RNA replication was quantified by measuring luminescence levels using the Luciferase Assay System (Promega, USA). The effective concentration of toxins that inhibit 50% (EC_{50}) of replication was calculated using GraphPad Prism software. Cytotoxicity assays were carried out in parallel to determine the CC_{50}, using a MTT-based system as described above. The values of CC_{50} and EC_{50} were used to calculate the selectivity index (SI = CC_{50}/EC_{50}).

2.5. Virus assays

JFH-1 HCVcc particles (WAKITA et al., 2005) were generated as described previously (JARDIM et al., 2015). Huh-7.5 cells were infected with virus at a multiplicity of infection (MOI) of 0.4 and toxins at 10 µg/mL were added at different time points, depending on the stage of HCV life cycle to be evaluated (as described below). To virus titration, HCVcc supernatants were 10-fold serial diluted in DMEM medium and used to infect Huh-7.5 cells. Cells were fixed with 4% paraformaldehyde (PFA) 48 h post-infection (hpi), washed with 100 mM Glicine (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 (MACDONALD et al., 2003) as primary antibody and anti-sheep IgG, Alexa Fluor 594 conjugated, as secondary antibody. Infectivity was expressed as focus-forming units per milliliter of supernatant (FFU/mL). All assays were performed in triplicates and repeated a minimum of three times.

2.5.1 Antiviral activity against HCV Replication

Huh-7.5 cells were seeded the day before the assay was carried out. Toxins were diluted to the stated final concentrations in DMEM media. Cells were infected with infectious supernatant for 4 hours, washed extensively with PBS to remove non-endocytosed virus particles and toxins were added. After 48 h intracellular virus was titrated. PBS was used as untreated control and cyclosporine A (CsA, Sigma-Aldrich) as control of inhibition of replication (CIESEK et al., 2009).

2.5.2. Inhibitory effects on Entry steps

Infectious supernatant containing JFH-1 HCVcc was used to infect naive Huh-7.5 cells in the presence of toxins for 4 h at 37°C. Cells were extensively washed with PBS and replaced by fresh complete medium.

For virucidal assay, infectious supernatant was prior incubated with toxins for 1 h at 37 °C and then used to infect naive Huh-7.5 cells. Virus and toxin were incubated with cells for 4 h at 37 °C. The inoculum was removed; cells were washed three times with PBS to completely remove virus and toxins, and replaced by fresh media.

For both assays, virus was titrated 48 hpi. PBS and (-)-epigallocatechin gallate (EGCG, Sigma-Aldrich, USA) (CALLAND et al., 2012a) were used as controls.

2.6. Pre-treatment assay

Huh-7.5 cells were incubated with toxins for 1 hour at 37 °C in a humidified 5% prior to infection. After incubation, cells were washed extensively and incubated with HCVcc JFH-1 virus for 4 h. Infectious supernatant was removed, additional washes were performed to completely

remove non-endocytosed virus and fresh media was added. Virus was titrated 48 hpi as described above. PBS and EGCG were used as controls.

2.7. Toxins activity on viral release

Huh-7.5 cells infected with HCVcc JFH-1 were seed 48 h prior the treatment. Then, fresh media with toxins at 10 µg/mL was added following a previously described protocol (NAHMIAS et al., 2008). The plate was gently agitated 24 h post-treatment to mechanically release particles bound to cells and the supernatant was collected, filtered, and stored at - 80°C. Intracellular RNA was also extracted by using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and stored at - 80°C. Both procedures followed Nahmias et al. (2008) protocol for HCV secretion analysis.

Extracted RNA was used on cDNA synthesis using High-Capacity cDNA Archieve (Applied Biosystems, Foster City, CA, USA). HCV expression analysis was performed for detection of the HCV5'UTR region using TaqMan Universal PCR Master Mix no AmpErase UNG (Applied Biosystems, Branchburg, NJ, USA). The amplification of the endogenous gene GAPDH was used to normalize levels of expression. PBS was used as negative control and naringenin (NR) at 400 µM was used as positive control of HCV secretion inhibition.

2.8. dsRNA Intercalation assay

A dsRNA intercalation assay was performed based on the protocol described by (KRAWCZYK et al., 2009). HCV JFH-1 3' untranslated region (UTR) (accession no. AB047639) was amplified by a PCR reaction using specific primers flanked by T7 promoter region (forward: 5´TAATACGACTCACTATAGGGGGCACACACTAGGTACA3´; reverse: 5´TAATACGACTCACTATAGGGACATGATCTGCAGAGAG3´; T7 sequences are underlined). The PCR product of 273 bp was purified using

the Zymoclean Gel DNA recovery Kit (Zymo Research) and used as template for *in vitro* transcription with the T7 RiboMAX™ Express Large Scale RNA Production System kit (Promega). The synthetized dsRNAs were treated with RNase A for 2 h in a 0.3 M NaCl solution and confirmed by RNA denaturation agarose gel (1%) analysis (RNase A does not cleave dsRNA in 0.3 M NaCl solution). To investigate the dsRNA intercalation properties of the compounds, 15 mM dsRNA were incubated with each toxin at 10 µg/mL for 45 minutes and submitted to agarose gel analysis. PBS and Doxorubicin (DOX) at 400 µg/mL (KRAWCZYK et al., 2009) were used as negative and positive control, respectively.

2.9. Western blot analysis

Cells were lysed in CelLytic™ lysis buffer (Sigma-Aldrich) added of protease inhibitors (Sigma-Aldrich). Ten micrograms of protein were resolved by SDS/PAGE and transferred to a PVDF membrane. Membranes were blocked in 10% (w/v) dried skimmed milk powder in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Membranes were probed with anti-NS5A IgG (Macdonald et al., 2003) or mouse anti-GAPDH IgG (AbCam) in 5% (w/v) dried skimmed milk in TBS-T. The antibodies were detected with the secondary horseradish peroxidase-conjugated antibody and in-house enhanced chemiluminescent reagent.

2.10. Statistical analysis

Differences between means of readings were compared using analysis of variance (one-way or two-way ANOVA) or Student *t* test 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 toxins on HCV replication

To evaluate the potential effect of the CX, CP and PLA_2-CB toxins on HCV replication, we first used a subgenomic replicon system. Huh-7.5 cell line stably expressing SGR-FEO-JFH-1 (Huh-7.5-SGR-FEO-JFH-1) were treated with two fold serial dilutions (100 - 1.56 µg/mL) of each toxin for 48 h to assess the effect of these compounds on both HCV replication and cell viability **(Figure 2)**. The results showed that PLA_2-CB significantly ($p < 0.001$) inhibited HCV replication at non-cytotoxic concentrations (**Figure 2A**); while CX did not demonstrate any effect on SGR replication (**Figure 2B**). In contrast, cells treated with 100, 50 and 25 µg/mL of CP significantly ($p< 0.001$) increased HCV replication rates (**Figure 2C**). Treatment of cells with PLA_2-CB decreased HCV replication in a dose-dependent manner with EC_{50} of 6.08 µg/mL, CC_{50} of 17.84 µg/mL and a SI of 2.93 (**Figure 2D**). As shown in **Figure 2E**, expression of HCV NS5A protein was also gradually reduced in the presence of increasing non-cytotoxic doses of PLA_2-CB (20 - 4 µg/mL). Subsequent assays were performed with toxins at 10 µg/mL final concentration (favorable ratio of cytotoxicity to antiviral potency).

We next analyzed the effects of the toxins CX, CP and PLA_2-CB on genome replication in the context of full length virus. Huh-7.5 cells were infected with JFH-1 HCVcc at MOI 0.4 for 4 h, washed to remove non-endocytosed virus particle and added of toxins. Replication levels were assessed by the focus formation units assay 48 h post-infection. Consistent with the SGR data, PLA_2-CB effectively blocked ($p< 0.001$) virus replication **(Figure 3A)**. No significant differences on virus replication rates were observed by treating infected cells with CX or CP ($p< 0.001$). These results suggest that only the isolated form of PLA_2-CB is able to reduce HCV replication, whereas it has no effect on replication when associated with CP to form the CX complex.

In an attempt to elucidate the mechanism in which PLA_2-CB inhibits HCV replication, we performed a standard methodology to analyze the potential of the toxins to intercalate into dsRNA. As shown in figure 3B, PLA_2-CB strongly intercalated into dsRNA as observed by 86 % reduction of dsRNA stain by ethidium bromide **(Figure 3B)**. CX showed an intermediate capacity of intercalation (58 %); while CP demonstrated no intercalation property. The positive control doxorubicin presented 77% of intercalation to dsRNA and demonstrated to be less efficient than PLA_2-CB **(Figure 3B)**. These results suggest that the inhibition of replication by PLA_2-CB may be due to its dsRNA intercalation properties.

3.2. Crotoxin complex and its subunit PLA2-CB inhibited HCV entry

The effect of the toxins CX, CP and PLA_2-CB on different stages of HCV entry was investigated. First, Huh-7.5 cells were infected with JFH-1 HCVcc and toxins were immediately added. Cells were incubated with virus plus toxin inoculums for 4 h, washed to completely remove the inoculum and replaced with fresh medium for 48 h. Intracellular virus were titrated by using focus formation units assay. The results demonstrated that CX and PLA_2-CB were able to block 85% and 97.3% of HCV entry, respectively. Contradictory, CP showed no inhibitory effect on viral entry **(Figure 4A)**. It suggests that the inhibition of HCV entry might be related to the catalytic action of PLA_2-CB and the activity observed in CX treatment may be due to PLA_2-CB be part of CX complex.

To characterize the antiviral effect of CX and PLA_2-CB on virus entry, we next assessed the impact of these toxins on the HCV viral particle by performing a virucidal assay. JFH-1 HCVcc supernatant was incubated with each toxin for 1 h prior to the cells infection. The inoculum was then used to infect Huh-7.5 cells for 4 h. Cells were washed with PBS to remove the inoculum and replaced with fresh media. CX and PLA_2-CB demonstrated a significant virucidal activity, blocking 75.5% and 93% of virus entry,

respectively ($p < 0.0001$) **(Figure 4B)**. CP also demonstrated no virucidal activity on HCV virus. This result strongly suggests that the antiviral effect of CX and PLA_2-CB observed was due to a direct action of these toxins on the virus particle structure.

We further investigated whether the blockage of viral entry by the toxins is influenced by its activity on the host cells. Huh-7.5 cells were previously treated with toxins for 1 h and then infected with JFH-1 HCVcc virus for 4 h. Intracellular virus was titrated 48 hpi. The analysis showed that treatment with PLA_2-CB prior to infection significantly inhibited 73.5% of HCV infectivity **(Figure 4C)**. Therefore, PLA_2-CB may also act somehow on the host cells to decrease infectivity. In contrast, previous treatment on cells with CX and CP had not effect on HCV infectivity **(Figure 4C)**, suggesting that PLA_2-CB probably lacks the antiviral activity when associated with CP to form CX.

3.3. HCV release is inhibited by Crotapotin and Crotoxin but not by PLA2-CB

Finally, we investigated the effect of toxins on HCV release. HCVcc JFH-1 infected cells were treated with each toxin at 10 µg/mL for 24 hours. Supernatants were collected and cells were harvested, and viral RNA was quantified by qPCR. Intra and extracellular virus tires were analyzed in order to evaluate the amounts of virus produced (intracellular) and released (extracellular). Surprisingly, the results showed that CX and CP but not PLA_2-CB inhibited HCV release. The treatment with CP and CX reduced 50 and 78 % of HCV release, respectively **(Figure 5).** The reduction of both intra and extracellular viral RNA observed for PLA_2-CB was the consequence of its effect on HCV replication. Interestingly, the inhibitory action of CX on HCV release seems to be due to its subunit CP, and its effect it is not influenced by the interaction of subunits to form CX complex.

4. DISCUSSION

The current available therapy for Hepatitis C is not effective for all treated patients, is limited by side effects and is expensive. Therefore, there is an evident need to develop new therapeutic approaches that result in optimal response rates, milder side effects and lower cost of production.

Several studies have described compounds isolated from snake venoms with anti-bacterial, anti-inflammatory, anticancer and antiviral activity (CECILIO et al., 2013; PETRICEVICH; MENDONÇA, 2003; SANT'ANA et al., 2008; SLOTTA, K. H. FRAENKEL-CONRAT, 1938). Since the discovery of captopril, a potent anti-hypertensive drug based on snake venom protein (FERREIRA, 1965), many toxins are being investigated due to their therapeutic potential. Toxins isolated from *Crotalus durissus terrificus* were previously screened for their activity against other viruses of *Flaviviridae* family and demonstrated to be effective on blocking the early steps of the life cycle of these viruses (MULLER et al., 2012). CX was initially described with neurotoxic and myotoxic activity however over the years new activities like anti-inflammatory, antimicrobial and antitumor have been described for this complex (FAURE; BON, 1988; SAMPAIO et al., 2010).

In this study, we investigated the antiviral activities of the heterodimeric complex CX, and its subunits CP and PLA_2-CB, isolated from *Crotalus durissus terrificus* venom against HCV infection *in vitro*. We were able to demonstrate the multiple antiviral effects of these toxins which inhibited different stages of the HCV life cycle.

Our data showed that PLA_2-CB blocked viral entry by both the action on the virus particle and somehow on the host cells. It is consistent with findings of a previous study which demonstrated that the PLA_2-CB inhibits the early stages of replication cycle of two members of the *Flaviviridae* family, DENV and YFV (MULLER et al., 2012). The authors showed that PLA_2-CB reduced virus infection by acting on the host cells and/or on the viral particles.

PLA$_2$-CB is a phospholipase which belongs to the group II of secreted enzymes that hydrolyze glycerophospholipids at the sn-2 position, producing lysophospholipids and fatty acids (KINI, 2003). They represent a versatile class of enzymes which play a key role in various biological activities, such as lipid digestion, host defense and homeostasis of cellular membranes (DENNIS et al., 2011; KINI, 2003). More recently, the effect of PLA$_2$-CB against DENV was associated to its catalytic activity, inactivating virus particle probably by cleavage of glycerophospholipid of the virus envelope (MENJON MULLER, 2014). These results were also described for other enveloped virus such as *Rocio virus*, *Oropouche virus*, *Mayaro virus* (MULLER et al., 2014) and *Human Immunodeficiency Virus (*HIV) (MEENAKSHISUNDARAM; SWENI; THIRUMALAIKOLUNDUSUBRAMANIAN, 2009). Since HCV is an enveloped virus (BURLONE; BUDKOWSKA, 2009) and viral particles are also associated with lipoproteins (GASTAMINZA et al., 2008), it is plausible to suggest that the virucidal effect observed in our analysis could be due to the phospholipase activity attributed to PLA$_2$-CB.

Our results also demonstrated that PLA$_2$-CB reduced HCV replication in either subgenomic reporter SGR-Feo-JFH1 (EC_{50}= 6.08 µg/mL) or the full-length JFH1 systems. When the inhibitory effects were further investigated, we found that PLA$_2$-CB strongly intercalated into dsRNA, suggesting a possible mechanism of action in which PLA$_2$-CB inhibits HCV replication. The association of PLA$_2$-CB with CP decreased the intercalation property, consistent with the results observed for CX and CP. This result also could explain the discrete but not significant reduction of full length virus replication by CX when compared to CP treatment. This mode of actions was also previously described for other natural compounds as amidinoanthracyclines (KRAWCZYK et al., 2009) and acridones (STANKIEWICZ-DROGOŃ et al., 2010) against HCV.

Despite CX did not significantly inhibit HCV replication, this complex was effective in blocking the HCV entry and release stages. The observed data suggests that the effect on HCV entry is mainly due to its virucidal activity. CX is characterized as a heterodimeric protein complex consisting of two subunits, an acidic component (CP) and a basic subunit (PLA_2-CB) (FAURE; XU; SAUL, 2011; HENDON; FRAENKEL-CONRAT, 1971; SANTOS et al., 2007; SLOTTA, K. H. FRAENKEL-CONRAT, 1938). Therefore, the virucidal effect of CX is probably due to the presence of PLA_2-CB in this complex. Altogether, our data shows a stronger virucidal effect when cells are treated with the isolated form of PLA_2-CB, and a significant but reduced effect when it is in the complex form. This pattern of activity was similarly found concerning other biological activities of the CX. According to Sampaio *et al*., the enzymatic activity related to CX was also associated with the presence of the subunit PLA_2-CB (SAMPAIO et al., 2010).

Surprisingly, our data demonstrated that CP possesses inhibitory effect on HCV release. CP was initially described with no catalytical or cytotoxic activities and acts mainly preventing PLA_2-CB to perform unspecific interactions (BON, 1982; BREITHAUPT, 1976; FAURE; SAUL, 2012). This is the first report of CP inhibiting HCV life cycle, but the mechanism in which CP interferes on HCV release remains unclear. CP has already been described to possess anti-inflammatory and immunomodulatory properties (GARCIA et al., 2003; LANDUCCI et al., 2000), that could influence on the anti-HCV activity of this toxin. However, further analyses are need for a better understand of its action. We also believe that the inhibition of viral release by CX may be related to the presence of CP in the complex.

5. CONCLUSION

In summary, we demonstrated that toxins isolated from *Crotalus durissus terrificus* can inhibit different stages of HCV life cycle as entry, replication and release. The mechanisms of blocking HCV entry and replication seems to be by the direct action of PLA_2-CB on the viral particle and/or interference on the host cells, and by intercalating to the intermediate dsRNA formed during the replication process, respectively. CP was effective against HCV release and seems to be responsible for the antiviral effect caused by CX. These results may be useful for the development of future therapies and for a better understand of how these toxins act inhibiting virus machinery.

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7. FIGURES AND LEGENDS

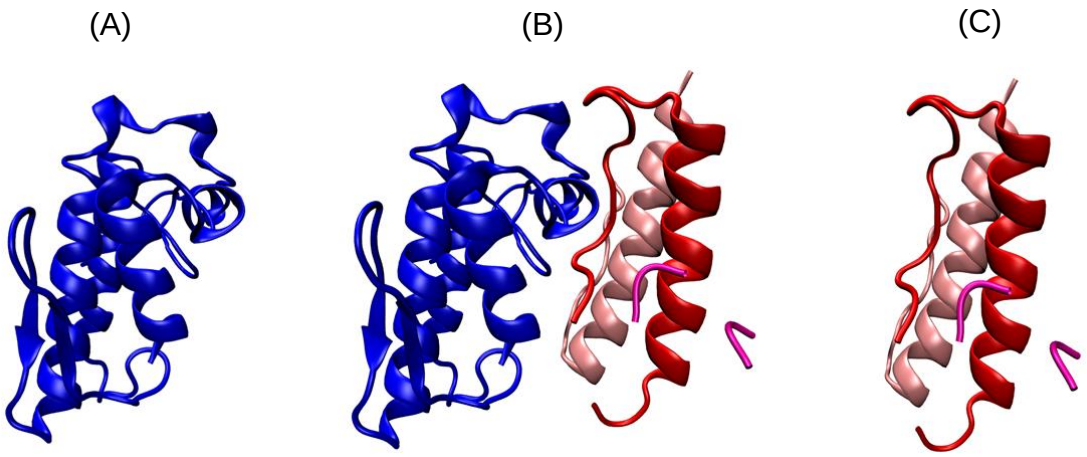

Figure 1. Crystal structure of the complex crotoxin from Crotalus durissus terrificus venom. The basic subunit (PLA_2-CB) is displayed in blue (A). The overall structure of crotoxin complex (B). The acid subunit (crotapotin) is shown in pink, red and light pink (C) (PDB ID: 3R0L).

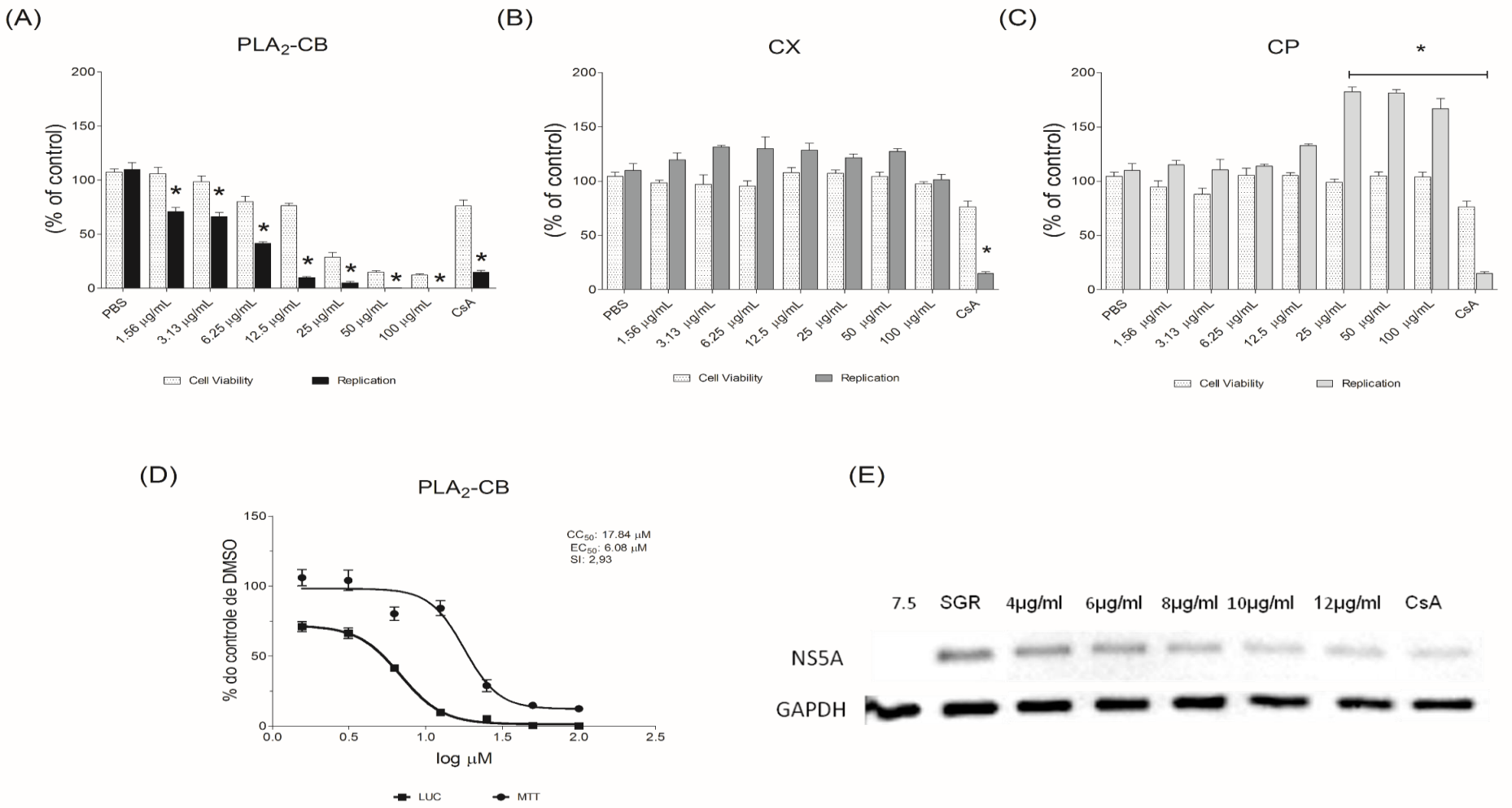

Figure 2. Inhibitory activity of the toxins on HCV replication. Huh-7.5 cell line stably expressing SGR-luc-JFH-1 were treated with PLA_2-CB (A), CX (B), CP (C) at specific concentrations for 48 h. the effective concentration of inhibition (EC_{50}), the cytotoxic concentration of 50% (CC_{50}), and the selectivity index (SI = CC_{50}/EC_{50}) were calculated (D). Expression of HCV NS5A protein was measured 48 h post-treatment using western blotting assays (E). Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.0001$ was considered significant.

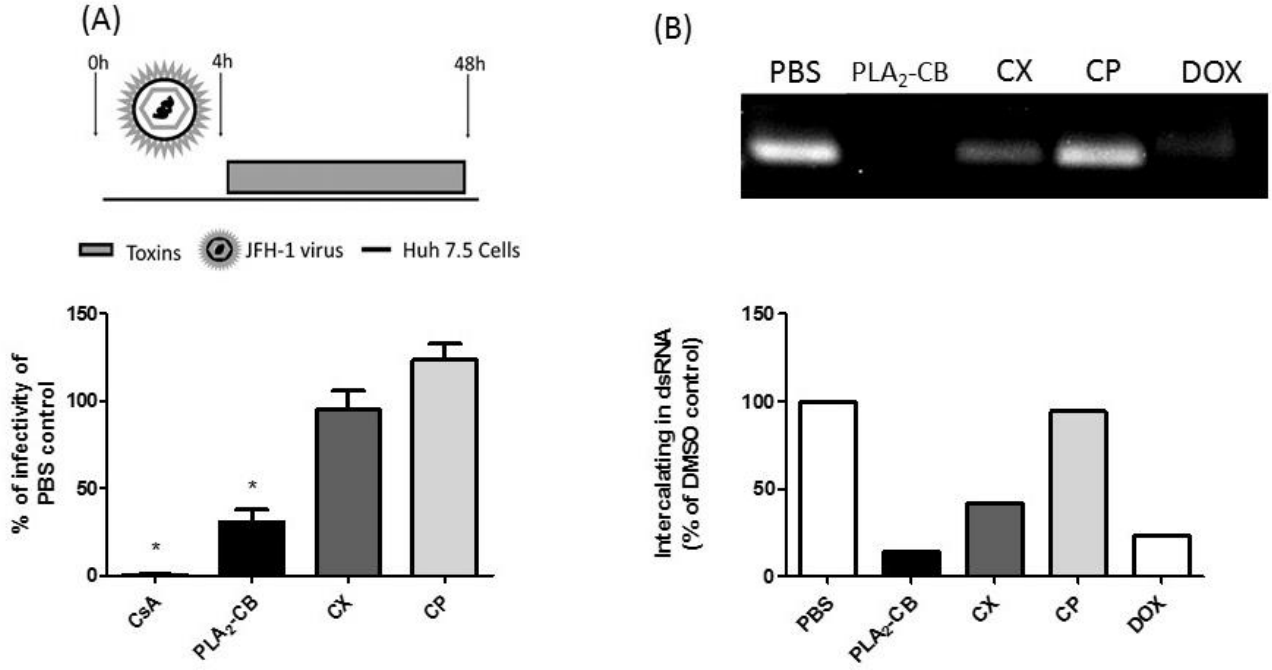

Figure 3. Effect of toxins on HCV replication. Huh7.5 cells were infected with JFH-1 HCVcc for 4 h, Then cells were washed extensively to remove virus and treated with toxins. Replication levels were assessed by performing the focus formation units assay 48 h post-infection. PBS was used as negative control and CsA as positive control for replication inhibition. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.001$ was considered significant (A). In an attempt to determine de antiviral mechanism of action of these toxins, syntethazed dsRNA was incubated with each toxin for 45 min and submitted to electrophoresis in 1% agarose gel. PBS wasused as negative control and Doxorubicin (DOX) was used as intercalating positive control (B).

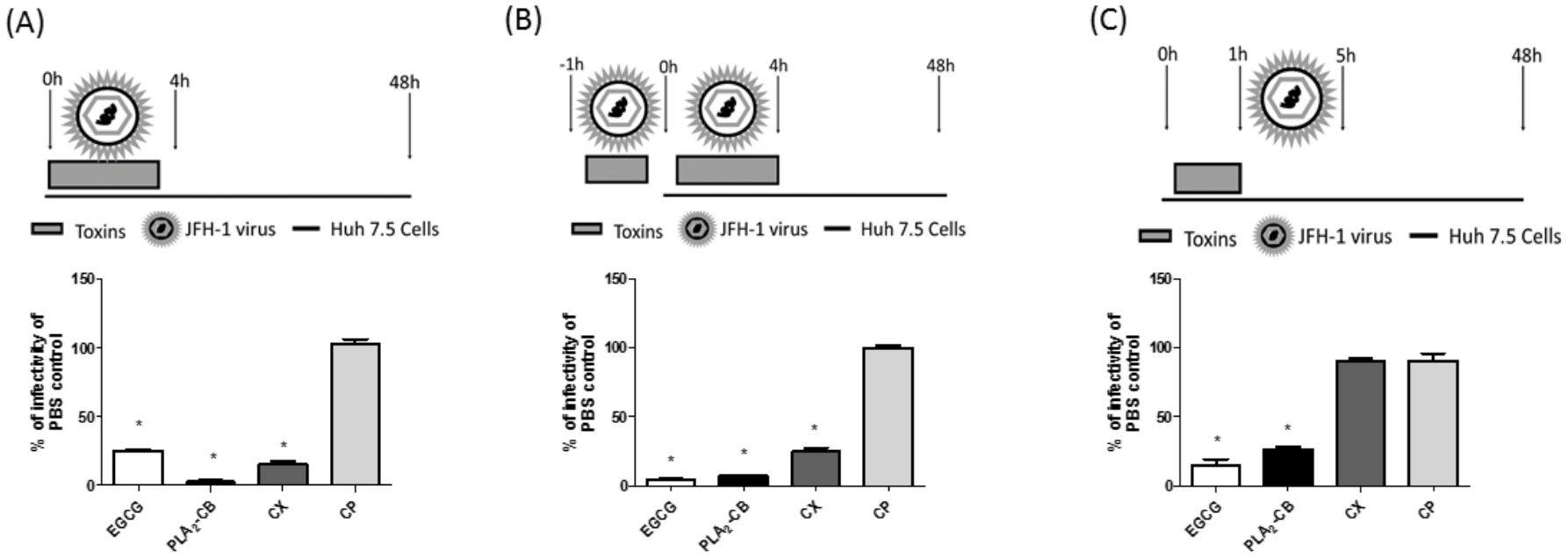

Figure 4. Effect of the toxins on HCV infectivity. Infectious supernatant and toxins were added in different times to the cells and intracellular virus was titrated 48 h post-infection by analyzing focus-forming units per milliliters (Ffu/mL). For entry assay, Huh-7.5 cells were infected with JFH-1 HCVcc and toxins were immediately added. After 4 h, the supernatant was replaced by fresh medium after repeated washes with PBS to remove completely the inoculum (A). For virucidal assay, JFH-1 HCVcc particles were incubated with toxins for 1 h prior to the infection. After that, the inoculum was used to infect naïve Huh-7.5 cells for 4 h. Cells were extensively washed and medium was added (B). In the pre-treatment assay, cells were previously treated with toxins for 1 h, washed to completely remove toxins andinfected with JFH-1 virus for 4 h. Cells were then washed to virus removal and replaced with fresh media for up to 48 h post-infection (C). PBS was used as negative control and EGCG as control of entry blockage. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.001$ was considered significant.

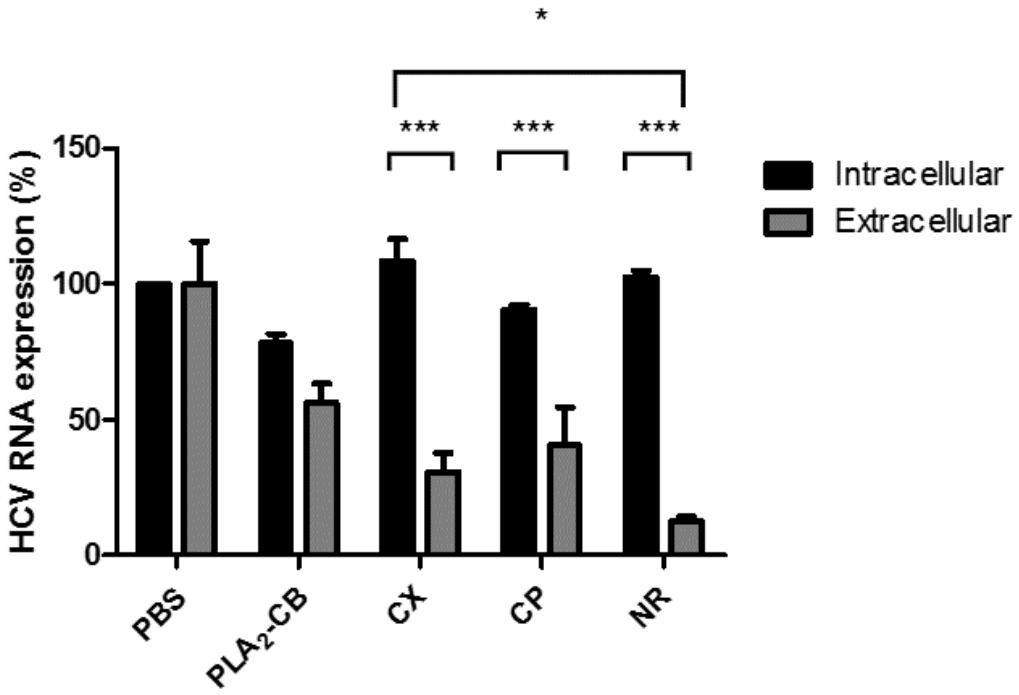

Figura 5. Antiviral activity of toxins on HCV release. Huh-7.5 cells previously infected with JFH-1 virus was plated 48 h prior treatment. The toxins were added at 10 µg/mL and incubated for 24 h. Supernatant was collected and cells were harvested, and intra and extracellular RNA were quantified by qPCR. PBS was used as negative control and naringenin (NR) at 400 µM was used as positive control of HCV secretion. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.001$ was considered significant.

CAPÍTULO III:

NATURAL COMPOUNDS ISOLATED FROM *PTEROGYNE NITENS* INHIBIT HEPATITIS C VIRUS ENTRY

*Este capítulo está formatado como manuscrito com algumas alterações estruturais para melhor se adequar ao formato da dissertação. Apresenta dados em fase de elaboração, a serem submetidos à revista “Antiviral Research”

NATURAL COMPOUNDS ISOLATED FROM *PTEROGYNE NITENS* INHIBIT HEPATITIS C VIRUS ENTRY

Shimizu, J.F. [1]; Lima, C. S. [2]; Bittar, C. [1]; Regasini, L. O.[2]; Rahal, P.[1]; Jardim, A.C.G. *[1,3].

Contact details:

Jacqueline Farinha Shimizu[1]
Email: jacquelineshimizu@gmail.com

Luis Octávio Regasini[2]
Email: regasini@ibilce.unesp.br

Caroline Lima Sprengel[2]
Email: carolsprengel@mail.com

Paula Rahal[1]
Email: rahalp@yahoo.com.br

Cintia Bittar[1]
Email: cibittar@gmail.com

Ana Carolina Gomes Jardim *[3,1]
Email: caroljardim@gmail.com

[1]Genomics Study Laboratory, São Paulo State University, IBILCE, S. José do Rio Preto, SP, Brazil

[2]Green and Medicinal Chemistry Laboratory, São Paulo State University, IBILCE, S. José do Rio Preto, SP, Brazil

[3]Institute of Biomedical Science, ICBIM, Federal University of Uberlândia, Uberlândia, MG, Brazil

*Corresponding author. Tel.: +55 (34) 3225-8679

Address: Avenida Amazonas, Bloco 4C – sala 216. Umuarama. Uberlândia, MG, Brazil, CEP: 38405-302

E-mail address: acgjardim@icbim.ufu.br

ABSTRACT

Hepatitis C virus (HCV) represents a public health problem worldwide. It is one of the leading causes of liver diseases and transplantation. The current available therapy for Hepatitis C is based on interferon-α, ribavirin and the new direct-acting antivirals (DAAs) such as the NS3 protease inhibitors. However, the high costs of development, severe side effects and the described viral resistance presented by the existing treatments demonstrate the need for developing more efficient antivirals against HCV. The naturally occurring flavonoids such as (-)-epigallocatechin gallate (EGCG), naringenin, silibin and ladanein have demonstrated to inhibit different stages of HCV life cycle. Therefore, natural flavonoids can provide an alternative source for the identification of products with therapeutic potential. This study aimed to evaluate the antiviral effects of the flavonoids sorbifolin (PN2) and pedalitin (F18) isolated from *Pterogyne nitens* on HCV life cycle. These compounds were investigated by their anti-HCV activities using subgenomic replicons and infectious systems. The data obtained demonstrated that F18 and PN2 at non-cytotoxic concentration blocked up to 78.7% and 45% of virus entry, respectively. The mechanism in which F18 blocked virus entry showed to be both the direct action on virus particles and the interference on the host cells. Alternatively, PN2 activity was restricted to its virucidal effect. Additionally, no inhibitory effect on HCV replication was observed by treating cells with these flavonoids. Our data are the first description of PN2 and F18 possessing antiviral activities against HCV *in vitro.* Further analyses are being performed in order to investigate the mode of action of those natural flavonoids extracted from Brazilian plants on HCV life cycle.

Keywords: Hepatitis C Virus, *Pterogyne nitens*, antivirals, natural compounds

1. INTRODUCTION

Hepatitis C virus (HCV) was identified in 1989 as the causative agent of hepatitis C (CHOO et al., 1989). 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 hepatitis C (WHO, 2015).

HCV is an enveloped, single stranded positive-sense RNA virus which belongs to the Flaviviridae family, genus *Hepacivirus* (SIMMONDS et al., 2005). There is no effective vaccine for prevention of the HCV infection.

Until recently, HCV infected patients were treated with pegylated interferon and ribavirin (pegIFN–RBV) (ROSEN, 2011). The availability of new, direct antivirals agents (DAAs) as simeprevir and sofosbovir have increased rates of sustained virological response (SVR) with treatment efficacies as high as 90% for most common HCV genotypes (GAETANO, 2014; LAWITZ et al., 2013; MCQUAID; SAVINI; SEYEDKAZEMI, 2015; SORIANO; PETERS; ZEUZEM, 2009). However, the available treatments present several side effects, costs are still high (BARTH, 2015) and resistant variants were described even for the recent therapies approved by Food and Drugs Administration (FDA) (POVEDA et al., 2014; WYLES, 2013). Despite the introduction of interferon-free regimens, the current therapy in many countries is still based on pegIFN–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 compounds have demonstrated to provide a wide source of molecules which can be evaluated for their antiviral properties (BRAHMACHARI, 2012).

Flavonoids represent an important class of compounds, mainly produced by plants as a response to microbial infections (DIXON; DEY; LAMB, 2006). Some naturally occurring flavonoids have been described to

possess antiviral activities. The compound Epigallocatechin isolated from green tea demonstrated to inhibited the life cycle of Enterovirus 71 (HO et al., 2009), Chikungunya virus (WEBER et al., 2015) and HCV (CALLAND et al., 2012a). The grapefruit-extracted flavonoid naringenin showed antiviral effect against Herpes simplex virus type 1 (HSV-1) (LYU; RHIM; PARK, 2005) and HCV (GOLDWASSER et al., 2011). Silibin and ladanein have also demonstrated anti-HCV activities by inhibition of entry step (BLAISING et al., 2013; HAID et al., 2012).

Pterogyne nitens is a native tree in Brazilian Cerrado biome which belongs to Fabaceae family (LORENZI, 2008). Compounds extracted from *P. nitens* have demonstrated antimicrobial activity against the opportunistic fungi (REGASINI et al., 2010) and multi-drug-resistant strains (MRSA) (COQUEIRO et al., 2014) of *Candida sp.*. Those compounds also showed to induce apoptosis in human breast cancer cells (DUARTE et al., 2010) and presented antioxidant properties (DOS SANTOS et al., 2009).

In this study, we investigated the effects of pedalitin (F18) and sorbifolin (PN2) isolated from leaves extract of *P. nitens* on HCV life cycle. The data obtained showed that these compounds inhibited HCV entry to the host cells, but had no effect on virus replication.

2. MATERIAL AND METHODS

2.1. Natural compounds

The flavonoid compounds sorbifolin (PN2) and pedalitin (F18) (Figure 1) were isolated from the leaves extract of *Pterogyne nitens*. The leaves of P. nitens was collected in the city of São José do Rio Preto (São Paulo State, Brazil, at 20°47'02.4"S; 49°21'36.0"W) in March 2013. The plant was identified by Profª. Drª. Andréia Alves Rezende from Department of Biology and Animal Science, Faculty of Engineering, Unesp – Ilha Solteira. A

voucher specimen (10291) has been deposited in the Herbarium of Ilha Solteira (HISA), Faculty of Engineering, Ilha Solteira, São Paulo, Brazil. Dry leaves were extracted by maceration in ethanol for 48 hours. Subsequently, the extraction solution was separated by simple filtration in filter paper. The filtrate was dried under rotary evaporation. The crude ethanol extracts were further submitted to reverse phase chromatography column (ODS- C18; 4 cm x 22 cm), yielding 13 fractions among them PN2 and F18. The molecular structures of compounds were elucidated by nuclear magnetic resonance (NMR). The purification procedures were performed by Caroline Lima Sprengel under supervision of Prof.º Dr.º Luís Octávio Regasini, from Green and Medicinal Chemistry Laboratory, São Paulo State University, IBILCE, São José do Rio Preto, SP, Brazil.

Lyophilized compounds were dissolved in dimethyl sulfoxide (DMSO) in stock solutions 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%. For all the assays performed, non-treated control was added of DMSO at the same final concentration. The flavonoid (-)-epigallocatechin gallate (EGCG, Sigma-Aldrich) was used as positive control for entry, pre-treatment and virucidal assays (CIESEK et al., 2011)(CALLAND et al., 2012a)(CALLAND et al., 2012a)(CALLAND et al., 2012a). Cyclosporine A (CsA) was used as control of inhibition of replication (WATASHI et al., 2003).

2.2. HCV replicons

Antiviral activity was evaluated by two HCV replicon systems. The full-length HCV genotype 2a JFH-1 replicon (WAKITA et al., 2005) was used to perform virus assays as described below. The subgenomic replicon SGR-FEO-JFH-1, based on the non-structural proteins NS3-NS5B of JFH-1

genotype 2a strain inserted of a firefly luciferase-neomycin phosphotransferase fusion protein, was used to performed Luciferase-based replication assay (WYLES et al., 2009).

2.3. Cell Culture

The human hepatoma cell line (Huh-7.5) was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 100 U/mL penicillin (Gibco Life Technologies), 100 mg/mL streptomycin (Gibco Life Technologies), 1% non-essential amino acids (Gibco Life Technologies), 1 % HEPES (Gibco Life Technologies, USA) and 10% fetal bovine serum (FBS; Cultilab) at 37 ºC in a humidified 5% CO_2 incubator. Huh-7.5 cells stably harboring theSGR-FEO-JFH-1 were cultured under the same condition, added of 500 µg/mL of G418 (Sigma-Aldrich, USA).

2.4. Cell viability assay

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich) method. Huh-7.5 or SGR-FEO-JFH-1 Huh-7.5 cells were seed to 96-well microplates and incubated at 37 °C in a humidified 5% CO_2 incubator overnight. Drug-containing medium at different concentrations added to the cell culture. After 72 hours incubation, DMEM containing MTT at the final concentration of 1 mg/mL was added to each well, incubated for 1 hour and replaced with 100 µl of DMSO to solubilize the formazan crystals. The absorbance was measured at a wavelength of 562 nm on plate reader (FLUOstar Omega/BMG LABTECH, Offenburg, BW, DE). Cells viability was calculated according to the equation (T/C) × 100%, where T and C represent the mean optical density of the treated group and control group, respectively. DMSO was used as non-treated control.

2.5. Luciferase-based replication assay.

Huh-7.5 cells stably harboring the SGR-FEO-JFH-1 were seeded into 96-well plates at a density of 5 x 10^3 per well and compounds at 50 µM, 10 µM, 2 µM and 0.4 µM were added. After 72 h, cells were harvested by lysis with Passive Lysis Buffer (Promega) and HCV RNA replication was quantified by measuring luminescence levels using the Luciferase Assay System (Promega) and a plate reader (FLUOstar Omega/BMG LABTECH, Offenburg, BW, DE).

2.6. Virus assays

For virus assays, HCVcc particles were generated as described previously (JARDIM et al., 2015). Briefly, 8 × 10^6 Huh-7.5 cells were electroporated with 10 µg of RNA JFH-1 (WAKITA et al., 2005). Naïve huh7.5 cells were seeded the day before the assay was carried out. Cells were infected with JFH1 virus (Wakita et al., 2005) at a multiplicity of infection (MOI) of 0.4 and compounds at 50 µM were added at different times, depending on the stage of HCV life cycle to be evaluated (as described below).

2.7. Inhibitory effects on Entry steps

For virus entry experiments, JFH-1 HCVcc was used to infect Huh-7.5 cells in complete medium with compounds for 4 h. The supernatant plus virus was removed, cells were washed three times with PBS to completely remove the inoculum, replaced with fresh complete medium and incubated at 37 °C in a humidified 5% CO_2 incubator, After 72 hours, supernatant was removed, cells were fixed and intracellular virus was titrated.

2.7. Pre-treatment assay

Huh-7.5 cells were pre-treated with each flavonoid for 1 hour at 37 °C in a humidified 5% CO_2 incubator prior to the infection. After incubation, cells were washed extensively to remove compounds and were infected with HCVcc JFH-1 virus for 4 hours. Infectious supernatant was removed, additional washes were performed to virus removal and fresh media was added. Virus was titrated 72 hpi.

2.8. Virucidal assay

For virucidal assay, infectious supernatant containing HCVcc JFH-1 virus was previously incubated with each compound for 1 hour at 37 °C. Then, the mixture was used to infect Huh-7.5 cells for 4 hours at 37 °C in a humidified 5% CO_2 incubator prior to the infection. The inoculums was removed and cells were washed three times with PBS, replaced by fresh media and incubated 72 hours at 37 °C in a humidified 5% CO_2 incubator. Virus was titrated 72 hpi as described below.

2.9. Virus titration

Huh-7.5 cells infected with HCVcc JFH-1 particles were fixed with 4% paraformaldehyde (PFA) 72 hpi , washed with 100 mM Glicine (Applichem), semi-permeabilized with 0.1% Triton X-100 (Vetec Labs) and stained for NS5A using sheep anti-NS5A (MACDONALD et al., 2003) and Alexa Fluor anti-sheep 594 secondary antibody. Infectivity was expressed as focus-forming units per milliliter of supernatant (FFU/mL).

2.10. Statistical analysis

Individual experiments were performed in triplicate and all assays were performed a minimum of three times in order to confirm the reproducibility of the results. Differences between means of readings were compared using analysis of variance (one-way or two-way ANOVA) and Student t test. P values of less than 0.05 (indicated by asterisks) were considered to be statistically significant.

3. Results

3.1. The flavonoids pedalitin and sorbifolin inhibit HCV entry

To investigate the effect of F18 and PN2 compounds on HCV entry to the host cells, we first evaluated whether F18 and PN2 compound had likewise a cytotoxic effect in näive Huh-7.5 cell line. We treated Huh-7.5 cells with 50 µM of each compounds for 4 and 72 hours and cell viability was measured by the MTT method. The results showed a higher tolerance of Huh-7.5 cell line to the treatment with compounds **(Figure 2)**.

Next, huh-7.5 cells were infected with JFH-1 HCVcc in the presence of 50 µM of each compounds. Therefore, virus and compounds were simultaneously added to the naïve cells and incubated for 4 hours at 37 °C in a humidified 5% CO_2 incubator. Cells were extensively washed with PBS to remove any remained virus or compounds, and replaced with fresh medium for 72 hours. Intracellular virus was titrated by using focus formation units assay. The results demonstrated that either F18 or PN2 were able to block HCV entry up to 78.7% and 45%, respectively **(Figure 3A)**.

3.2. F18 and PN2 block HCV entry by a direct action on the virus particle

Once our data showed an inhibitory activity of F18 and PN2 against the HCV entry, we wished to investigate whether the observed antiviral effect was due to the compounds action on the viral particle. Supernatant containing JFH-1 HCVcc was incubated with 50 µM of each compound for 1 hour at 37 °C in a humidified 5% CO_2 incubator, prior to the infection of Huh-7.5 cells. The inoculums of virus plus compound was transferred to the naïve cells and incubated for 4 hours. Cells were washed with PBS to the complete removal of the inoculum and replaced with fresh media for 72 hour when virus was titrated. A significant virucidal activity was observed for both F18 and PN2 which blocked 86% and 38.2% of virus entry, respectivitely ($p < 0.0001$) **(Figure 3B)**. These data suggest that an anti-HCV mechanism of action of F18 and PN2 might be related to a direct action on the virus particle structure.

3.3. Pedalitin blockage of HCV entry by an its effect on the host cells

We further evaluated whether the antiviral activity of F18 and PN2 on viral entry is influenced by an effect on the host cells. For this, huh-7.5 cells were previously treated with each compound for 1 hour at 37 °C in a humidified 5% CO_2 incubator, followed by repeated washes with PBS to remove any trace of drugs. Cells were infected with JFH-1 HCVcc virus for 4 hours, and then supernatant was removed and replaced by fresh media after PBS washes to the virus removal. Intracellular virus was titrated 72 hpi. The analysis showed that F18 but not PN2 significantly inhibited infectivity when cells were previously treated with this compound, blocking 72.5% of virus entry to the host cells **(Figure 3C)**. These results strongly suggest that the inhibitory anti-HCV effect observed for PN2 was due to a direct action on the virus particle, while F18 appear to act on both virus particle and host cells.

3.4. Pedalitin and sorbifolin showed no effect on HCV replication.

The effects of F18 and PN2 on HCV replication were evaluated by using a firefly luciferase HCV subgenomic replication system (SGR-FEO-JFH-1). Huh7.5 cells stably harboring the SGR-FEO-JFH-1 were treated with 50, 10, 2 and 0.4 µM of each compound and incubated for 72 h to assess both the cytotoxicity and antiviral effects of the compounds. Cells viability and HCV replication levels were measured by MTT and luciferase assays, respectively. The results demonstrated that treatment of cells with PN2 or F18 compounds at non-toxic concentrations had no effect on inhibiting HCV replication **(Figure 4)**. Treatment of cells for 72 h with F18 for any tested concentrations demonstrated no cytotoxicity, but PN2 at 50 µM showed to interfere in cell viability (55% compared to the non-treated control) **(Figure 4)**.

4. **DISCUSSION**

HCV is still one of the leading causes of liver disease and transplantation, and is related to more than 350,000 people die worldwide. Despite the advances in recent years, current therapy for Hepatitis C is not effective for all treated patients, presents many side effects and high cost of development.

Flavonoids are a group of chemical substances that have been described with many activities as antioxidant (LEOPOLDINI et al., 2006), anti-inflammatory (SERAFINI; PELUSO; RAGUZZINI, 2010), anticancer (YI et al., 2005), hepatoprotective (ZHU et al., 2012) and antimicrobial (PANDEY, 2007; REGASINI et al., 2010). Antiviral activity was also documented against a range of viruses including Enterovirus 71 (HO et al., 2009), Chikungunya virus (WEBER et al., 2015) and Herpes simplex virus type 1 (HSV-1) (LYU; RHIM; PARK, 2005). In the literature, various flavonoid compounds as naringenin (GOLDWASSER et al., 2011; NAHMIAS et al.,

2008), silibin (BLAISING et al., 2013), ladanein (HAID et al., 2012) and EGCG (CALLAND et al., 2012a) are described to possess anti-HCV activity against different stages of the replicative cycle. Therefore, naturally occurring flavonoids can provide an alternative source for the identification of products with therapeutic potential.

In this study, we investigated the anti-HCV potential of the natural flavonoids PN2 and F18 isolated from *P. nitens.* This is the first description of PN2 and F18 isolated from a Brazilian plant which were tested against HCV and demonstrated antiviral effect by blocking entry stage of HCV life cycle. The PN2 and F18 were previously documented to be isolated from other species of plants as *Ruellia tuberosa* (LIN et al., 2006), *Mentha pulegium* and *M. suaveolens* (ZAIDI et al., 1998a), and mainly described with antioxidant activity (FERNANDES et al., 2008).

We found that treatment of human hepatocarcinoma cells with PN2 and F18 blocked HCV entry to the host cells but had no effect on virus replication. The activity of natural flavonoids on virus entry was previously described by other authors. By using quantum chemical and chemometric methods, Souza *et al.* demonstrated the antipicornavirus activity of flavones on virus particle (SOUZA et al., 2004). Haid *et al.,* isolated the ladanein from *Marrubium peregrinum,* a flavone with potent and broad-spectrum antiviral activity against entry of all HCV genotypes into human hepatocytes (HAID et al., 2012).

Our results suggest that the decrease on viral infectivity observed by treating cells with F18 is due to its effects on both virus particles and host cells, since this compound significantly inhibited infectivity when cells were treated prior to the infection with JFH-1 virus or when virus and compound were incubated and then inoculated to the cells. Calland *et al.* showed that the flavonoids delphinidin and EGCG acted directly on HCV envelope glycoproteins E1 and E2 causing structural modifications, which prevented virus attachment to the cell surface (CALLAND et al., 2015). Colpitts and

Schang also demosntrated that EGCG inhibited viral entry of HCV and HSV-1 by blocking binding of virus and heparan sulfate on the cell surface (COLPITTS; SCHANG, 2014). Another possible mechanism of action was observed for silibinin, a flavonoid reported to block the HCV post-attachment step by alters clathrin mediated endocytosis and endosomal trafficking, and resulting in a protective effect on host cells (BLAISING et al., 2013). Anggakusuma *et al.* verified the anti-HCV activity of turmeric curcumin, a natural compound that inhibits HCV entry of all major genotypes by affecting virion envelope fluidity and also by preventing HCV cell-to-cell transmission. However, the mechanism in which turmeric curcumin inhibits cell-to-cell transmission remains unclear (ANGGAKUSUMA et al., 2014).

Alternatively, PN2 had no protective effect on host cells but acted on virus particles to block HCV entry. In 2004, Erlejman *et al* demonstrated that the protective effects of flavonoids on the cells membranes were mainly associated to their hydrophilicity and the number of hydroxyl groups present in the molecules (ERLEJMAN et al., 2004). The Brazilian flavonoid F18 presents only one hydroxyl group more than PN2. This difference could explain the results observed in our pre-treatment assays.

In summary, our data showed that two natural flavonoids isolated from the Brazilian plant *P.nitens* inhibited HCV entry *in vitro.* Further analyses are need to clarify the mechanisms in which these flavonoids act on viral particle, and to a better understand of how F18 protects cells against HCV infection. In our knowledge, this is the first description of natural compounds isolated from *P.nitens* possessing antiviral activity against HCV, and these finds may be useful to the development of new anti-HCV approaches.

5. CONCLUSIONS

Our data suggest that the flavonoids PN2 and F18 isolated from *P. nitens* possess antiviral activity against HCV infection by blocking the entry

of virus into the host cells. The mechanism in which PN2 inhibited HCV entry is by a direct action on the virus particle, while F18 appear to act on both virus particle and host cells. No inhibition of HCV replication was observed for these compounds.

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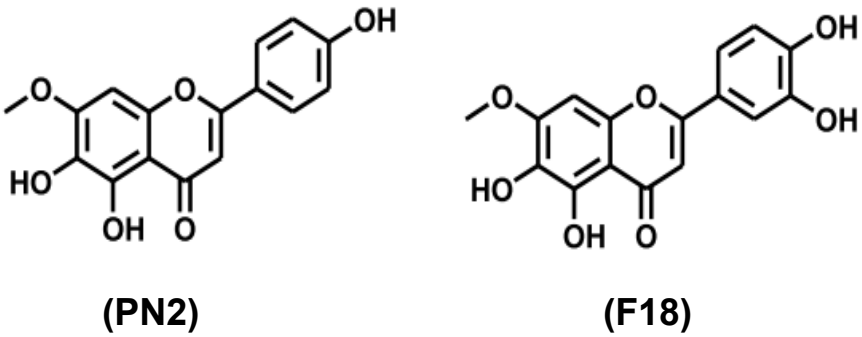
7. FIGURES AND LEGENDS



(PN2) **(F18)**

Figure 1. Chemical structure of flavones PN2 (left) and F18 (right) isolated from *Pterogynes nitens* (REGASINI, 2015).

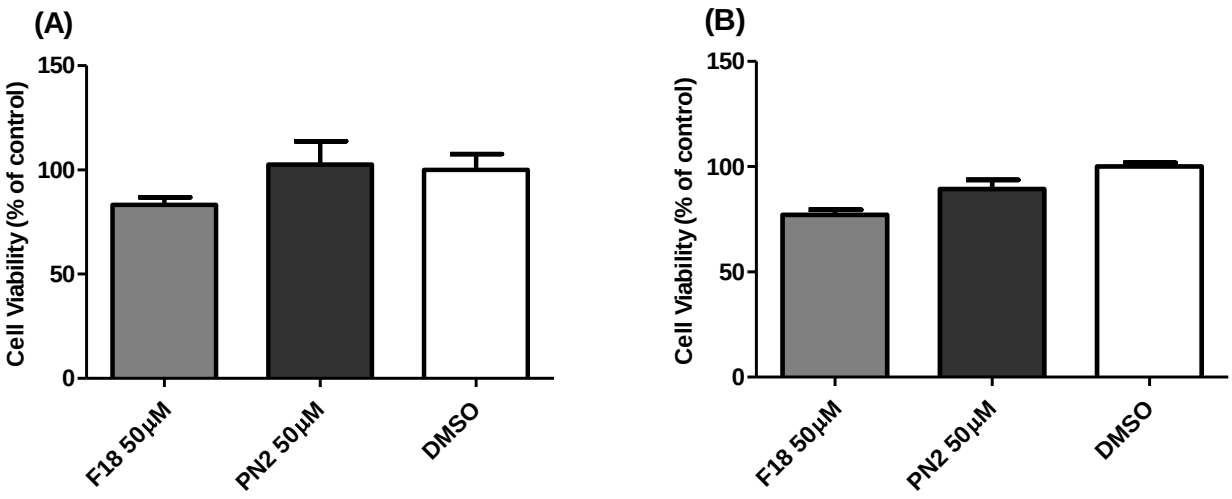


Figure 2. Cell viability of natural compounds in näive Huh-7.5 cell line. Cells were treated with F18 and PN2 at specific concentrations for 4h (A) and 72 h (B). DMSO were used as non-treated control. Mean values of experiments are shown and $P < 0.001$ was considered significant (*).

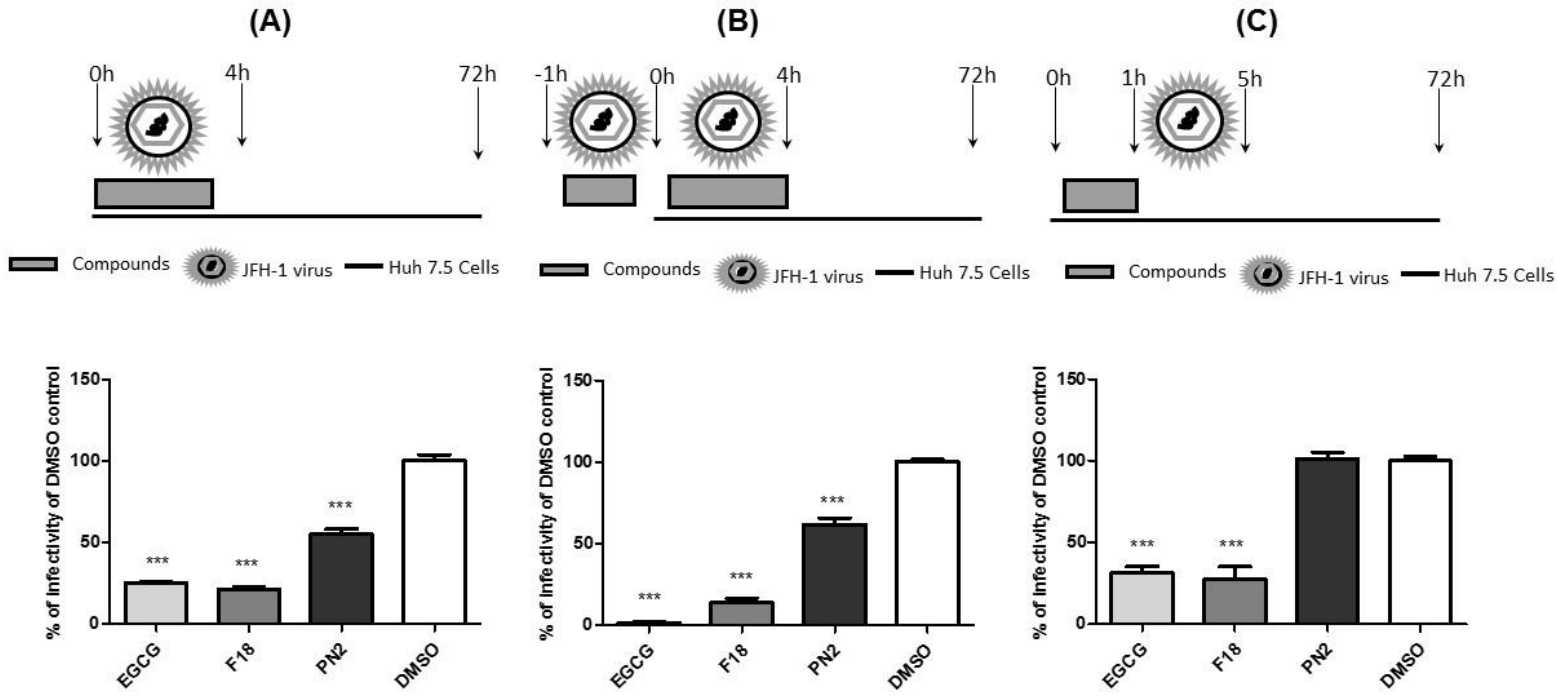


Figure 3. Effects of natural flavonoids on HCV infectivity. Infectious supernatant and natural compounds were added in different times to the cells, and intracellular virus was titrated 72 h post-infection by analyzing focus-forming units per milliliters (Ffu/mL). For entry assay, Huh-7.5 cells were infected with JFH-1 HCVcc and compounds were immediately added. After 4 h the supernatant was removed and replaced with fresh medium after repeated washes with PBS to remove the inoculum (A). For virucidal assay, JFH-1 HCVcc were incubated with natural compounds for 1 h prior to the infection. After that, the inoculum was used to infect naïve Huh-7.5 cells for 4 h. Cells were extensively washed and medium replaced (B). In the pre-treatment assay, cells were previously treated with compounds for 1 h prior to the infection. Cells were washed to remove compounds and infected with JFH-1 virus for 4 h. Supernatants was removed, cells were washed to complete virus removal and were incubated with fresh media for up to 72 h post-infection (C). DMSO was used as negative control and EGCG as control of entry blockage. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.001$ was considered significant.

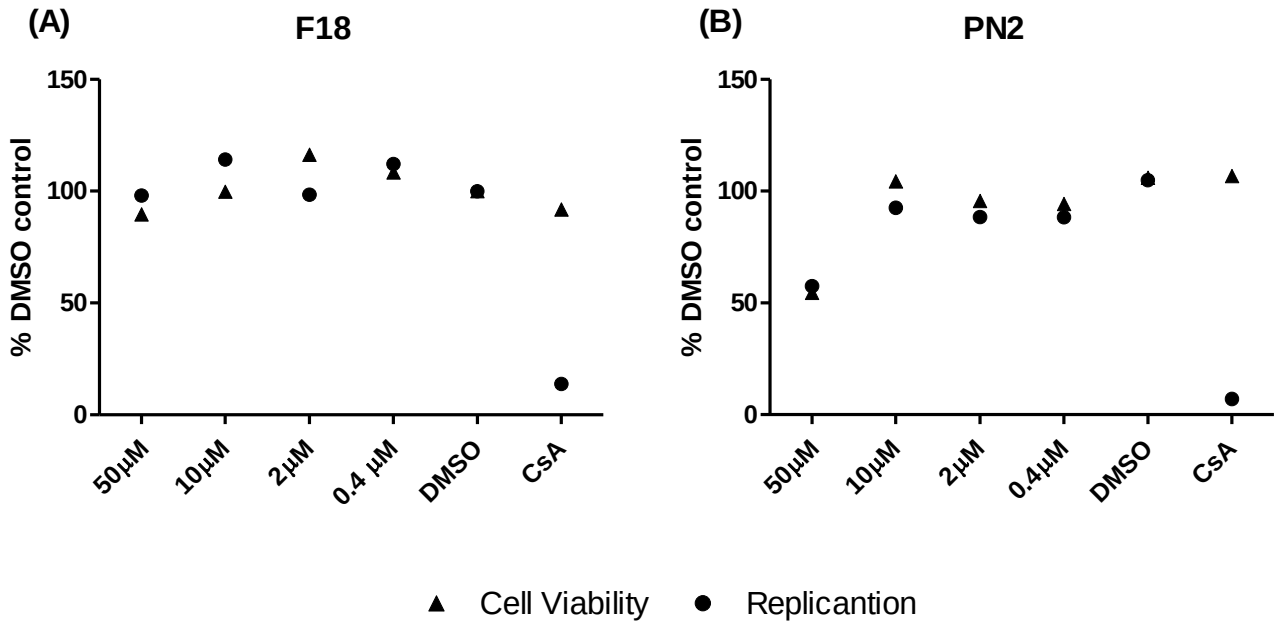


Figure 4. Effect of natural compounds on HCV replication. Huh-7.5 cells stably harbouring subgenomic replicon SGR-FEO-JFH-1 were treated with F18 (A) and PN2 (B) at specific concentrations for 72 h. Cell viability (▲) and replication (●) were evaluated by MTT and luciferase assays, respectively. DMSO used as non-treated control and CsA as control of inhibition of replication. Mean values of experiments are shown and $P < 0.001$ was considered significant.

CAPÍTULO IV:

NATURAL COMPOUNDS ISOLATED FROM BRAZILIAN PLANTS ARE POTENT INHIBITORS OF HEPATITIS C VIRUS REPLICATION IN VITRO

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Natural compounds isolated from Brazilian plants are potent inhibitors of hepatitis C virus replication in vitro

A.C.G. Jardim [a,b,*], Z. Igloi [c], J.F. Shimizu [b], V.A.F.F.M. Santos [d], L.G. Felippe [d], B.F. Mazzeu [b], Y. Amako [e], M. Furlan [d], M. Harris [c,1], P. Rahal [b,1]

[a] UFU – Federal University of Uberlândia, Institute of Biomedical Science – ICBIM, Uberlândia, MG, Brazil
[b] UNESP – São Paulo State University, Institute of Bioscience, Language and Exact Science – IBILCE, Department of Biology, São José do Rio Preto, SP, Brazil
[c] School of Molecular and Cellular Biology, Faculty of Biological Sciences and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom
[d] UNESP – São Paulo State University, Institute of Chemistry, Department of Organic Chemistry, Araraquara, SP, Brazil
[e] Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

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ABSTRACT

Compounds extracted from plants can provide an alternative approach to new therapies. They present characteristics such as high chemical diversity, lower cost of production and milder or inexistent side effects compared with conventional treatment. The Brazilian flora represents a vast, largely untapped, resource of potential antiviral compounds. In this study, we investigate the antiviral effects of a panel of natural compounds isolated from Brazilian plants species on hepatitis C virus (HCV) genome replication. To do this we used firefly luciferase-based HCV sub-genomic replicons of genotypes 2a (JFH-1), 1b and 3a and the compounds were assessed for their effects on both HCV replication and cellular toxicity. Initial screening of compounds was performed using the maximum non-toxic concentration and 4 compounds that exhibited a useful therapeutic index (favourable ratio of cytotoxicity to antiviral potency) were selected for extra analysis. The compounds APS (EC_{50} = 2.3 μM), a natural alkaloid isolated from *Maytrenus ilicifolia*, and the lignans 3*43 (EC_{50} = 4.0 μM), 3*20 (EC_{50} = 8.2 μM) and 5*362 (EC_{50} = 38.9 μM) from *Peperomia blanda* dramatically inhibited HCV replication as judged by reductions in luciferase activity and HCV protein expression in both the subgenomic and infectious systems. We further show that these compounds are active against a daclatasvir resistance mutant subgenomic replicon. Consistent with inhibition of genome replication, production of infectious JFH-1 virus was significantly reduced by all 4 compounds. These data are the first description of Brazilian natural compounds possessing anti-HCV activity and further analyses are being performed in order to investigate the mode of action of those compounds.

1. Introduction

Hepatitis C virus (HCV) infection is a worldwide public health problem and it is estimated that the virus infects around 3% of the world population (Shepard et al., 2005). Chronic infection can progress to liver cirrhosis with risk of the development of hepatocellular carcinoma, and causes around 500,000 deaths per year (Alter, 2007; Chevaliez and Pawlotsky, 2007; Saito et al., 1990). There is no effective vaccine for prevention of HCV infection; however a number of drugs are available for the treatment of infection. Until recently, the standard therapy was based on pegylated interferon (IFN) plus ribavirin (RBV), resulting in a sustained virological response in approximately 50% of patients infected with HCV genotypes 1a/1b and 80% of those infected with genotypes 2 or 3 (Fried et al., 2002; Hadziyannis et al., 2004; Manns et al., 2001). The availability of new, direct-acting antivirals targeting the NS3 protease, NS5B polymerase and NS5A protein have dramatically improved therapeutic options (Pawlotsky, 2014). However, the high costs and potential for development of resistance presented by existing treatment demonstrate the need for the development of more efficient new antivirals, or combination of therapies for HCV treatment.

* Corresponding author at: Avenida Amazonas, Bloco 4C sala 216, Umuarama, Uberlândia, MG CEP 38405-302, Brazil. Tel.: +55 34 3218 2557.
E-mail addresses: caroljardim@gmail.com, acgjardim@icbim.ufu.br (A.C.G. Jardim), z.Igloi@leeds.ac.uk (Z. Igloi), jacquelineshimizu@gmail.com (J.F. Shimizu), vaniaapf@gmail.com (V.A.F.F.M. Santos), lidiane_iq@yahoo.com.br (L.G. Felippe), bruna.mazzeu@gmail.com (B.F. Mazzeu), amako-yt@igakuken.or.jp (Y. Amako), mayfurlan@gmail.com (M. Furlan), m.harris@leeds.ac.uk (M. Harris), prahal@ibilce.unesp.br (P. Rahal).
[1] These authors contributed equally to this work.

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Traditional medicines have a long history and there is now a great interest in discovering new molecules from natural sources for the treatment of many human diseases. An extensive variety of natural compounds has demonstrated antiviral action worldwide, including anti-HCV activity (Calland et al., 2012). In this context, compounds extracted from plants can provide an alternative approach to new therapies. Natural compounds present characteristics such as high chemical diversity, lower cost of production and milder or non-existent side effects than conventional treatment (Kitazato et al., 2007). Additionally, most of the drugs used today in the clinic were first discovered from plants and microorganisms (Mann, 2002). Therefore, they present a great opportunity to find novel compounds that can act as antiviral drugs.

The Brazilian flora represents a vast, largely untapped, resource of potential therapeutic compounds. The wide distribution of natural resources in Brazil and the natural diversity of chemical components provide the country with potential bioactive materials (Duarte et al., 2005). Here we investigate the antiviral effects of a panel of Brazilian natural compounds consisting of extracts, fractions and isolated compounds on HCV replication. These data are the first description of Brazilian natural compounds possessing anti-HCV activity.

2. Materials and methods

2.1. Natural compounds

Compounds were extracted from *Maytrenus ilicifolia* (APS, C, P and M), *Peperomia blanda* (5-362, 3-20, 3-43, 48-3, F3 and F6) and *Piper fuligineum* (F8–40). The root bark of *M. ilicifolia* was collected in the city of Ribeirão Preto (São Paulo State, Brazil, at 21°11′56.1″S; 47°46′42.2″W) in March 2006. The plant was identified by Rita Maria de Carvalho. A voucher specimen (HPM-BR 0059) has been deposited in the Herbarium of the University of Campinas, São Paulo, Brazil (Santos et al., 2012). The aerial parts of *P. blanda* were collected at the Reserva da Ripasa, Ibaté – SP, Brazil in January of 2005 and identified by Dr. Elsie Franklin Guimarães. A voucher specimen (Kato-547) has been deposited at the Herbarium of the Institute of Bioscience, São Paulo University, São Paulo – SP, Brazil (Felippe et al., 2008). The *Piper fuligineum* species was identified by Dr. Agnes Lamb of the Institute of Botany (IBt of São Paulo, SP, Brazil) and their voucher specimens are deposited in the Herbarium of the Institute of Botany (USP – SP) under the voucher Kato-0720.

The full details of compound extraction and purification was described previously (Costa et al., 2008; Dos Santos et al., 2013; Felippe et al., 2008, 2012; Gullo et al., 2012; Santos et al., 2012) and the structures of isolated compounds are shown in Fig. 1. The compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich) as stock solutions stored at −20 °C. Dilutions of the compounds in complete medium were made immediately prior to the experiments to reach a maximum final concentration of 0.5% DMSO. For all the assays performed, control cells were treated with medium added with DMSO at the final concentration of 0.5%. Cyclosporin A (CsA, Sigma–Aldrich) was used as a positive control for inhibition of replication.

2.2. Cell culture

Huh7.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 10% fetal calf serum, 100 IU penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 1%

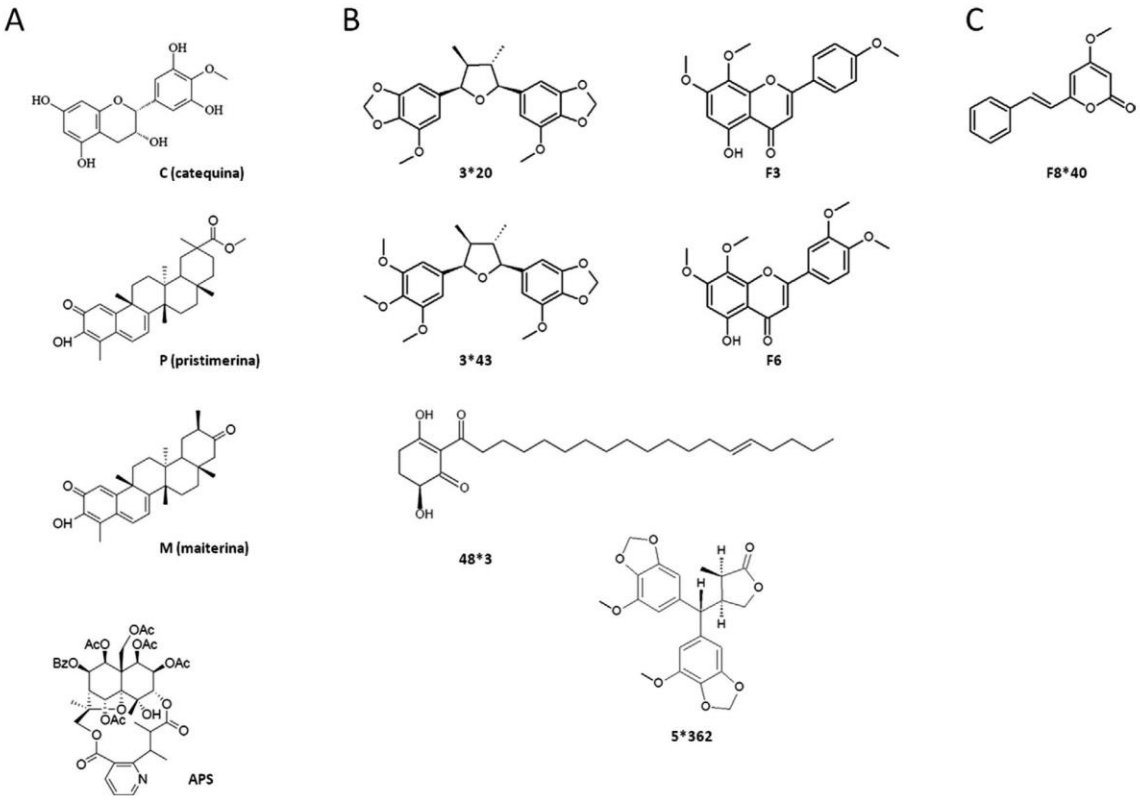

Fig. 1. Chemical structure of Brazilian natural compounds. Compounds isolated from *Maytrenus ilicifolia* (A), *Peperomia blanda* (B) and *Piper fuligineum* (C).

non-essential amino acids at 37 °C in a humidified 5% CO_2 incubator. Subgenomic replicon (SGR) harboring cell lines (genotype 2a SGR-Feo-JFH-1 (Wyles et al., 2009), genotype 1b SGR-Feo-BM4-5 (Wyles et al., 2007) and (genotype 3a – Genbank GU814264 (Saeed et al., 2012)) were maintained in DMEM with 300 μg/mL G418.

2.3. Cytotoxicity assay

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich) method. Huh7.5 cells or SGR-harboring cell lines were cultured in DMEM medium in a 96-multi-well plate and incubated at 37 °C in a humidified 5% CO_2 incubator overnight. Drug-containing medium at different concentrations was added to the cell culture being replaced every 24 h. After 48 h incubation at 37 °C, DMEM containing MTT at the final concentration of 1 mg/mL was added to each well, incubated for 1 h and replaced with 100 μl of DMSO to solubilize the formazan crystals. Surviving cells were measured by optical density (OD) of each well at 570 nm, using a spectrophotometer. Cells viability was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean optical density of the treated group and control group, respectively. All experiments were performed in triplicates and repeated at least three times. Further assays were performed considering 80% viability of treated cells.

2.4. Luciferase-based replication assay

T7 transcripts were generated from linearized DNA templates of SGR-luc-JFH-1, SGR-luc JFH-1 containing the NS5A Y93H Daclatasvir (DCV) resistance mutation or SGR-luc-JFH-1/GND luciferase subgenomic replicons (SGR) (Targett-Adams and McLauchlan, 2005). 4×10^6 Huh7.5 cells were washed and resuspended in diethylpyrocarbonate (DEPC)-treated PBS, and electroporated with SGR RNA (2–5 μg) in 0.4 cm cuvettes at 950 μF, 270 V. Cells were seeded into 96-well plates at a density of 8×10^3 per well and compounds were added at 2–4 h post-electroporation. Cells were harvested by lysis with Passive Lysis Buffer (Promega) at 4, 16, 24 and 48 h post-electroporation and HCV RNA replication was quantified by measuring luciferase activity using the Luciferase Assay System (Promega). The same assays were performed with SGR-harboring cell lines (genotype 2a SGR-Feo-JFH-1) for comparison. The effective concentration 50% (EC_{50}) was calculated using Prism (GraphPad) and cytotoxicity assays were carried out in parallel to determine the cytotoxic concentration 50% (CC_{50}) using a MTT-based system as described below. 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. SI with value of four or higher suggests that a compound have a promising antiviral activity that merit further studies.

Huh7.5 cells stably harboring the SGR-Feo-BM4-5 (Wyles et al., 2007) (genotype 1b) or SGR-Feo-S52 (genotype 3a) culture adapted mutants AII (T1056A, T1429I and S2204I) or SHI (P1220S, D1430H and S2204I) (Saeed et al., 2012) were seeded in a 96 well plates at the same cell density. Cells were treated at 4 h post seeding for 48 h with the previously determined concentration of compounds or DCV, harvested and luciferase measured.

2.5. Virus assays

For virus replication assays, 8×10^6 Huh7.5 cells were electroporated with 10 μg of Rluc-J6/JFH1 (mFL-J6/JFH-5′C19Rluc2AUbi) (Tscherne et al., 2006). Compounds were added at 2–4 h post-electroporation. Samples were harvested in Renilla lysis buffer (Promega) at 48 h post-electroporation and virus replication was quantified by measuring luciferase activity using the Renilla Luciferase Assay System (Promega).

For infection assays, Huh7.5 cells were seeded the day before the assay was carried out. Compounds were diluted to the stated final concentrations in DMEM media. Two types of experiments were carried out; Cells were infected with Rluc-J6/JFH1 virus and compounds were added. After 48 h samples were harvested and luminescence was measured. Alternatively, cells were infected with JFH1 virus (Wakita et al., 2005) for 4 h, washed extensively to remove virus and treated with the compounds. After 48 h extra cellular virus was titrated. The titer plate was fixed with 4% PFA after 48 hpi and stained for NS5A using sheep anti-NS5A (Macdonald et al., 2003) and Alexa Fluor anti-sheep 594 secondary antibody.

2.6. Western blot analysis

Cells were lysed in Glasgow lysis buffer [GLB; 10 mM Pipes–KOH (pH7.2), 120 mM KCl, 30 mM NaCl, 5 mM $MgCl^2$, 1% Triton X-100 (Sigma), 10% glycerol] (Ross-Thriepland and Harris, 2014) plus protease and phosphatase inhibitors (2 mM Na_3VO_4, 5 mM NaF, 5 mM $Na_4P_2O_7$). Fifty micrograms of protein were resolved by SDS/PAGE and transferred to a PVDF membrane using a semidry transfer apparatus. Membranes were blocked in 10% (w/v) dried skimmed milk powder in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Membranes were probed with anti-NS5A (Macdonald et al., 2003) or mouse anti-GAPDH (AbCam) in 5% (w/v) dried skimmed milk in TBS-T. The antibodies were detected with the relevant secondary horseradish peroxidase-conjugated antibody and in-house enhanced chemiluminescent reagent.

2.7. Statistical analysis

Individual experiments were performed in triplicate and all assays were performed a minimum of three times in order to confirm the reproducibility of the results. Differences between means of readings were compared using analysis of variance (one-way or two-way ANOVA) and Student t test. P values of less than 0.05 (indicated by asterisks) were considered to be statistically significant.

3. Results

3.1. Screening of compounds isolated from Brazilian plants for effects on HCV replication

To evaluate whether a panel of Brazilian natural compounds (Fig. 1) could inhibit HCV replication, we performed a screening assay using a firefly luciferase SGR construct (SGR-luc-JFH1). Initially, Huh7.5 cells were treated with 100, 10, 1 or 0.1 μM of each compound and incubated for 48 h to assess the cytotoxicity of the compounds (Fig. S1). Then, Huh7.5 cells were electroporated with SGR-luc-JFH1 and compounds were added to the cells at 4 h post-electroporation. Replication levels were assessed 48 h later by luciferase assay. The initial data showed that the purified compounds APS, 3*43, 3*20, 5*362, F3 and F8*40 (Fig. 1) significantly inhibited HCV SGR replication ($p < 0.05$) (Fig. 2A). Expression of NS5A was also significantly reduced in the presence of APS, 3*43, 3*20, 5*362, F3 and F8*40 as shown in Fig. 2B. This analysis also revealed that the compounds had no significant effect on the phosphorylation profile of NS5A, as both basal and hyper phosphorylated forms could be seen. Intriguingly, treatment of cells with compound C appeared to significantly enhance replication ($p < 0.05$) with a concomitant increase in protein expression

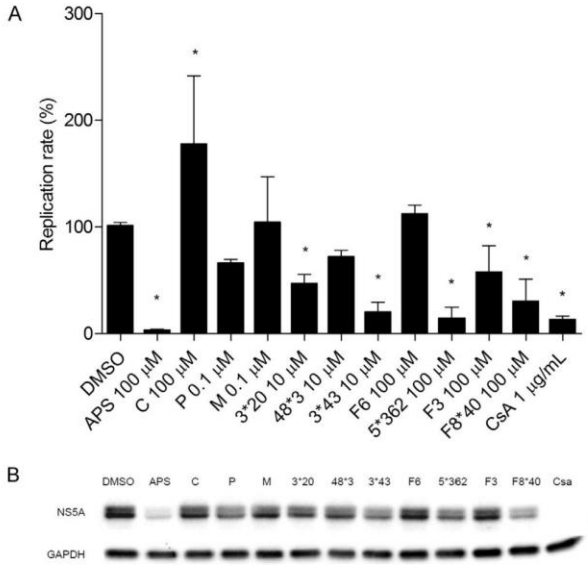

Fig. 2. Screening of plant-derived compounds for activity against HCV replication. Huh7.5 cells were electroporated with SGR-luc-JFH1, and 4 h later, specific concentrations of compounds were added. Replication efficiency was measured 48 h post-electroporation using luciferase (A) and western blotting assays (B). DMSO and cyclosporine A were used as negative and positive controls respectively. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.05$ was considered significant.

(Fig. 2B). These results demonstrated that most of the selected Brazilian natural compounds are able to inhibit HCV replication.

3.2. Inhibitory effect of Brazilian natural compounds on HCV replication

For further analysis we selected four compounds, APS, 3*43, 3*20 and 5*362, as these showed significant inhibition of HCV genome replication at non-cytotoxic concentrations. An Huh7.5 cell line stably harboring the SGR-Feo-JFH-1 replicon was treated with increasing doses of compounds and replication efficiency and cell viability were measured 48 h after compound addition. The results indicated that all four compounds APS, 3*43, 3*20 and 5*362 decreased HCV replication in a dose-dependent manner with EC_{50} of 2.3, 4.0, 8.2 and 38.9 µM, respectively (Table 1; Figs. 3 and S2). We also assayed the compounds F3 and F8*40 however we were not able to establish EC_{50} for those compounds. They reduced replication only when cytotoxic concentrations were used (data not shown) and were therefore excluded from further analysis. Subsequent studies focused on compounds APS, 3*43, 3*20 and 5*362.

3.3. Effect of the compounds on HCV IRES driven translation

We next assessed the impact of natural compounds on HCV-RNA translation, also considering compounds which did not present effects on replication in the previous assays. To this end, we transfected Huh7.5 cells with in vitro transcribed RNA of SGR-luc-JFH1 or the SGR-luc-JFH1 (GND) polymerase-defective construct (containing a mutation of the conserved GDD motif to GND) and compounds were added immediately. Luciferase values of both WT and GND constructs are shown at 4 h, which was representative of input translation. The results demonstrated that the treatment with most of the compounds did not affect HCV IRES driven translation (Fig. 4). As an exception, the compound F8*40 showed a modest yet significant reduction of luciferase levels to 80.6% ($p < 0.05$), suggesting that this compound can have a slight effect on IRES-directed translation. These data corroborate with a reduction in protein levels observed in the presence of F8*40 (Fig. 2B).

3.4. Compounds APS, 3*43, 3*20 and 5*362 prevent replication complex formation

We wished to investigate whether compounds APS, 3*43, 3*20 and 5*362 acted either on pre-existing replication complexes (RC), or by inhibiting their formation. Huh7.5 cells were electroporated with SGR-luc-JFH-1 RNA and compounds were added to the cells at 2 h post-electroporation at the defined concentrations. RNA replication was monitored for 48 h by luciferase assay in order to detect the ability of compounds to prevent RC formation. In parallel, Huh7.5 cells stably expressing SGR-Feo-JFH-1 replicons were treated with compounds and harvested at the same time points to evaluate the activity on pre-existing RCs. No significant reduction of replication levels was observed in either assay at 4 h. For both transient and stable replicons, replication decreased significantly compared to DMSO control from 16 h post-electroporation but there was no difference between the two assays (Fig. 5). In contrast, at 24 h there was marked difference between the levels of inhibition observed in the transient and stable assay formats. Specifically, the compounds were more effective on the transient replicons. At a later time point (48 h) again no difference was observed. These data are consistent with the hypothesis that these compounds block formation of RCs and have a lesser effect on pre-existing RCs. In the transient assay the luciferase levels at 4 h reflect translation from input RNA whereas luciferase activity at 24 h is a measure of RNA produced by newly formed RCs. After this time point, replication was gradually reduced over time up to 48 h, showing that the compounds were preventing replication.

The luciferase levels detected at 4 h in the stable replicon cells reflects replication by pre-existing active RCs and did not respond to treatment with the compounds. Values at 16 and 24 h reflect both pre-existing and newly formed RCs and are not affected as efficiently as the corresponding values in the transient assay, con-

Table 1
Inhibitory effect of Brazilian natural compounds on HCV replication.

Compound	EC_{50} (µM)	SI (CC_{50}/EC_{50})	Concentration assays (µM)	SGR-luc-JFH1 RC assay	SGR-JFH1 FEO RC assay	SGR-BM4-5 assay	SGR-Feo-S52 AII – SHI assay	J6/JFH1 HCVcc infection assay	JFH1 virus infection assay
				% Inhibition					
APS	2.3	58.8	50	100	94	88	78–87	99	96
3*43	4.0	4.7	12.5	92	88	75	96–98	92	100
3*20	8.2	4.0	25	87	82	32	80–86	95	100
5*362	38.9	1.9	50	68	52	62	88–88	83	38
CsA	NT	NT	1 µg/µL	93	95	94	100–100	100	100

EC_{50}, effective concentration 50%; CC_{50}, cytotoxic concentration 50%; SI, selective index; RC, Replication Complex; HCV, hepatitic C virus; NT, not tested; CsA, Cyclosporin A.

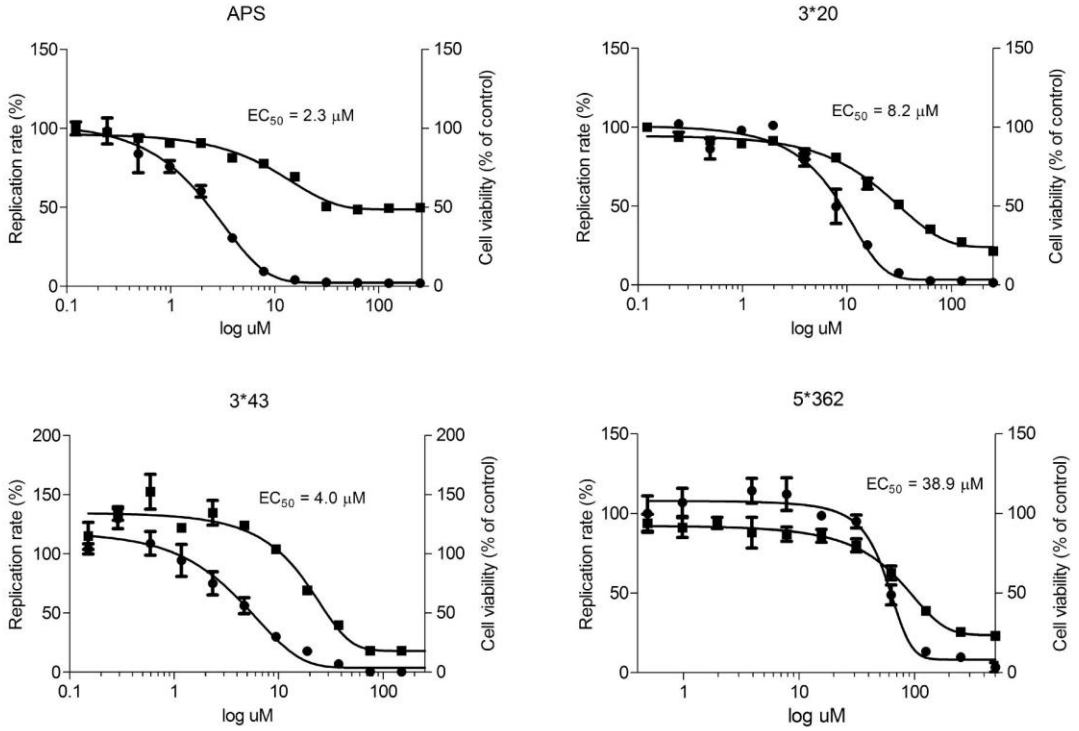

Fig. 3. Determination of EC_{50} for compounds APS, 3*20, 3*43 and 5*362. Huh7.5 stably harboring SGR-Feo-JFH-1 were incubated with compounds at concentrations over a 3-log range for 48 h. Replication efficiency was measured by luciferase assay (indicated by ●) and cellular viability measured using an MTT assay (indicated by ■). Mean values of three independent experiments each measured in triplicate including the standard deviation are represented.

sistent with the hypothesis that the compounds are predominantly inhibiting RC formation. However, we acknowledge that interpretation of these experiments is challenging and we therefore cannot rule out the possibility that these compounds inhibit both RC formation and activity.

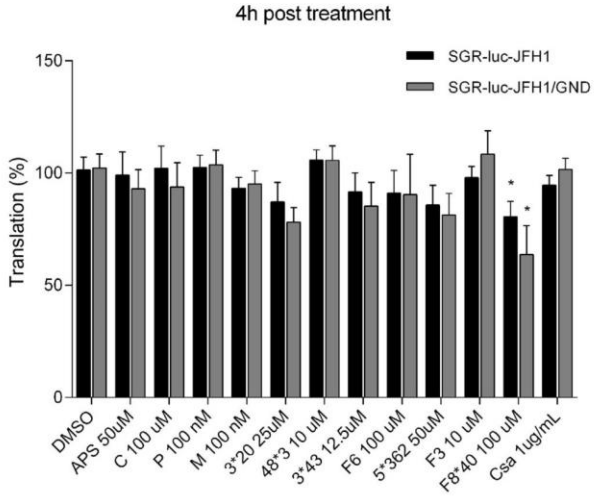

Fig. 4. Effects of compounds APS, 3*20, 3*43 and 5*362 on HCV IRES-mediated translation. Huh7.5 cells were transfected with in vitro transcripts of the SGR-luc-JFH1 or the SGR-luc-JFH-1 (GND) and compounds were added immediately. Translation levels of WT and mutant constructs are shown at 4 h post-electroporation. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.05$ was considered significant.

*3.5. Activity of compounds APS, 3*43, 3*20 and 5*362 against a DCV resistant JFH-1 SGR and genotypes 1b/3a SGRs*

Next, we investigated whether compounds APS, 3*43, 3*20 and 5*362 were able to block replication of an SGR that was resistant to one of the DAAs in current use. The most potent of these is daclatasvir (DCV) with an EC_{50} against HCV replication of less than 100 ρM (Gao et al., 2010) – however a single point mutation (Y93H in NS5A) results in ~1000-fold loss of sensitivity to DCV. Huh7.5 cells were therefore electroporated with SGR-luc-JFH1 WT or Y93H RNA and seeded in a 96 well plate. Cells were incubated from 4 to 48 h post seeding in the presence of either APS, 3*43, 3*20, 5*362 or DCV (17.6 pM), prior to lysis and measurement of luciferase activity. Reassuringly, all 4 compounds significantly inhibited both WT and Y93H SGR replicon to similar levels ($p < 0.05$) (Fig. 6).

We also evaluated the ability of the compounds to inhibit the replication of alternative genotypes of HCV. To do this we chose genotype 3a as this is increasingly common and is inherently more resistant to the new DAAs. As transient SGR for genotype 3a are not available, we utilized Huh7.5 cells stably harboring the genotype 3a derived SGR-Feo-S52 containing either the AII (T1056A, T1429I and S2204I) or SHI (P1220S, D1430H and S2204I) set of culture adaptive mutations (Saeed et al., 2012). These cells were incubated with the 4 compounds or DCV (AII: 5.2 ηM and SHI 2.4 ηM) for 48 h and harvested. Both genotype 3a SGRs were effectively inhibited by all 4 compounds ($p < 0.05$) (Fig. 6, Table 1). Additionally, we assayed the 4 compounds against genotype 1b by using Huh7.5 cells stably harboring the SGR-BM4-5 (Wyles et al., 2007). The 4 compounds were also able to significantly reduce HCV genotype 1b replication ($p < 0.05$) (Table 1). No significant differences were observed after treatment in replication with different genotypes (Fig. 6).

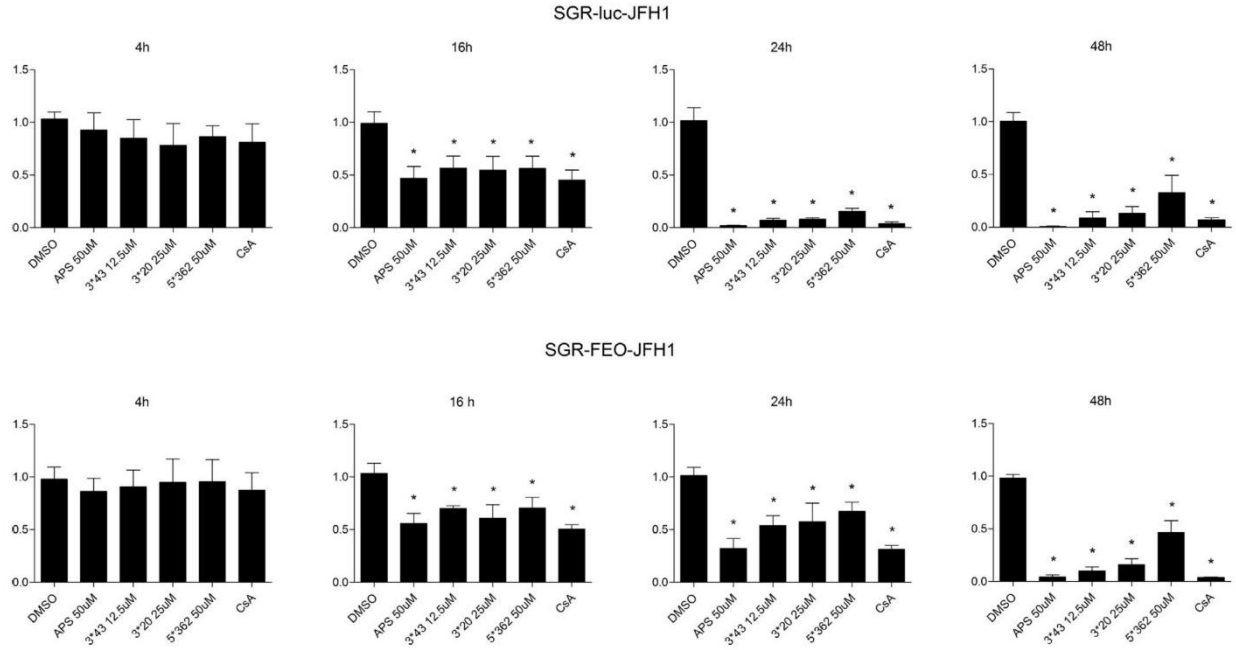

Fig. 5. Effects of compounds APS, 3*20, 3*43 and 5*362 on formation of HCV genome replication complexes. Huh7.5 cells were electroporated with SGR-luc-JFH1, and 2 h later, specific concentrations of compounds were added. Replication efficiency was measured at 4, 16, 24 and 48 h post-electroporation using luciferase to assess the effect of compounds in preventing the formation of RCs (upper panel). Huh7.5 harboring SGR-Feo-JFH-1 were treated with indicated concentrations of compounds for 48 h. Replication efficiency was assessed to check the effect of compound on pre-existing RCs (lower panel). Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.01$ was considered significant.

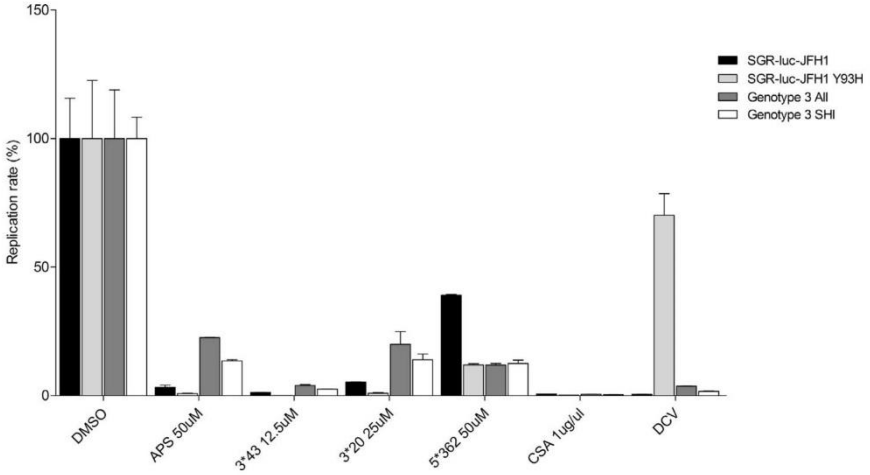

Fig. 6. Activity of compounds APS, 3*43, 3*20 and 5*362 against a DCV resistant mutant and genotypes 1b/3a SGRs. Huh7.5 cells electroporated with SGR-luc-JFH-1 or a corresponding NS5A Y93H DCV resistance mutation containing RNA, or Huh7.5 cells stably harboring the SGR-Feo genotype 3a AII (T1056A, T1429I and S2204I) or SHI (P1220S, D1430H and S2204I) (Saeed et al., 2012) culture adaptive mutations were treated 4 h post electroporation/seeding for 48 h with the previously determined concentration of compounds or of DCV (JFH1: 17.6 pM; genotype 3a AII: 5.2 nm and SHI 2.4 nm), harvested and luciferase measured. $P < 0.05$ was considered significant.

3.6. HCVcc infection is inhibited by Brazilian natural compounds

To determine the effect of the compounds APS, 3*43, 3*20 and 5*362 on genome replication in the context of full length virus, we first used the Rluc-J6/JFH1 (FL-J6/JFH-5′C19Rluc2AUbi reporter) – a genotype 2a J6/JFH1 chimeric virus with *Renilla* luciferase fused to the HCV Core protein (Tscherne et al., 2006). Huh7.5 cells were electroporated with in vitro transcribed Rluc-J6/JFH1 RNA prior to incubation with the 4 compounds at 4 h. Replication was assessed by measuring *Renilla* luciferase levels at 48 h post-electroporation. Consistent with the SGR data, these compounds effectively blocked Rluc-J6/JFH1 replication (Fig. 7A). Protein expression levels were also significantly reduced in the presence of the compounds (Fig. 7B). APS was the most effective inhibitor of HCVcc replication, reducing replication by 500 fold at a concentration of 50 μM. CsA was included as a control for inhibition of genome replication.

To confirm that the compounds inhibited genome replication in the context of virus infection (as compared to RNA electroporation)

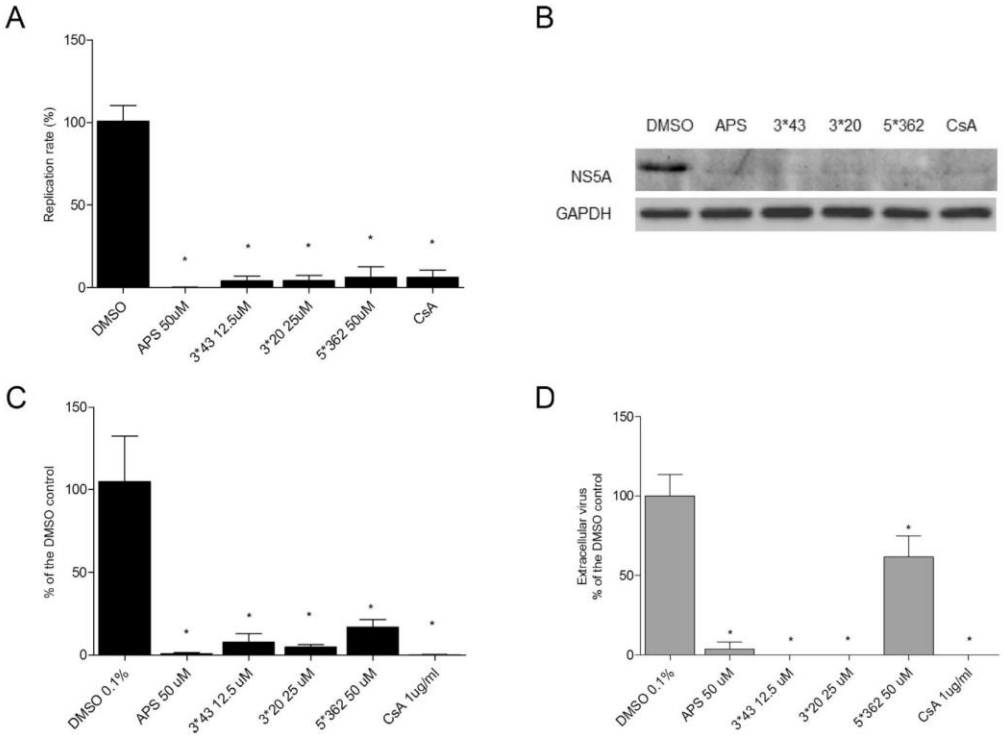

Fig. 7. Effects of compounds APS, 3*20, 3*43 and 5*362 on the HCV virus production. Huh7.5 cells were electroporated with Rluc-J6/JFH1 RNA, and 4 h later, specific concentrations of compounds were added. Replication efficiency was measured 48 h post-electroporation by measuring *Renilla* levels (A) and western blotting assays (B). Huh7.5 cells were infected with Rluc-J6/JFH1 virus and compounds were immediately added. Samples were harvested after 48 h and luminescence was measured (C). Cells were infected with JFH1 virus for 4 h, washed extensively to remove virus and treated with the compounds. After 48 h samples were harvested, and extracellular virus titrated (D). Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. $P < 0.05$ was considered significant.

we infected Huh7.5 cells with Rluc-J6/JFH1 HCVcc virus in the presence or absence of compounds for 48 h and again measured *Renilla* luciferase. As expected, HCVcc infection was significantly reduced in the presence of APS, 3*43, 3*20 and 5*362 (Fig. 7C, Table 1).

We further confirmed the anti-HCV activity of the compounds by quantifying extracellular levels of virus after incubation of infected cells with the compounds. In this case Huh7.5 were infected with JFH1 virus for 4 h and subsequently treated for 48 h. Levels of released virus were significantly reduced by all 4 compounds (Fig. 7D, Table 1), although in this context 5*362 had a less dramatic effect.

4. Discussion

HCV infection is a serious health problem and the new therapeutic regimes for the treatment of patients are very expensive and are associated with significant risk for the development of resistance. Therefore, the search for alternative therapies against HCV remains a valid aim, particularly in the context of low and middle-income countries that will not be able to afford the new drugs.

In this study, we screened a set of compounds extracted from Brazilian plants and we identified four compounds with potent inhibitory activity on HCV replication. These compounds are APS (EC_{50} = 2.3 μM), a natural alkaloid isolated from *M. ilicifolia*, the tetrahydrofuran lignans 3*43 (EC_{50} = 4.0 μM) and 3*20 (EC_{50} = 8.2 μM) and the secolignan 5*362 (EC_{50} = 38.9 μM) from *P. blanda*. Our data demonstrated that HCV RNA and protein levels were dramatically reduced when the inhibitory effects of these compounds on HCV replication were analyzed using either subgenomic reporter SGR-Feo-JFH1 and the full-length Rluc-J6/JFH1.

The antiviral activity of alkaloids and lignans on HCV life cycle was previously described. Honokiol, a lignan isolated from leaves of *Magnolia officinais*, showed to have multiple effects on HCV infection, inhibiting entry, translation and replication in Huh7.5 cells using HCVcc, HCVpp, and subgenomic replicons (Lan et al., 2012). The reduction of protein and RNA levels was also shown by the treatment of cells in a subgenomic replicon system with 3-hydroxy caruilignan C (3-HCl-C) isolated from *Swietenia macrophylla* stems, which also increased the replication suppression when combined with IFN-α and protease or polymerase inhibitors (Wu et al., 2012). The flavonolignan Silymarin extracted from *Silybum marianum* (milk thistle) has shown recently to block virus entry, RNA and protein expression, virus production and cell to cell spread of virus (Wagoner et al., 2010). Additionally, this compound demonstrated a hepatoprotective effect on treated cells (Polyak et al., 2010). Myriberine A is an alkaloid isolated from *Myrioneuron faberi* and demonstrated inhibition against the HCV life cycle in vitro with a good therapeutic index (CC_{50}/EC_{50}) of greater than 12.0 in vitro for non-cytotoxic concentration (Huang et al., 2013). Oxymatrine and matrine are the two major alkaloid aqueous extracts from the *Sophora* root. Oxymatrine is reported to have antiviral activity against HCV in cell cultures and has shown hepatoprotective activity in an animal study (Chen et al., 2001; Liu et al., 1994). In a clinical perspective, the components Oxymatrine and matrine found in sophora roots have shown to reduce viral load and inhibition of liver fibrosis (Hussein et al., 2000; Kitazato et al., 2007). All these studies showed that natural lignans and

alkaloids have potential for development as new bioactive molecules against HCV. Moreover, the extra effects of those compounds on HCV life cycle and clinical data demonstrated that further Brazilian compounds can present extra mode of action which need to be investigated.

Our results demonstrated that the compounds APS, 3*43, 3*20 and 5*362 decreased HCV replication in a dose-dependent manner and acted to prevent RC formation. Using an Huh7.5 cell line stably harboring a subgenomic reporter we were able to demonstrate that treatment with compounds for 4 h did not inhibit RCs. In contrast, replication levels were reduced from 16 h of treatment when new RCs were formed, similar to transient assay performed with subgenomic reporter, suggesting that these compounds are acting on new RCs. In a previous study, Lyn et al. demonstrated that the treatment of Huh7.5 with lipid metabolism inhibitors disrupted the replication complexes by changing density and distribution of lipid droplets and consequently changing HCV RNA location which inhibited HCV replication (Lyn et al., 2009). However, the action of the compounds on pre-existing RCs was not clearly addressed.

In this context, reduction of HCV RNA and protein levels observed in our data could be consequence of the direct inhibition of viral enzymes (Ahmed-Belkacem et al., 2010; Bachmetov et al., 2012; Wagoner et al., 2010), the interference of these compounds with cellular factors involved in virus replication, or by inducing cellular antiviral effectors as has been shown previously (Gonzalez et al., 2009; Polyak et al., 2007; Rinck et al., 2001; Yi et al., 2011).

We were also able to show that the antiviral activity of Brazilian naturally occurring compounds was independent of HCV genotype and was not affected by variants described to confer resistance to Daclatasvir, a highly potent direct-acting antiviral drug targeting NS5A (Gao et al., 2010; Guedj et al., 2013; Lemm et al., 2010). Other plant-derived compounds have showed to be active on HCV life cycle independently of viral genotype or subtype (Choi et al., 2014; Haid et al., 2012), presenting an additional benefit to the current interferon-based HCV therapies or to the directly target antivirals which efficacy depend on viral genotypes. Haid et al. also demonstrated that viral resistance did not compromise the antiviral activity of a synthetic flavonoid-like compound against wild-type and mutant virus (Haid et al., 2012).

Moreover, most of the compounds did not affect HCV IRES driven translation indicating that the major antiviral mechanism is to directly inhibit virus genome replication. As an exception, the compound F8*40, a natural kavalactone isolated from *Piper fuligineum*, showed significant but not dramatic effect on IRES-directed translation and corroborated with protein levels reduction in the presence of this compound. This data can suggest that the mode of action of this compound is related to the inhibition of IRES-mediated translation. The effect in baseline IRES translation was earlier showed by Gonzalez et al. by treating cells with the plant-derived flavonoid Quercetin which also had a strong inhibitory effect at 50 μM on HCV production in cell culture (Gonzalez et al., 2009).

In summary, our data demonstrate that natural alkaloids and lignans isolated from Brazilian plants dramatically inhibited HCV replication in vitro. Further analyses are in progress to elucidate other modes of action of those compounds. These data are the first description of Brazilian natural compounds possessing anti-HCV activity and as such may be useful in the development of future antiviral interventions for HCV and possibly other viral infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.12.018.

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CAPÍTULO V:

CONCLUSÕES GERAIS

CONCLUSÕES GERAIS

- Compostos naturais isolados de diferentes origens possuem atividade antiviral no ciclo replicativo do HCV.

- A análise da atividade dos compostos no ciclo replicativo viral demonstrou que um composto natural pode agir em mais de uma etapa do ciclo realizada pelo vírus durante a infecção das células hospedeiras.

- Os compostos isolados de *C. durissus terrificus* apresentaram inibição das etapas de entrada, replicação e liberação do HCV.

- Os compostos isolados de *P. nitens* apresentaram efeito na entrada dos vírus nas células hospedeiras, por mecanismos de ação direta nas partículas virais e/ou por efeito protetor ainda não elucidado nas células hospedeiras.

- Compostos naturais podem ser uteis para investigar as interações do HCV com as células hospedeiras, bem como no desenvolvimento de futuras terapias para o tratamento da hepatite C.

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