



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
Campus de São José do Rio Preto

Daniel Oliveira Silva Martins

**Atividade antiviral *in vitro* de derivados de sulfadoxina contra o vírus
Chikungunya**

São José do Rio Preto
2024

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor 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.

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RESUMO

O vírus chikungunya (CHIKV) é um arbovírus transmitido através da picada de mosquitos do gênero *Aedes*, responsável pela Febre Chikungunya, uma doença com sintomas semelhantes aos da dengue, como febre alta, artralgia e dores nas articulações. Os pacientes infectados pelo CHIKV ainda podem desenvolver outras condições clínicas como a síndrome de Guillain-Barré, ou artralgias crônicas altamente debilitantes. O CHIKV já foi detectado em mais de 100 países, e até o final de junho de 2023 mais de 200 mil casos de Febre Chikungunya foram reportados no mundo. A febre chikungunya foi a doença que mais resultou em incapacitação dos indivíduos infectados, dentre as doenças mais prevalentes no Brasil, resultando em um gasto de aproximadamente R\$ 2,3 bilhões no manejo de CHIKV e outras arboviroses. Atualmente, não existem antivirais licenciados contra a infecção por CHIKV, sendo o tratamento baseado em analgésicos e anti-inflamatórios para aliviar os sintomas, demonstrando a necessidade de descobrir compostos com atividade anti-CHIKV. Neste trabalho nos demonstramos a atividade antiviral de Sulfadoxina (SFX), um fármaco aprovado pela Agência Reguladora de Alimentos e Medicamentos dos Estados Unidos (FDA - *the United States of America Food and Drug Administration*), e seus derivados complexado com prata(I) (Ag-SFX), com salicilaldeído *Schiff* base (SFX-L) e com prata(I) e salicilaldeído *Schiff* base (AgSFX-SL), utilizando células BHK 21 infectadas com CHIKV-*nanoluc*, um vírus carregando o gene *reporter nanoluciferase*. Os resultados demonstraram que SFX a 50 μ M inibiu 34% da replicação do CHIKV, enquanto AgSFX e SFX-SL a 2 μ M apresentaram atividade antiviral de 84% e 89%, respectivamente. A maior atividade antiviral foi observada com a molécula AgSFX-SL a 2 μ M, que reduziu 95% da replicação do CHIKV. Ensaio de tempo de adição dos compostos mostraram que AgSFX-SL inibiu os estágios de entrada e pós-entrada em 79% e 81% respectivamente, e também protegeu as células da infecção em 51%. AgSFX e SFX-SL também reduziram significativamente a atividade antiviral na entrada e pós-entrada. Coletivamente, nossos achados sugerem que a adição de Ag e/ou SL à SFX aprimorou a atividade antiviral deste fármaco contra CHIKV.

Palavras-chave: Antivirais. Arbovírus. Vírus chikungunya. Sulfadoxina. Prata(I). *Schiff* base.

ABSTRACT

Chikungunya virus (CHIKV) is an arbovirus transmitted through the bite of *Aedes* mosquitoes, responsible for Chikungunya fever, a dengue-like disease characterized by high fever, arthralgia, and joint pain. The CHIKV infected patients can also develop other clinical condition as the Guillain-Barré syndrome or strongly disabling chronic arthralgias. CHIKV was identified in over 100 countries, and up to June 2023 more than 200 000 cases of Chikungunya fever were reported worldwide. Chikungunya fever was the disease that resulted in the highest incapacitation of infected individuals, among the most prevalent diseases in Brazil, resulting in an expenditure of approximately R\$ 2.3 billion in the management of CHIKV and other arboviruses. Currently, there is no antiviral licensed to treat CHIKV infection, and the treatment is based on analgesic and anti-inflammatory drugs to relief symptoms, urging the need to discover compounds with anti-CHIKV activity. Here we demonstrated the antiviral activity of Sulfadoxine (SFX), a drug approved by the United States of America Food and Drug Administration (FDA), and its derivatives complexed with silver(I) (Ag-SFX), with salicylaldehyde Schiff base (SFX-SL), and with silver(I) plus salicylaldehyde Schiff base (AgSFX-SL), using BHK-21 cells infected with CHIKV-*nanoluc*, a marker virus carrying *nanoluciferase* reporter. The results demonstrated that SFX at 50 μ M inhibited 34% of CHIKV replication, while AgSFX and SFX-SL at 2 μ M increased anti-CHIKV activity to 84% and 89%, respectively. The highest anti-CHIKV action was observed to AgSFX-SL at 2 μ M, which reduced 95% of CHIKV replication. Time of drug-addition assays showed that AgSFX-SL inhibited viral entry and post-entry stages in 79% and 81%, respectively, and protected cells from infection in 51%. AgSFX and SFX-SL also significantly decreased viral entry and post-entry. Collectively, our findings suggest that the addition of Ag(I) ion and/or SL to SFX improved its antiviral activity against CHIKV.

Keywords: Antiviral. Arboviruses. Chikungunya virus. Sulfadoxine. Silver(I). Schiff base.

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1. INTRODUÇÃO

A relação da humanidade com os vírus é longa, mas pode-se dizer que foi intensificada no período neolítico, cerca de 12 mil anos atrás, quando os humanos passaram a se organizar em grupos agrícolas fixos. Estes grupos passaram a crescer em termos populacionais e, em consequência, na apresentação de doenças, inclusive aquelas causadas por vírus (BOCQUET-APPEL, 2011). Os principais vírus conhecidos por afetarem as primeiras grandes sociedades humanas são os vírus da varíola e do sarampo, que causaram grandes impactos sociais e mudaram o curso da história mundial (FURUSE; SUZUKI; OSHITANI, 2010; LI et al., 2007). Com os avanços tecnológicos, revolução industrial e o aumento da população mundial nas últimas décadas, as mudanças ambientais e climáticas decorrentes das ações antropogênicas, tais como, urbanização, desmatamentos, monoculturas, e construção de grandes estradas e hidrovias, promoveram um aumento no contato com novos microrganismos (LINDAHL; GRACE, 2015). Associado a este processo, a globalização e o aumento das relações comerciais e expansão da movimentação humana ao redor do globo favoreceu a disseminação de patógenos (FINDLATER; BOGOCH, 2018).

Neste contexto, os vírus são grandes causadores de doenças humanas, e incluindo os que são transmitidos por vetores artrópodes hematófagos, os arbovírus. Os principais arbovírus identificados e reportados em países subdesenvolvidos, ou países entre os trópicos, incluem os vírus da dengue (DENV), Chikungunya (CHIKV), Orthoflavivirus zikaense (ZIKV), Oropouche (OROV) e da febre amarela (YFV), que provocam importantes doenças humanas com grande impacto social e econômico (DONALISIO; FREITAS; ZUBEN, 2017; FIGUEIREDO, 2007b; MADEWELL, 2020; SAKKAS et al., 2018).

Apesar dos impactos dos arbovírus para a população mundial, não existem atualmente antivirais licenciados para o tratamento destas infecções, resultando na necessidade de estudos científicos na busca de substâncias candidatas a fármacos. Outro ponto importante é que, com exceção da febre amarela (HANSEN; BARRETT, 2021), dengue (TORRES-FLORES; REYES-SANDOVAL; SALAZAR, 2022) e chikungunya (SCHNEIDER et al., 2023), também não há vacinas licenciadas contra esses vírus. O quadro clínico inicial das arboviroses é semelhante, caracterizando-se por febre, dores no corpo e dores de cabeça. Entretanto, as doenças causadas por esses vírus diferem-se na patologia clínica. Na febre chikungunya a infecção pode se tornar crônica, caracterizada por dores incapacitantes nas articulações em cerca de 25 a 40% dos acometidos (LÁZARI et al., 2023). De acordo com o estudo realizado por Teich e colaboradores, a febre chikungunya foi a doença que mais resultou em incapacitação dos indivíduos infectados, dentre as doenças mais prevalentes no Brasil, resultando em um

gasto de aproximadamente R\$ 2,3 bilhões no manejo de CHIKV e outras arboviroses (TEICH; ARINELLI; FAHAM, 2017). Desta forma, além dos óbitos resultantes da infecção por CHIKV, o Brasil também enfrenta impactos econômicos relacionados à circulação desse arbovírus. Traz-se à ciência o desafio para a busca de múltiplos manejos para o combate da doença, como vacina, combate aos vetores e descoberta de antivirais, com o objetivo de mitigar os impactos da prevalência desse vírus em território brasileiro.

1.1. Arboriões

Arboviroses são infecções virais que são transmitidas por artrópodes, como mosquitos e carrapatos hematófagos (DAVIS; BECKHAM; TYLER, 2008). O termo arbovírus é uma abreviação de "vírus transmitidos por artrópodes" (do inglês, *Arthropod-borne viruses*). Essas doenças são causadas por uma variedade de vírus pertencentes a diferentes famílias, como *Flaviviridae*, *Togaviridae* e *Bunyaviridae*, dentre outras. Os principais exemplos de arbovírus são: DENV, ZIKV, YFV e CHIKV (GUBLER, 2006; WEAVER et al., 2018).

Por serem vírus obrigatoriamente vetorizados na infecção humana, o controle e prevenção das arboviroses frequentemente envolvem medidas para reduzir a população de vetores e educação ambiental e sanitária sobre medidas de proteção pessoal, como o uso de repelentes, roupas de manga comprida e a eliminação de criadouros de mosquitos (KOERICH; SANT'ANNA; HUIITS, 2022). Em áreas onde essas doenças são endêmicas, a vigilância e o monitoramento são essenciais para detectar surtos precocemente e implementar medidas de controle eficazes (LORENZ et al., 2017).

O Brasil é o maior país em extensão territorial e população da América do Sul, com aproximadamente 204 milhões de habitantes (IBGE, 2022). O país possui cerca de 30% do território coberto por florestas e outros ecossistemas naturais, que propiciam a manutenção de diferentes ciclos zoonóticos de arboviroses. Até o ano de 2017 foram isolados 200 tipos de arbovírus no país, sendo que 40 destes podem causar doenças em humanos (FIGUEIREDO, 2007a). Apesar dessa quantidade, os arbovírus que mais impactam o país são DENV, ZIKV e CHIKV (DONALISIO; FREITAS; ZUBEN, 2017).

1.2. A Febre Chikungunya

O CHIKV é o patógeno causador da Febre chikungunya, doença que teve seu primeiro relato descrito em 1952 na atual Tanzânia no continente africano (LUMSDEN, 1955). Apesar de possuir sintomas semelhantes aos da dengue, como febre, dores de cabeça e dores no corpo, a doença se diferencia por causar fortes dores nas articulações, o que pode levar os indivíduos

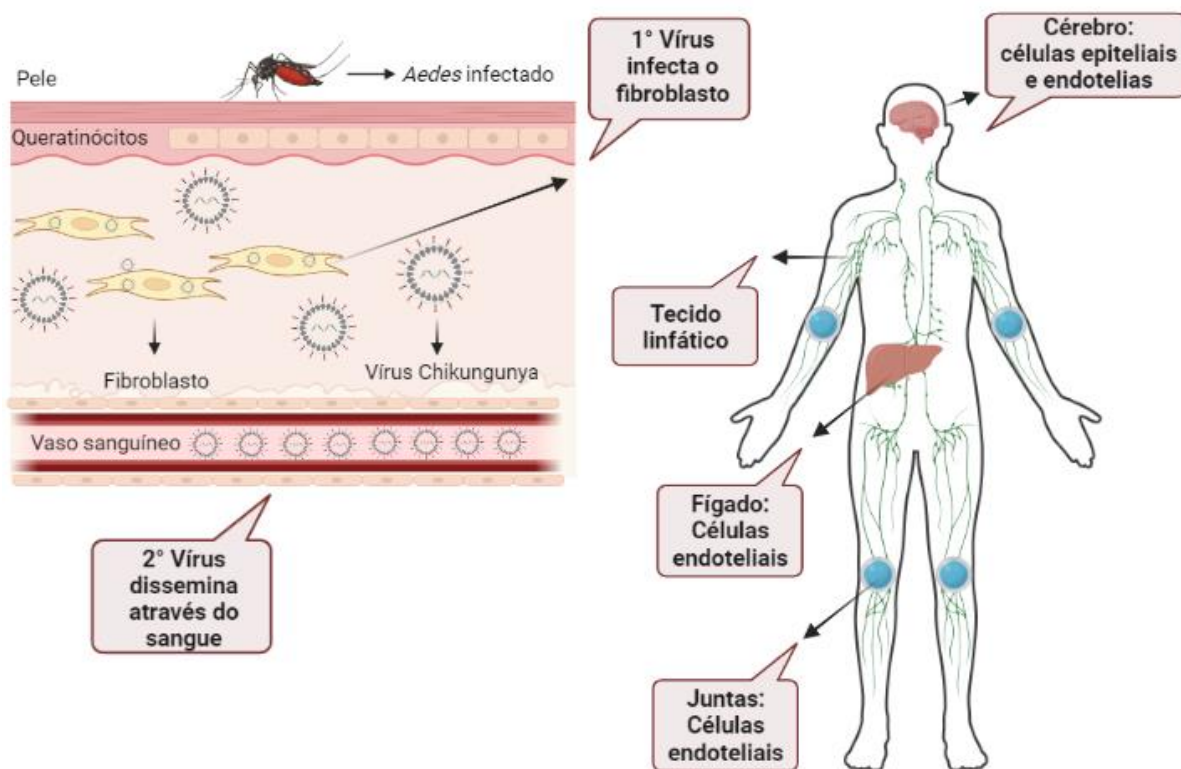
infectados a assumirem uma postura curvada/contorcida/dobrada. A palavra chikungunya vem do dialeto Makonde e significa “aqueles que se dobram”, referindo-se à aparência curvada dos pacientes devido às fortes dores nas articulações (WEAVER; LECUIT, 2015).

O CHIKV foi responsável por pequenos surtos na África e Ásia durante a década de 1960, e por milhares de casos nos países entre os trópicos e subtropicais (MAVALANKAR; SHASTRI; RAMAN, 2007; NJENGA et al., 2008; NUNES et al., 2015; SCHWARTZ; ALBERT, 2010a; VAN BORTEL et al., 2014; WATSON, 2007). De acordo com o Centro Europeu de Prevenção e Controle das Doenças (ECDC), até julho 2023 foram registrados cerca de 300 mil casos de Febre chikungunya no mundo (ECDC, 2023). O Brasil é responsável por aproximadamente 60% dos casos, seguido por Paraguai, Argentina, Bolívia, Ásia e Tailândia (ECDC, 2023).

A Febre chikungunya é uma doença viral, a qual a transmissão do vírus ocorre principalmente pela picada de mosquitos do gênero *Aedes*. Os pacientes infectados apresentam como sintomas a febre elevada ($>38.5^{\circ}\text{C}$), calafrios, dores musculares e nas articulações, fraqueza, dor de cabeça, erupções cutâneas e náuseas (CAGLIOTI et al., 2013; MOHAN et al., 2010). De modo geral, esse quadro clínico dura de 4 a 14 dias, seguido de uma completa recuperação do paciente (SCHWARTZ; ALBERT, 2010b). Porém, a infecção aguda pode progredir para uma condição crônica da Febre Chikungunya, que representa um dos maiores agravantes da infecção desse vírus quando comparado a outras arboviroses. Os sintomas da infecção são caracterizados por dores intensas, principalmente nas articulações e músculos (MORO et al., 2012; PAIXÃO et al., 2018). Apesar de não ser considerado um vírus neurotrópico, há relatos de complicações neurológicas decorrentes da infecção por CHIKV, como meningites, convulsões, síndrome de Guillain-barré e paralisia flácida aguda (ARPINO; CURATOLO; REZZA, 2009; MAZAUD et al., 1971; MEHTA et al., 2018).

A patogênese inicia-se assim que a fêmea infectada do mosquito do gênero *Aedes* pica o ser humano e transmite o vírus (GÉRARDIN et al., 2008). Primariamente, o vírus infecta as células epiteliais e endoteliais, multiplicando-se principalmente nas células da pele e fibroblastos, e se espalha pelo sangue e em seguida para os linfonodos, fígado e para as articulações, instalando uma infecção aguda entre o segundo e quinto dia de infecção (**Figura 1**) (GANESAN; DUAN; REID, 2017; ROBIN et al., 2010; TALARMIN et al., 2007). A doença então pode ser dividida em uma fase aguda e uma crônica, sendo que a primeira ocorre durante as duas primeiras semanas de infecção (THIBERVILLE et al., 2013a) e a segunda quando o paciente manifesta artralgia por períodos mais longos que duas semanas, podendo chegar a anos (MORO et al., 2012).

Figura 1. Patogênese da infecção pelo CHIKV.



Fonte: Adaptado de (SCHWARTZ; ALBERT, 2010b) e criado com BioRender.com

Legenda: Figura representando a infecção do CHIKV e os principais locais onde o vírus se replica.

A E2 é a principal proteína do vírus envolvida no processo de adsorção do vírus aos receptores celulares, para que ocorra a infecção. (VAN DUIJL-RICHTER et al., 2015). Os principais receptores celulares em humanos que o vírus interage são: proibitina I (PHB1), uma proteína de membrana expressa em vários tecidos e tipos celulares (WINTACHAI et al., 2012b); glicosaminoglicanos (GAGs), polissacarídeos carregados negativamente que são expressos em membranas celulares e domínios de mucina (MCALLISTER et al., 2020); e imunoglobulina I de célula T (TIM-I), uma glicoproteína também expressa em uma variedade de tipos celulares (KIRUI et al., 2021). Em células de mosquito, o principal receptor que o vírus interage são do tipo Adenosina Trifosfato (ATP) sintase β (ATPS β) (FONGSARAN et al., 2014).

Depois que o CHIKV reconhece os receptores e infecta as células é observado uma indução da resposta exacerbada de interferon do tipo I (IFN-I, IFN- α e IFN β), que irão por sua vez ativar uma cadeia de fatores da atividade imune inata para combater a infecção viral (FROS; PIJLMAN, 2016). Também é observada a elevação de Interleucina 6 (IL-6), IL- β e Fator de Necrose Tumoral alfa (TNF α), que são pirogênicos e estão diretamente associadas aos sintomas

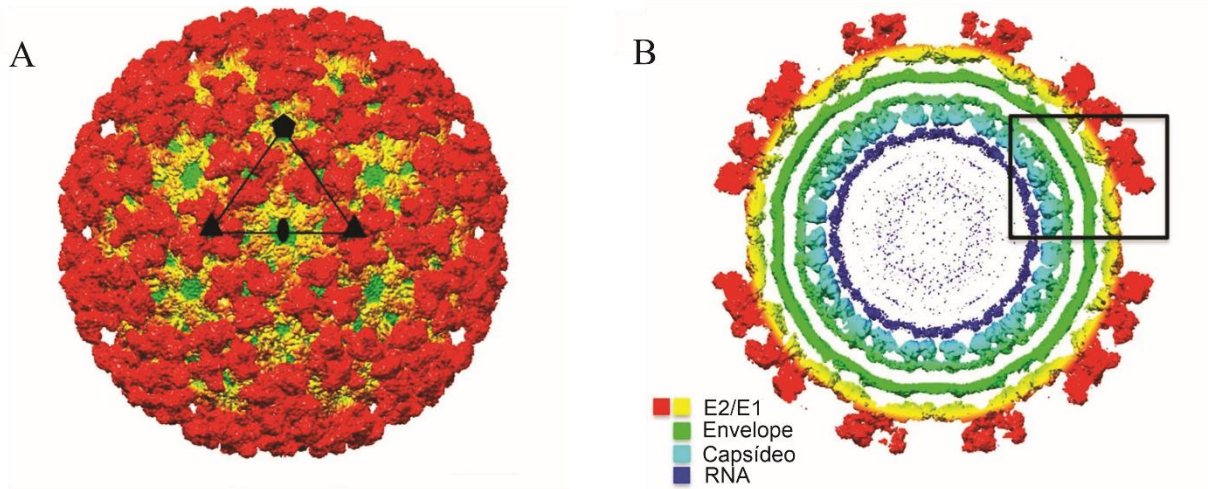
de febre e artralgia (NG et al., 2009). Além disso, células NK (*natural killer*) estão diretamente envolvidas na patogênese da febre chikungunya, principalmente na sua fase aguda. Aparentemente, estas células invadem o líquido sinovial das articulações e promovem um processo inflamatório, que está diretamente associado à artralgia, um dos principais e mais característicos sintomas da infecção por CHIKV (HOARAU et al., 2010).

A resposta imune adaptativa é mediada por células B e T. De modo geral, observa-se uma resposta imune humoral robusta que resulta na produção de anticorpos IgG e IgM contra o CHIKV (LUM; NG, 2015), sendo que a maioria dos anticorpos IgG são direcionados a porção N-terminal da proteína E2 (KAM et al., 2012; SMITH et al., 2015). No início fase aguda da doença observa-se uma elevação no número de células T CD8⁺, que são responsáveis por destruir as células infectadas, enquanto no fim da fase aguda ou na fase crônica, ocorre um maior perfil de células T CD4⁺ (WAUQUIER et al., 2011).

1.3. CHIKV

O CHIKV pertence à família *Togaviridae* e ao gênero *Alphavirus* (POWERS et al., 2001). A partícula viral de aproximadamente 70 nm de diâmetro é caracterizada pela presença de um envelope viral formado pelo heterodímero E1-E2 associado a proteína 6K (**Figura2**) (GAEDIGK-NITSCHKO; SCHLESINGER, 1990; PHILLIPS, 2015). A proteína E1 é responsável pela fusão do envelope viral com a membrana da célula hospedeira, enquanto a E2 se liga aos receptores presentes nessa membrana. A proteína 6k atua como uma viroporina viral (*viraporin*), que modifica a membrana da célula hospedeira e serve como uma facilitadora para a liberação dos vírus resultantes da nova progênie viral (DEY et al., 2019; YAP et al., 2017a). O capsídeo viral icosaédrico é formado pela combinação de monômeros da proteína de capsídeo (C), de aproximadamente 30 kDa, que se ligam ao RNA viral (THOMAS et al., 2013) (**Figura2**).

Figura 2. Estrutura da partícula viral do Chikungunya.

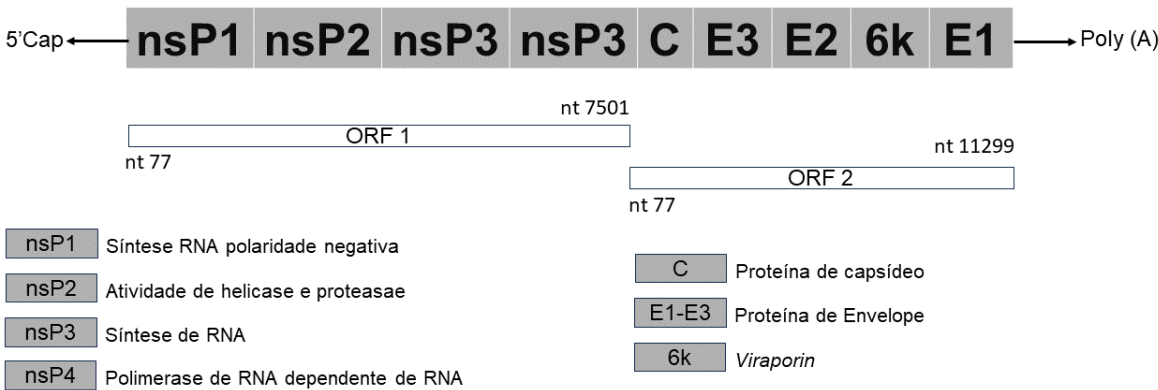


Fonte: Adaptado de (YAP et al., 2017b).

Legenda: Figura apresentando a estrutura do CHIKV. (A) Estrutura tridimensional cristalizada da partícula viral do CHIKV com estrutura icosaédrica marcada com setas pretas. (B) Corte transeccional com estruturas indicadas de acordo com a cor na legenda.

O material genético do vírus é constituído de uma fita simples de RNA polaridade positiva, com aproximadamente 12 kb (**Figura 2**), dividida em duas regiões abertas de leitura (ORFs – *open reading frames*): ORFs 1 e 2. A ORF1 codifica as proteínas não estruturais (nsPs) 1, 2, 3 e 4, e a ORF2 codifica as proteínas estruturais do capsídeo (C), E3, 6k e E1 (KHAN et al., 2002; THIBERVILLE et al., 2013b) (**Figura 3**). Além das regiões traduzíveis, o genoma viral possui 3 regiões não traduzíveis (UTRs – *non-translated regions*).

Figura 3. Genoma do CHIKV.



Fonte: Adaptado de (JAIN et al., 2017; SOLIGNAT et al., 2009).

Legenda: Representação do genoma do CHIKV, contendo as ORFs 1 e 2, que codificam as proteínas não estruturais e estruturais do vírus, respectivamente.

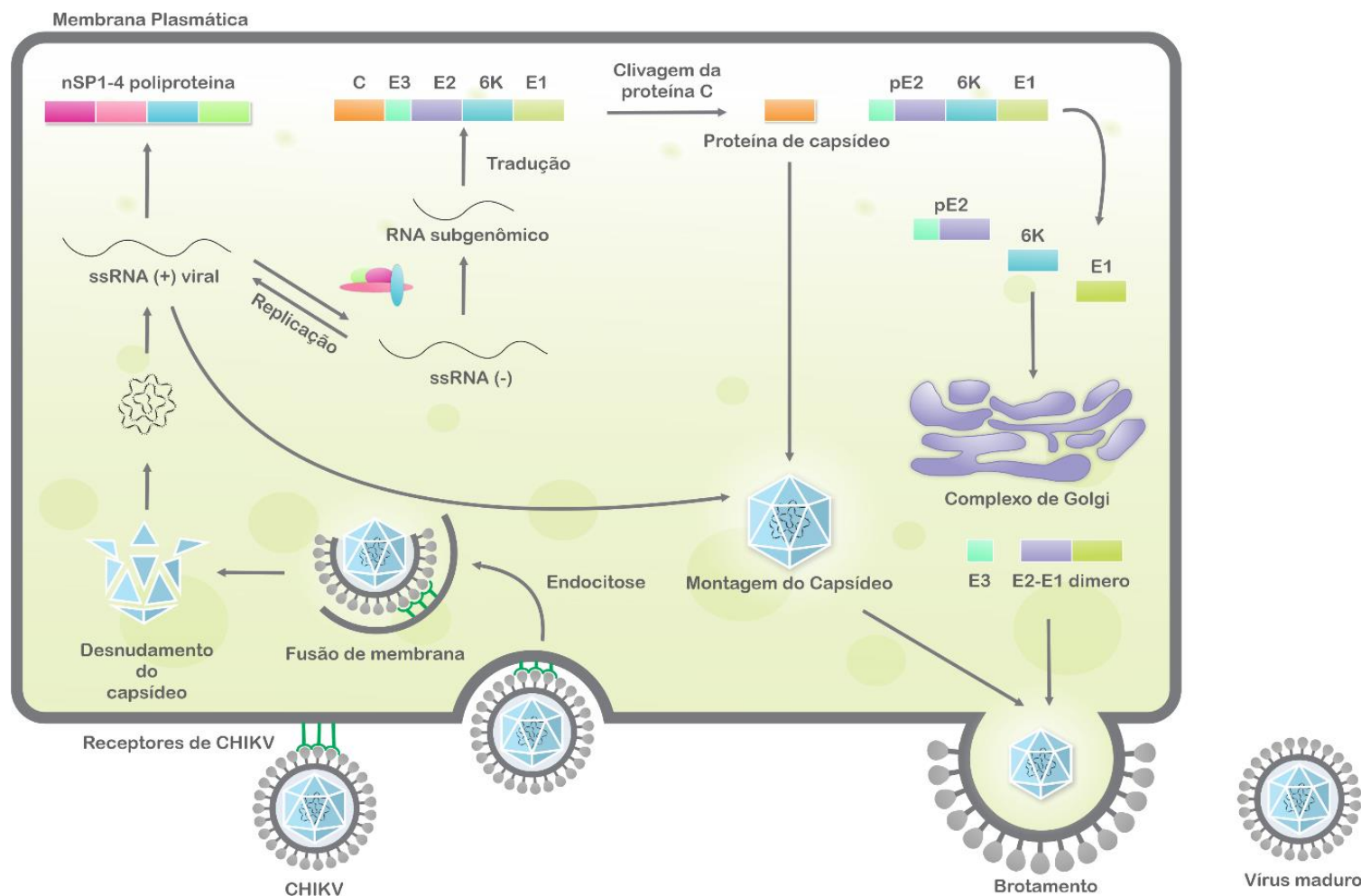
1.4. O ciclo replicativo do CHIKV

O CHIKV inicia o seu ciclo replicativo a partir da adsorção aos receptores celulares glicosaminoglicanos (GAGs) (ACHARYA et al., 2015), proibitina I (PHB1) (WINTACHAI et al., 2012a) e domínio de mucina e imunoglobulina de célula T 1 (TIM-1) (KIRUI et al., 2021), em células de mamíferos, e ATP sintase beta (ATPS β) (FONGSARAN et al., 2014), em células de mosquito. Após adsorção, o vírus é internalizado por endocitose mediada por clatrin, por invaginações da membrana citoplasmática com a consequente formação de endossomos, ocorrendo então a penetração viral. Além desse mecanismo, o CHIKV também penetra nas células hospedeiras, mais especificamente em células musculares, através de macropinocitose (LEE, CHING HUA; MOHAMED HUSSAIN; CHU, 2019).

Ocorre então um processo de acidificação dos endossomos, resultante da fusão da proteína E1 com a membrana endossomal, que libera o capsídeo viral no citoplasma, onde ocorre desnudamento viral, liberando o RNA viral de fita simples e polaridade positiva no citoplasma celular (HOORNWEG et al., 2016). Inicia-se a etapa de replicação viral, quando a maquinaria celular inicia a tradução da ORF1 do RNA viral em poliproteína nP1–4, que sofre um processo de autoclivagem pela nsP2. As proteínas nsPs formam então o complexo replicase viral que catalisa a síntese de uma fita de RNA negativa, como molde para sintetizar o genoma completo de sentido positivo e o mRNA subgenômico 26S (ORF2). O mRNA subgenômico é traduzido em uma poliproteína, que produzirá as proteínas estruturais C, E3, E2, 6k e E1. (ABDELNABI et al., 2016).

As proteínas estruturais recém traduzidas formam heterodímeros PE2-E1, os quais são direcionados para o complexo de Golgi, onde sofrerá uma clivagem por uma furina para formar separadamente a E2 e E1, que em seguida são direcionadas para membrana celular interna. A proteína E3, que não é encontrada associada com vírus na sua fase madura (SIMIZU et al., 1984), funciona como uma translocadora da poliproteína (E3-E2-6K-E1), formada logo após a tradução da ORF2, para o retículo endoplasmático (SNYDER; MUKHOPADHYAY, 2012). As proteínas de capsídeo se associam ao RNA viral, a partir de um dobramento de α -hélice, para ser incorporado na nova progênie viral. Há então a formação de um complexo E1, E2, proteína de capsídeo e ssRNA(+). Seguido dessa complexação inicia-se o processo de maturação e liberação da nova progênie viral, que no caso do CHIKV ocorre principalmente por brotamento (**Figura 4**) (REIS et al., 2022; SILVA; DERMODY, 2017).

Figura 4. O ciclo replicativo do CHIKV.



Fonte: Adaptado de (MARTINS et al., 2020)

Legenda: Representação esquemática do ciclo de replicação do vírus chikungunya (CHIKV): Compostos naturais com atividade antiviral contra CHIKV são indicados em cada etapa do ciclo de replicação do vírus (entrada, replicação e liberação).

1.5. Variabilidade viral e Epidemiologia

De acordo com estudos filogenéticos, o CHIKV possui três genótipos: Oeste Africano (WA - West African), África Oriental/Central/Sul (ECSA - East-Central-South-African) e Asiático (POWERS et al., 2000). O genótipo ECSA é associado a surtos na África oriental e se espalhou por vários continentes como o Asiático, Oceania e América do Sul (CHARLYS DA COSTA et al., 2017). Esse mesmo genótipo foi subdividido na linhagem do Oceano Índico (IOL), responsável pelo surto nas Ilhas do Oceano Índico, como na La Reunion, e na Índia, onde foi responsável por mais de um milhão de casos no ano de 2006 (ARANKALLE et al., 2007; SCHUFFENECKER et al., 2006). Estes genótipos estão distribuídos pelo globo, sendo o Asiático e o ECSA os mais prevalentes (WAHID et al., 2017).

O CHIKV foi isolado pela primeira vez em um surto na atual Tanzânia entre 1952 e 1953 (LUMSDEN, 1955; ROSS, 1956b). Do começo da década de 1960 até o início da década de 1970 alguns surtos esporádicos foram registrados na África e Ásia, principalmente na Tailândia e Índia (WEAVER; FORRESTER, 2015). (YERGOLKAR et al., 2006). O vírus permaneceu circulando pelo continente Asiático, sendo registrados casos no Camboja (CHASTEL, 1963), Vietnã (BEDEKAR; PAVRI, 1969) e Filipinas (SY et al., 2016). Nos anos de 2004 e 2005 foi registrado um surto no Quênia e nas Ilhas de Comoro (CHARREL; DE LAMBALLERIE; RAOULT, 2007). Em 2006, um dos maiores surtos de CHIKV foi reportado na Ilha de Reunião, no oceano Indico, quando aproximadamente 30% da população da ilha foi infectada pelo vírus, resultando em 1 óbito a cada 1000 infectados (JOSSERAN et al., 2006). Dados sugerem que durante este surto surgiu a linhagem IOL, que possui uma mutação na valina 226 da E1, o que fez com que o vírus infectasse mais eficientemente o *Aedes albopictus*, levando a uma maior disseminação do CHIKV para outros países. Em 2013, foram registrados os primeiros casos de CHIKV nas ilhas do caribe San Martin e Martinica (LEPARC-GOFFART et al., 2014). Já em 2015, o vírus se espalhou para o continente americano, com casos registrados em mais de 50 países, principalmente na Colômbia (MATTAR et al., 2015), Trindade e Tobago (SAHADEO et al., 2015) e Brasil (NUNES et al., 2015), sendo a linhagem asiática a principal responsável pelos casos.

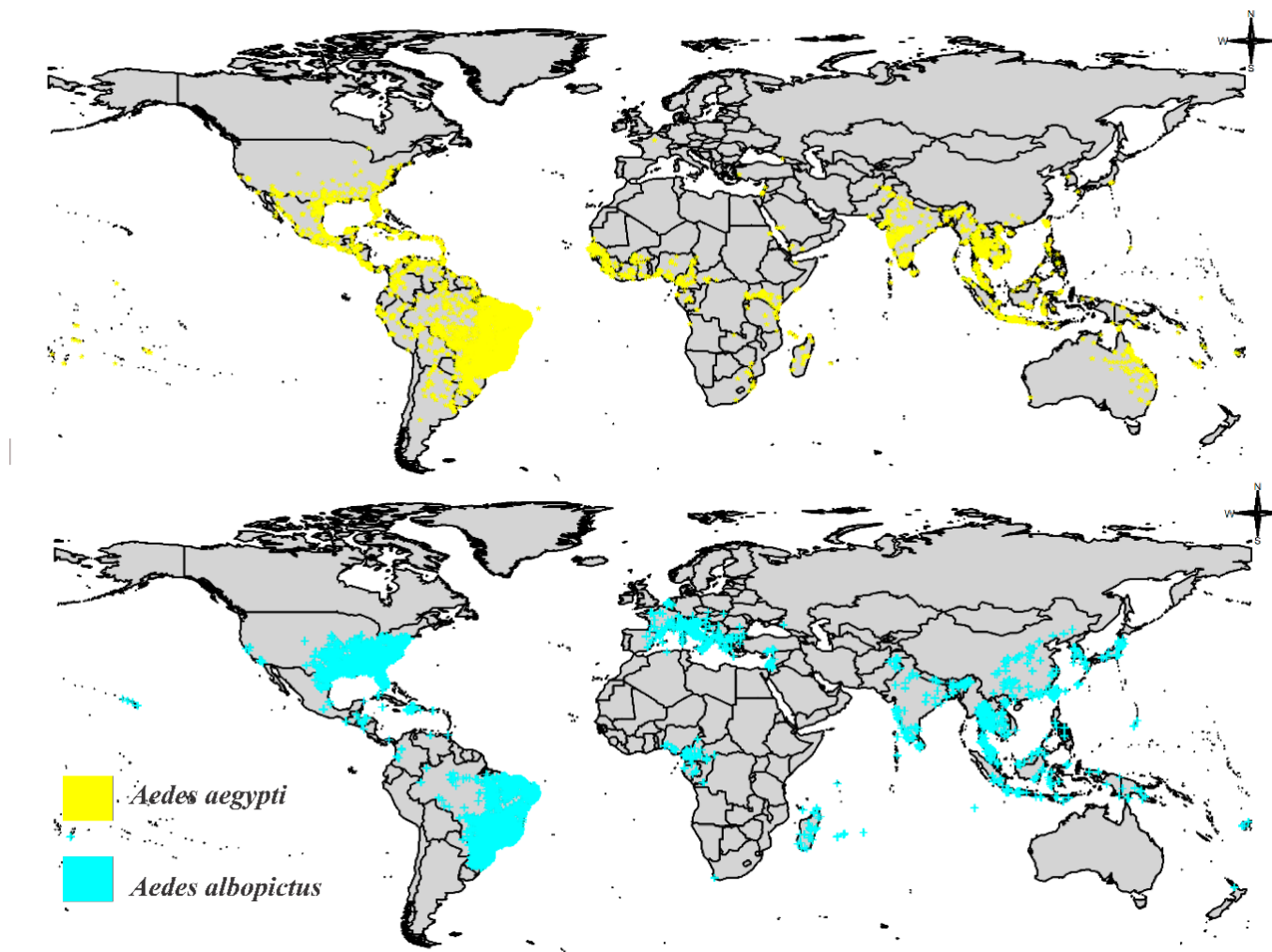
O CHIKV já foi registrado em vários países com transmissões autóctones, ou seja, a transmissão ocorreu dentro do próprio território, enquanto outros territórios registraram casos de Febre Chikungunya, mas de pacientes que haviam viajado para outros países, quando adquiriram a infecção e foram posteriormente diagnosticados em outro local, que não o de transmissão **(Figura 5)** (GRABENSTEIN; TOMAR, 2023).

1.6. Transmissão

O CHIKV possui dois ciclos de transmissão: o urbano e o selvagem. No ciclo selvagem, a transmissão ocorre de um animal não humano para insetos, com transmissão ocasional para humanos (EVANS et al., 2022). Enquanto que, no ciclo urbano, o vírus é transmitido para humanos pelas fêmeas do mosquito do gênero *Aedes*, principalmente *Aedes albopictus* e *Aedes aegypti*, que são amplamente encontrados em zonas urbanas (WEAVER, 2006). Inicialmente, o CHIKV era apenas transmitido pelo *Aedes aegypti*, (MOWATT; JACKSON, 2014), entretanto, a mutação A226V na proteína E1 permitiu que o vírus também fosse transmitido pelo *Aedes albopictus*. Indícios sugerem que essa mutação ocorreu devido à baixa de circulação de *Ae. aegypti* na ilha de Lá Reunion no surto de 2007 (VAZEILLE et al., 2007).

Por depender do mosquito para sua transmissão, a presença endêmica do CHIKV está muito relacionada a presença do vetor. Uma vez que esse vetor depende de água e temperaturas quentes para sua multiplicação, há maiores registros de casos de CHIKV nas regiões tropicais. Os mosquitos são frequentemente encontrados nessas regiões, com o *Ae. albopictus* sendo registrado em 85 países, e encontrado com mais frequência em regiões de alta latitude. Já o *Ae. aegypti*, foi registrado em 114 países (**Figura 6**) (LAPORTA et al., 2023).

Figura 6. Distribuição global do *Aedes aegypti* e *Aedes albopictus*.



Fonte: Adaptado de (LAPORTA et al., 2023)

Legenda: Mapa da ocorrência geográfica de mosquitos do gênero *Aedes* considerando adultos machos e fêmeas.

Os mosquitos são encontrados em regiões que podem ter temperaturas variando entre 10 e 30°C, precipitação mensal de 50 a 200 mm por mês, conseguem voar de 400 a 600 metros por dia, os ovos são estáveis em diferentes ambientes, mas dependem de umidade para eclosão (CHEVILLON et al., 2008; LAPORTA et al., 2023). Modelos matemáticos demonstraram que os mosquitos *Ae. aegypti* tem um potencial de aumento de distribuição global de 10 a 30% até o fim desse século, o que pode ser preocupante, uma vez que os mosquitos desse gênero são conhecidos por transmitirem diferentes arboviroses em torno do globo (LIU-HELMERSSON et al., 2019).

1.7. Diagnóstico e tratamento

No Brasil, o diagnóstico da Febre Chikungunya é baseado em uma combinação de fatores, incluindo os sintomas apresentados pelo paciente, histórico de viagem para áreas onde a doença é endêmica e exames laboratoriais específicos. De acordo com o manual de manejo clínico elaborado pelo Ministério da Saúde em 2017, os casos devem ser confirmados mediante isolamento viral positivo, detecção do RNA viral por Reação de transcriptase reversa seguida de reação de polimerase em cadeia (RT-PCR – *Reverse transcription Polymerase Chain Reaction*) ou qRT-PCR (*quantitative RT-PCR*), anticorpos IgM durante a fase aguda ou IgG em pacientes na fase crônica, a partir de ELISA e teste imunocromatográfico do tipo *Point of care*. As amostras utilizadas para tais exames incluem sangue, soro, líquido cefalorraquidiano e, em casos de óbito, a coleta de fragmentos de órgãos (SAÚDE, 2017).

Não existe um tratamento específico contra o CHIKV. A terapia compreende medidas para aliviar os sintomas e melhorar o conforto do paciente durante o período de infecção, sendo indicado repouso, hidratação, medicamentos para aliviar febre e dor, e anti-inflamatórios (CAI et al., 2023; SAÚDE, 2017).

1.8. A vacina

Em novembro de 2023 a Agência Reguladora de Alimentos e Medicamentos dos Estados Unidos (FDA - *the United States of America Food and Drug Administration*), aprovou uma vacina para prevenção da febre chikungunya, a Ixchiq (FDA, 2024). A vacina foi desenvolvida a partir de uma colaboração entre o Instituto Butantan e a empresa de biotecnologia Valneva (BUTANTAN, 2024).

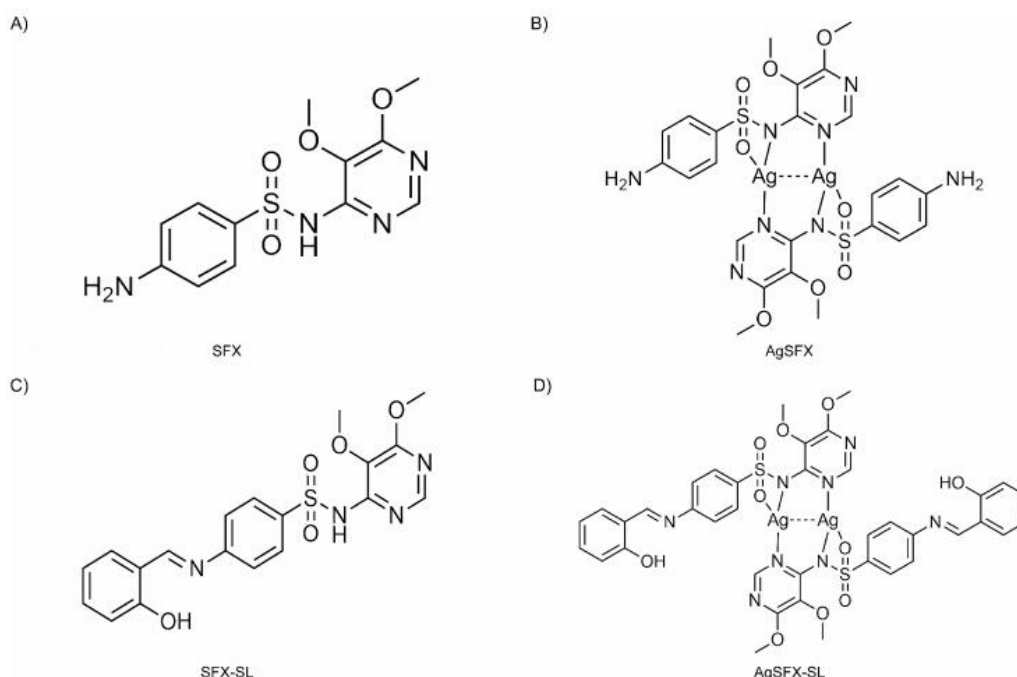
Utilizando a tecnologia de vírus atenuado, a vacina foi aplicada em 4 mil voluntários, com idade entre 18 e 65 anos, em um estudo de fase 3 nos Estados Unidos, em que 98.6% apresentaram produção de anticorpos com título médio de 3269,5 (SCHNEIDER et al., 2023). No Brasil, está sendo conduzido um estudo de fase 3 incluindo 750 jovens, com idade entre 12 e 17 anos, que residem em áreas consideradas endêmicas, e que já tiveram ou não contato prévio com o CHIKV. Depois de 29 dias da aplicação da dose única, 98.8 % dos voluntários que não tinham infecção prévia desenvolveram anticorpos anti-CHIKV, enquanto 100% dos que já haviam sido infectados apresentaram anticorpos, em ambos os grupos com título aproximado de 3889 (BUTANTAN, 2024).

1.9. As sulfonamidas

As sulfonamidas são uma classe de compostos químicos que incluem derivados sintéticos do ácido sulfanílico. Esses compostos são conhecidos por suas propriedades antimicrobianas e foram alguns dos primeiros antibióticos desenvolvidos para combater infecções bacterianas. Além das aplicações como antibióticos, as sulfonamidas também são utilizadas para tratar diurese, hipoglicemia, tireoidite, inflamação e glaucoma (OVUNG; BHATTACHARYYA, 2021).

A sulfadoxina (SFX) (**Figura 7**) é um composto químico que pertence à classe das sulfonamidas. É frequentemente combinada com a pirimetamina para formar uma combinação de medicamentos conhecida como SFX-pirimetamina, comercializada como Fansidar® (BELL et al., 2008). Essa combinação é usada principalmente como tratamento para a malária, podendo ser utilizada também para tratar determinadas infecções bacterianas. A sulfadoxina atua inibindo a síntese de ácido fólico nas células, o que interfere no crescimento e na reprodução dos parasitas causadores da malária, bem como de algumas bactérias. A pirimetamina, por sua vez, interfere na síntese de DNA e RNA, complementando o efeito da SFX (AKINTUNDE et al., 2021).

Figura 7. Estrutura molecular da SFX e seus derivados.



Fonte: próprio autor

Legenda: A estrutura molecular de SFX (A), AgSFX (B), SFX-SL (C) e AgSFX-SL (D) [23]. AgSFX Ag(I) coordenado com sulfadoxina, AgSFX-SL Ag(I) coordenado com sulfadoxina salicilaldeído base de *Schiff*, SFX sulfadoxina, SFX-SL sulfadoxina salicilaldeído base de *Schiff*.

A conjugação de sulfonamidas com prata (Ag) pode ser realizada para o desenvolvimento de compostos com propriedades antimicrobianas e antibacterianas potencializadas (AMIN et al., 2022). Ag tem sido reportada por suas propriedades antimicrobianas há muito tempo e usada historicamente para tratar infecções e promover a cicatrização de feridas (ALEXANDER, 2009). Possui a capacidade de interagir com as bactérias de várias maneiras, incluindo a interferência nas funções celulares, a inibição da divisão celular e a destruição de membranas celulares bacterianas. Isso torna a Ag eficaz contra um amplo espectro de microrganismos, incluindo bactérias, fungos e vírus (DAKAL et al., 2016; GHERASIM et al., 2020). A combinação de sulfonamidas com Ag pode resultar em compostos que capitalizam tanto as propriedades inibidoras de ácido fólico das sulfonamidas, quanto as propriedades antimicrobianas da Ag, como por exemplo no tratamento de infecções em pacientes queimados com sulfadiazina de prata (OAKS; CINDASS, 2023).

Uma base *Schiff* é um composto químico que resulta da reação entre uma amina primária e um grupo funcional contendo um carbonila (grupo C=O), geralmente um aldeído ou uma cetona. Esse tipo de reação é conhecido como condensação de *Schiff* (RACZUK et al., 2022). As bases *Schiff* têm sido estudadas em diferentes campos da química e da bioquímica, incluindo a síntese de produtos químicos, a ligação à metais em complexos, a catálise de reações, e até

mesmo em processos biológicos, como a ligação de íons metálicos em enzimas e proteínas. Portanto, as bases *Schiff* têm aplicações amplas e diversificadas na pesquisa e na indústria química (VICINI et al., 2002; ZOUBI, 2013).

Zanvettor e colaboradores demonstraram que a síntese de SFX complexada com Ag resultou em um complexo metálico denominado AgSFX (**Figura 7**), que mostrou ser ativo contra bactérias gram-positivas e gram-negativas *in vitro* (ZANVETTOR et al., 2015). Ainda com a SFX, Santos Oliveira e colaboradores também demonstraram que a coordenação de SFX com uma base Salicilaldeído *Schiff* (SL) e a prata resultou no composto AgSFX-SL (**Figura 7**), ativo contra *Staphylococcus aureus*, *S. epidermidis*, *Cutibacterium acnes*, *Pseudomonas aeruginosa* e *Burkholderia cepacia*, (SANTOS OLIVEIRA et al., 2022a).

Como citado anteriormente, não há tratamento antiviral específico contra CHIKV, uma infecção viral que pode progredir para uma condição clínica crônica da Febre Chikungunya, síndrome de Guillain-barré, e até ao óbito. Dessa forma o presente trabalho apresenta a avaliação do potencial antiviral da SFX e seus derivados AgSFX e AgSFX-SL contra o CHIKV *in vitro*.

2. OBJETIVOS

Avaliar o potencial antiviral da SFX e seus derivados AgSFX e AgSFX-SL contra o CHIKV *in vitro*.

2..2.Objetivos específicos

- Avaliar a citotoxicidade dos compostos SFX, SFX-SL, AgSFX e AgSFX SL em células BHK-21, a fim de determinar as concentrações não citotóxicas de cada composto, bem como determinar a concentração citotóxica de 50% (CC₅₀);
- Avaliar atividade antiviral dos compostos contra o CHIKV *in vitro*, e determinar a concentração efetiva de inibição de 50% da infecção (EC₅₀);
- Determinar o Índice de Seletividade (S.I) de cada composto, como objetivo de comparar a potência de inibição de cada composto;
- Investigar a atividade antiviral dos compostos nas diferentes etapas do ciclo replicativo viral através de ensaio de tempo de adição dos compostos;
- Analisar as potenciais interações dos compostos com a partícula viral, por meio de Espectroscopia no infravermelho por transformada de Fourier (FTIR).

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ARTIGO CIENTÍFICO

Exploring the antiviral activities of the FDA-approved drug
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Este capítulo está formatado como manuscrito e foi submetido para análise no periódico “*Pharmacological reports*”. Algumas alterações de formatação foram realizadas para melhor apresentação no formato da tese

Exploring the antiviral activities of the FDA-approved drug sulfadoxine and its derivatives against Chikungunya virus

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ABSTRACT

Background: Currently, there is no antiviral licensed to treat chikungunya fever, a disease caused by the infection with *Alphavirus chikungunya* (CHIKV). Treatment is based on analgesic and anti-inflammatory drugs to relieve symptoms. Our study aimed to evaluate the antiviral activity of sulfadoxine (SFX), an FDA-approved drug, and its derivatives complexed with silver(I) (AgSFX), salicylaldehyde Schiff base (SFX-SL), and with both Ag and SL (AgSFX-SL) against CHIKV.

Methods: The anti-CHIKV activity of SFX and its derivatives was investigated using BHK-21 cells infected with CHIKV-*nanoluc*, a marker virus-carrying *nanoluciferase* reporter. Dose-response and time of drug-addition assays were performed in order to assess the antiviral effects of the compounds, as well as in silico data and ATR-FTIR analysis for insights on their mechanisms of action.

Results: The SFX inhibited 34% of CHIKV replication, while AgSFX, SFX-SL, and AgSFX-SL enhanced anti-CHIKV activity to 84%, 89%, and 95%, respectively. AgSFX, SFX-SL, and AgSFX-SL significantly decreased viral entry and post-entry to host cells, and the latter also protected cells against infection. Additionally, molecular docking calculations and ATR-FTIR analysis demonstrated interactions of SFX-SL, AgSFX, and AgSFX-SL with CHIKV.

Conclusions: Collectively, our findings suggest that the addition of metal ions and/or Schiff base to SFX improved its antiviral activity against CHIKV.

Keywords: antiviral; arboviruses; chikungunya virus; sulfadoxine; silver(I); schiff base.

INTRODUCTION

Arboviruses affect mainly populations in tropical and subtropical regions, due to wide distribution of *Aedes* sp mosquitos that act as vectors for several arboviruses[1–4]. *Alphavirus chikungunya* (CHIKV) is an enveloped virus with an icosahedral capsid and positive sense RNA (+ssRNA) genome [5]. Transmitted by *Aedes* sp, it is the causative agent of chikungunya fever (CHIKF). CHIKF is an illness characterized by fever, body aches, headache, and joint pain. Aggravatingly, the infection can progress into Guillain-Barré syndrome [6], or a chronic disabling disease affecting infected patients for months to years [7].

CHIKV was first isolated in Tanzania in 1952 [8], and since its discovery, cases of CHIKF have been reported in Africa, Asia, Europe, and South America [9,10]. CHIKF continues to be a significant public health concern globally. As of April 30, 2024, approximately 240,000 CHIKF cases and over 90 deaths have been reported worldwide. Notably, most of these cases have been concentrated in South and Central America. Among these, countries reporting the highest number of cases are Brazil (243,024), Paraguay (3,121), Argentina (382), and Bolivia (268). Further, Brazil reported 99 CHIKV-associated deaths [11].

As there are no licensed antivirals available for the treatment of CHIKV infection, the current therapies are based on analgesic and anti-inflammatory drugs for the relief of symptoms. Recently, an attenuated virus vaccine was approved by The United States Food and Drug Administration (FDA) for the prevention of CHIKF cases, and it is currently under phase 3 of clinical trial in Brazil [12,13]. However, the limited availability of vaccines throughout tropical and subtropical regions, the increase of CHIKV spreading due to high human exposure, and the lack of antiviral drugs to manage the disease, highlight the need for new molecules that can be employed to abrogate symptoms and disease severity.

In drug discovery, drug repurposing stands out as an attractive approach to accelerate validation processes by unraveling novel clinical uses for FDA-approved and/or other regulatory agencies [14,15]. These drugs have already undergone a thorough evaluation of toxicity, mechanism of action, and pharmacokinetics, and were submitted to clinical trials [16]. Altogether, it contributes to accelerating the applicability of licensed drugs to new therapeutic applications for other diseases [17].

Interestingly, the coordination of metals to approved/repurposed drugs is a promising technique for enhancing pharmacological activities [18,19]. For instance, the coordination of valacyclovir with copper(II) enhanced its anti-bacterial activity [20], and silver(I) complexes with mafenide and ethyl-

mafenide improved the anti-CHIKV effect [21]. This approach can be even improved with the addition of organic fragments or functional groups to decrease cytotoxicity and target specific biomolecules [22]. Therefore, the use of chemical techniques opens a range of possibilities for the design of new molecules, becoming an interesting alternative to antiviral drug discovery.

Previously, Santos Oliveira and colleagues explored the antibacterial and antifungal activities of sulfadoxine (SFX) (**Figure 1A**), one of the active molecules in Fansidar® (SFX plus pyrimethamine), a drug used to treat patients infected with chloroquine-resistant *Plasmodium falciparum* [23,24]. In their work, new molecules were synthesized based on SFX, by coordinating SFX with a silver(I) ion (AgSFX) (**Figure 1B**), by adding a salicylaldehyde Schiff base to SFX (SFX-SL) (**Figure 1C**), or by coordinating the new SFX-SL with Ag(I) (AgSFX-SL) (**Figure 1D**). It is important to emphasize that these modifications enhanced the antimicrobial activity of the novel molecules compared to SFX [23].

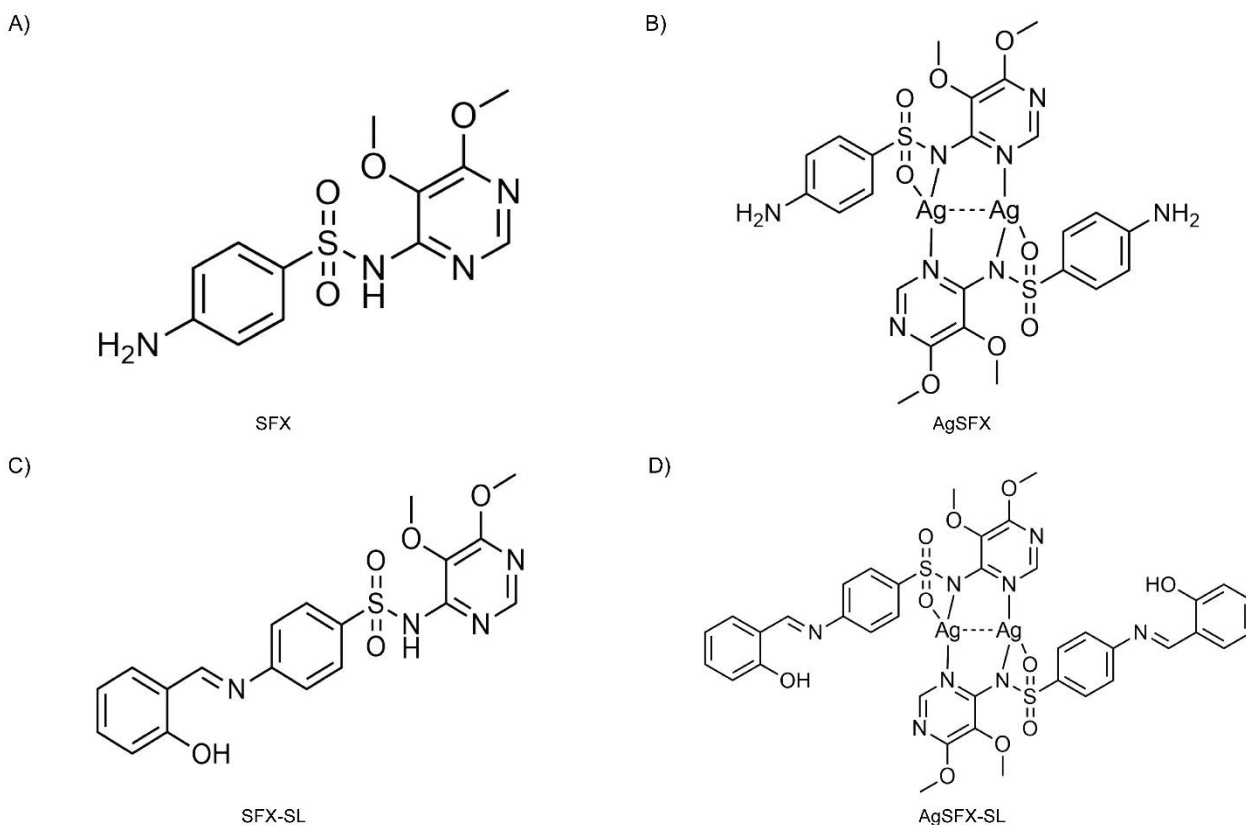


Figure 1. The molecular structure of SFX (A), AgSFX (B), SFX-SL (C), and AgSFX-SL (D) [23]. AgSFX Ag(I) coordinated with sulfadoxine, AgSFX-SL Ag(I) coordinated with sulfadoxine salicylaldehyde Schiff base, SFX sulfadoxine, SFX-SL sulfadoxine salicylaldehyde Schiff base.

Therefore, considering the biological potential of these new molecules and the lack of data encompassing their effects on virus infection, here we investigated the antiviral activity of SFX, and

its derivatives SFX-SL, AgSFX, and AgSFX-SL against CHIKV replication, and provided insights on their mode of action.

MATERIALS AND METHODS

Compounds

Sulfadoxine (SFX), its derivative with salicylaldehyde Schiff base (SFX-SL), and their coordinated Ag(I) analogs AgSFX and AgSFX-SL were synthesized as previously described [25]. The compounds were dissolved in dimethyl sulfoxide (DMSO) $\geq 99.9\%$ (ThermoFisher, Cat: J66650.AK) for a stock solution. Dilutions from a stock solution containing compounds in cell culture media were made immediately before the experiments (v/v). For all assays performed, DMSO 0.1% (v/v) was used as vehicle control (untreated control).

Cell culture

BHK-21 cells (fibroblasts derived from syrian golden hamster kidney; ATCC® CCL-10™) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Cat: D5030), supplemented with 1% of penicillin-streptomycin (Sigma-Aldrich, Cat: P4458), 1% of non-essential amino acids (Sigma-Aldrich, Cat: M7145) and 5% of fetal bovine serum (FBS; Sigma-Aldrich, Cat: F4135) in a humidified 5% CO₂ incubator at 37°C.

Cell viability assay

Cell viability assays were performed as previously described [26–28]. Briefly, 1×10^4 BHK-21 cells were seeded into 96 well plates overnight. Then, each compound at 50, 10, or 2 $\mu\text{mol.L}^{-1}$ was diluted in cell culture media and added to the cells for 24h. After treatment, media containing compound was replaced by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-aldrich, Cat: M5655) solution (1mg/mL) for 1 h at 37°C. The formazan crystals were dissolved with DMSO, and absorbance was measured at 560 nm in Microplate Reader & Luminometer Glomax® (Promega, USA). To assess cell viability rates, the following equation was used: $[(T/C) \times 100\%]$, where T and C represented the optical density of the treated well and DMSO vehicle control, respectively. The highest non-cytotoxic concentration tested was used for antiviral assays. The cytotoxic concentration of 50% (CC₅₀) was determined by treating cells with a medium containing two-fold serial dilutions of each compound at concentrations ranging from 400 to 3.12 $\mu\text{mol.L}^{-1}$ for

SFX-SL, and from 100 to 0.78 $\mu\text{mol.L}^{-1}$ for AgSFX and AgSFX-SL. Readings were performed as described for cell viability assays and CC_{50} values were calculated using GraphPad Prism 8.

Virus rescue and stock

CHIKV LR2006OPY1 (East/Central/South African genotype) infectious cDNA carrying the *nanoluciferase* reporter gene under the control of the CMV promoter (CMV-CHIKV-*nanoluc*) [29] was used to produce CHIKV-*nanoluc* (**Figure 2A**). To this, BHK-21 cells were transfected with 1.5 μg of CMV-CHIKV-*nanoluc* plasmid using lipofectamine 2000 (ThermoFischer, Cat: 11668027), as described previously [27,30,31]. The supernatant was collected 48 h post-transfection, centrifuged, and stored at $-80\text{ }^{\circ}\text{C}$. Viral titer was determined through plaque-forming assay as described previously [27,30]. The viral plaques were counted to determine viral titer which was presented in plaque-forming units per milliliter (PFU/mL).

Antiviral assays

For antiviral screening assays, 5×10^4 BHK-21 cells were seeded in 48 well plates overnight and then infected with CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 in the presence or absence of SFX, AgSFX, SFX-SL, or AgSFX-SL at the highest non-cytotoxic concentration defined by the cell viability assay. After 16h, the supernatant was removed and samples of cells were harvested, *renilla luciferase* lysis buffer and *luciferase* substrate were added (Promega®, Cat: E2810), and replication levels were measured through *nanoluciferase* activity, using *Renilla Luciferase* Assay Kit (Promega, USA). To establish the effective concentration of 50% (EC_{50}), cells were infected with CHIKV-*nanoluc* (MOI of 0.1) and treated with two-fold serial dilutions of SFX-SL at concentrations ranging from 3.12 to 400 $\mu\text{mol.L}^{-1}$, and AgSFX and AgSFX-SL at concentrations ranging from 0.78 to 100 $\mu\text{mol.L}^{-1}$. Samples were harvested 16 h post-infection (h.p.i.) and luminescence levels were assessed as described above to measure CHIKV replication rates. The EC_{50} values were calculated using the GraphPad Prism 8 software, and the selective index (SI), the ratio between $\text{CC}_{50}/\text{EC}_{50}$, was determined for each compound.

Time-of-drug addition assay

For all time-of-drug addition assays, cells were plated in 48 well plates (5×10^4 cells/well), infected with CHIKV-*nanoluc* (MOI of 0.1), and treated with SFX-SL at 50 $\mu\text{mol.L}^{-1}$, or AgSFX and

AgSFX-SL at $2\ \mu\text{mol.L}^{-1}$. Virus replication rates were measured at 16 h.p.i. through *nanoluciferase* activity using the Promega *Renilla Luciferase* Assay Kit (Promega, Cat: E2810).

For the pre-treatment assay, cells were incubated for 1 h with each compound at 37°C , followed by phosphate-buffered saline (PBS) washes (three times) to remove the compounds, and then cells were infected with CHIKV-*nanoluc*. For the entry assay, cells were treated with each compound and simultaneously infected with CHIKV-*nanoluc* for 1h at 37°C . Then, cells were washed with PBS (three times) to remove the inoculum and incubated with fresh cell culture media. Finally, for post-entry experiments, cells were incubated with CHIKV-*nanoluc* for 1 h at 37°C , washed with PBS (three times) to remove unbound virions, and then incubated with cell culture media containing each compound.

Infrared spectroscopy: spectral data analysis.

The virus and each compound were recorded in attenuated total reflection (ATR) Fourier transform infrared (FTIR) spectroscopy (Agilent Cary 630 FTIR, Agilent Technologies, Santa Clara, CA, USA). The diamond unit in the ATR system performs an internal reflection element to record the fingerprint infrared signature in a fingerprint at $1800\ \text{cm}^{-1}$ to $800\ \text{cm}^{-1}$ regions. The samples containing SFX-SL, AgSFX, or AgSFX-SL alone, virions of CHIKV-*nanoluc* (1×10^5 pfu/mL), or SFX-SL, AgSFX, and AgSFX-SL plus CHIKV-*nanoluc* virions were measured in the ATR-FTIR platform. The samples were prepared in PBS, as described previously [27,31,32]. A volume of $2\ \mu\text{L}$ of each sample in the same concentrations of the *in vitro* assay was dried directly on the ATR. The spectra were then recorded ($2\ \text{cm}^{-1}$ resolution, 64 scans). The second derivative spectra were created based on original data plotted in the Origin Pro 9.8.0.200 software (OriginLab, Northampton, MA, USA) and adjusted using the Savitzky-Golay algorithm with polynomial order 2 and 20 points of the window.

Molecular docking

The human matrix remodeling associated 8 (MXRA8) cellular receptor was prepared from PDB entry 6JO8. Coordinates for AgSFX were obtained from the previously published PXRD-solved crystal structure [18]. Coordinates for SFX-SL were obtained from a single-crystal structure [25]. Finally, coordinates for AgSFX-SL were obtained from the previously published DFT-optimized structure.

SFX, Ag-SFX, SFX-SL, and AgSFX-SL were docked on MXRA8 using the GOLD software package [33]. GOLD performs a search for the best-interacting pose of the chosen molecule in the receptor binding site using a genetic algorithm (GA) and the scoring function ChemPLP was selected

for ranking the poses. The center of the binding site was defined at the center of the two loops that connect D1 and D2 (x, y, and z coordinates 32.7184, 57.0255, and 51.5879, in this order) and a 50 Å sphere was used for a non-biased search. The GA search was set to slow. The M-L bonds were treated as zero-order bonds, and silver was set as a dummy atom. The best-ranked poses were exported as mol2 files, and final images were generated with The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.

Statistical analysis

Individual experiments were performed in triplicates and repeated a minimum of three times for the reproducibility of the results. Data were analyzed for normal distribution using D'Agostino-Pearson and Kolmogorov-Smirnov Tests. Data from cell viability screening, antiviral screening, and from time of drug-addition assays presented a normal distribution and were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. The AgSFX-SL treatment in time of addition assay did not follow a normal distribution, therefore, was analysed using the Kruskal–Wallis test and compared using Dunn's multiple comparisons test. P values differences < 0.05 were considered significant. EC₅₀ and CC₅₀ were calculated using a non-linear regression considering log(inhibitor) vs. response, with variable slope (four parameters). All data were processed in GraphPad Prisma 8 software.

RESULTS

Ag(I) and Salicylaldehyde Schiff base enhance SFX antiviral activity against CHIKV replication

Cell viability under the treatment with SFX, SFX-SL, AgSFX, and AgSFX-SL was evaluated by MTT assay. BHK-21 cells were treated with each compound at 50, 10, and 2 µmol.L⁻¹ for 24h, and data of cell viability were compared with the DMSO 0.1% vehicle control. As a result, the treatment with the different complexes resulted in different cytotoxicity profiles (one-way ANOVA, $F_{12,81} = 394.8$, $p < 0.0001$). When compared to DMSO control in Tukey's multiple comparison test, SFX and SFX-SL did not show significant cytotoxicity at the highest tested concentration ($p = 0.1299$ and $p = 0.9558$, respectively), with cell viability over 95%, while the lowest cytotoxicity observed for AgSFX and AgSFX-SL was at 2 µmol.L⁻¹ ($p = 0.0013$ and $p = 0.0007$, respectively), where the treatment with these complexes presented cell viability over 91% (**Supplementary Material 1**).

Then, concentrations of the compounds with cell viability of $\geq 90\%$ in MTT assays were selected for antiviral screening. BHK-21 cells were treated with SFX, AgSFX, SFX-SL, or AgSFX-

SL in the presence or absence of CHIKV-*nanoluc* (**Figure 2A**) at an MOI of 0.1. As expected, no significant cytotoxicity was observed (one-way ANOVA, $F_{4,40} = 0.7243$, $p = 0.5805$) (**Figure 2B**), but the treatment with all compounds was able to significantly decrease viral replication (**Figure 2C**) (one-way ANOVA, $F_{4,48} = 102.4$, $p < 0.0001$). As revealed by Tukey's multiple comparisons test, the free ligand SFX ($50 \mu\text{mol.L}^{-1}$) inhibited 34% of CHIKV replication ($p = 0.0009$ vs. DMSO control), while SFX-SL ($50 \mu\text{mol.L}^{-1}$), AgSFX ($2 \mu\text{mol.L}^{-1}$), and AgSFX-SL ($2 \mu\text{mol.L}^{-1}$) decreased 89% ($p < 0.001$ vs. DMSO control), 85% ($p < 0.0001$ vs. DMSO control), and 95% ($p < 0.0001$ vs. DMSO control) of viral replication, respectively (**Figure 2C**), suggesting that Ag(I) coordination and/or addition of salicylaldehyde base significantly enhanced SFX antiviral activity. Moreover, no significant differences were observed among the mean values from the antiviral activity of AgSFX, SFX-SL, and AgSFX-SL. However, SFX derivatives were significantly different compared to the free ligand SFX ($p < 0.0001$ vs. SFX) (**Figure 2C**). Thus, AgSFX, SFX-SL, and AgSFX-SL were selected for further antiviral analyses.

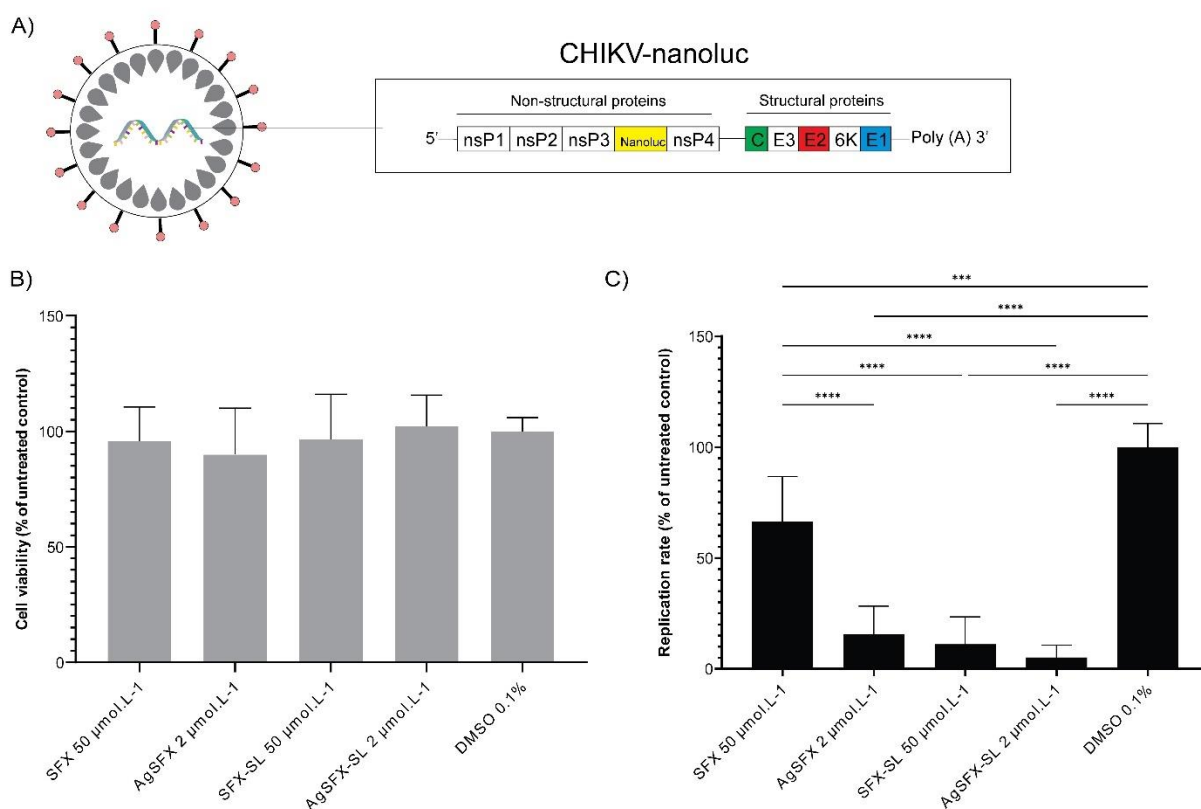


Figure 2. Antiviral activity of SFX, AgSFX, SFX-SL and AgSFX-SL on CHIKV-*nanoluc* replication. CHIKV-*nanoluc* genome sequence (A). BHK-21 cells were treated with SFX and SFX-SL at $50 \mu\text{mol.L}^{-1}$ or AgSFX and AgSFX-SL at $2 \mu\text{mol.L}^{-1}$ at the presence or absence of CHIKV-*nanoluc* (multiplicity of infection of 0.1). Cell viability was performed by MTT assay (B), and replication levels were measured by *nanoluciferase* activity 16 h.p.i (C). Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. Statistical comparison was

performed using one-way ANOVA, followed by Tukey's multiple comparisons test. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$ vs DMSO control group. All images were generated using GraphPad Prism 8 and CorelDrawGraphics Suite 2020. AgSFX Ag(I) coordinated with sulfadoxine, AgSFX-SL Ag(I) coordinated with sulfadoxine salicylaldehyde Schiff base, CHIKV *Alphavirus chikungunya*, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, SD standard deviation, SFX sulfadoxine, SFX-SL sulfadoxine salicylaldehyde Schiff base.

The selective index (SI) of SFX derivatives was assessed by performing a dose-response assay. To this, BHK-21 cells were treated with 2-fold serial dilutions containing SFX-SL at concentrations ranging from 400 to $3.12 \mu\text{mol.L}^{-1}$, or AgSFX and AgSFX-SL at concentrations ranging from 100 to $0.78 \mu\text{mol.L}^{-1}$, and simultaneously infected with CHIKV-*nanoluc* (MOI of 0.1) (**Figure 3A**). From the cell viability and replication rate data, a non-linear regression considering log(inhibitor) vs. response with variable slope (four parameters) was performed to identify the cytotoxic concentration of 50% (CC_{50}) and effective concentration of 50% (EC_{50}). As an outcome, SFX-SL displayed a CC_{50} of $457.90 \mu\text{mol.L}^{-1}$, EC_{50} of $42.17 \mu\text{mol.L}^{-1}$, and SI of 10.85 ($F_{2,140} = 455.5$) (**Figure 3B, Table 1**). On the other hand, AgSFX presented CC_{50} of $9.17 \mu\text{mol.L}^{-1}$, EC_{50} of $2.97 \mu\text{mol.L}^{-1}$, and SI of 3.08 ($F_{2,133} = 160.2$) (**Figure 3C, Table 1**), and AgSFX-SL demonstrated CC_{50} of $8.21 \mu\text{mol.L}^{-1}$, EC_{50} of $2.32 \mu\text{mol.L}^{-1}$ and, SI of 3.53 ($F_{2,149} = 174.7$) (**Figure 3D, Table 1**).

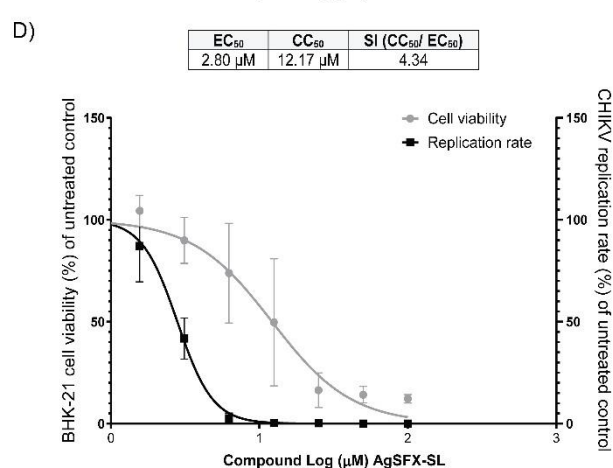
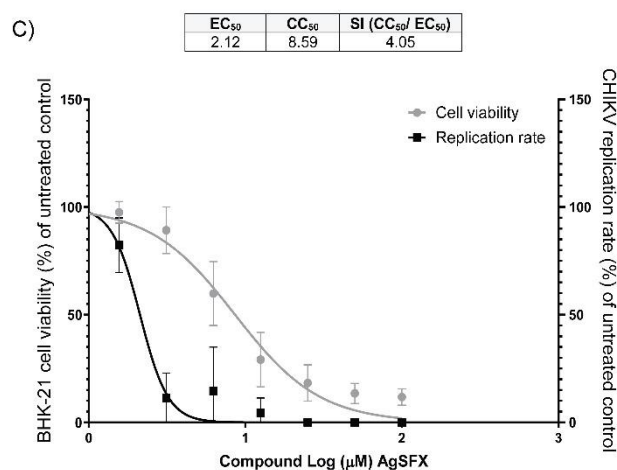
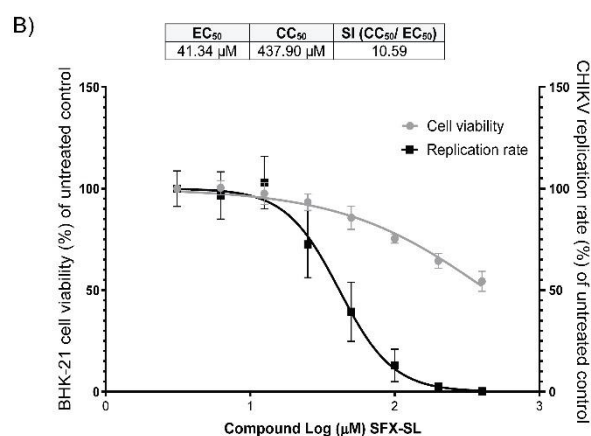
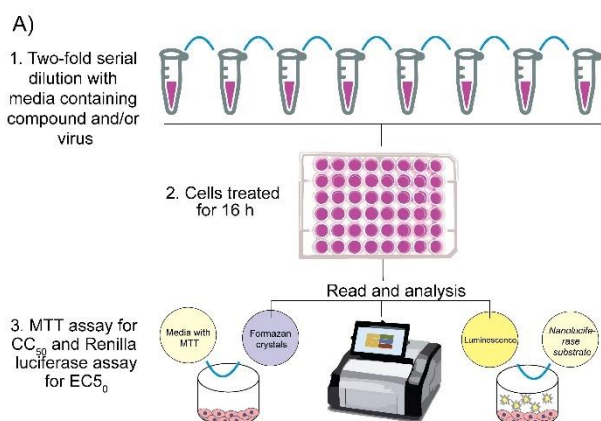


Figure 3. EC₅₀, CC₅₀, and SI of SFX-SL, AgSFX and AgSFX-SL. Representative schematic of dose-response assay (A). The EC₅₀ (indicated by black squares ■) and CC₅₀ (indicated by grey circles ●) of SFX-SL (B) at concentrations ranging from 400 to 3.12 $\mu\text{mol.L}^{-1}$, or AgSFX (C) and AgSFX-SL (D) at concentrations ranging from 100 to 0.78 $\mu\text{mol.L}^{-1}$. CHIKV-*nanoluc* levels of replication were measured by quantified *nanoluciferase* activity and cell viability through MTT assay. Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. The curves were created using a non-linear log(inhibitor) vs. response with a variable slope (four parameters). All images were generated using GraphPad Prism 8 and CorelDrawGraphics Suite 2020. AgSFX Ag(I) coordinated with sulfadoxine, AgSFX-SL Ag(I) coordinated with sulfadoxine salicylaldehyde Schiff base, CC₅₀ cytotoxic concentration of 50%, CHIKV *Alphavirus chikungunya*, EC₅₀ effective concentration of 50%, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, SD standard deviation, SFX sulfadoxine, SFX-SL sulfadoxine salicylaldehyde Schiff base, SI selective index.

Table 1. Cytotoxic concentration of 50% (CC₅₀), effective concentration of 50% (EC₅₀), and selectivity index (SI) values of metal complexes.

Compound	CC ₅₀ ($\mu\text{mol.L}^{-1}$)	EC ₅₀ ($\mu\text{mol.L}^{-1}$)	SI
SFX-SL	457.90	42.17	10.85
AgSFX	9.17	2.97	3.08
AgSFX-SL	8.21	2.32	3.53

SFX-SL, AgSFX, and AgSFX-SL impair multiple stages of the CHIKV replicative cycle

To further investigate the antiviral effects of these derivatives, time-of-drug addition assays were carried out (**Figure 4A**), and one-way ANOVA was performed to evaluate differences in the mean values from different treatment protocols.

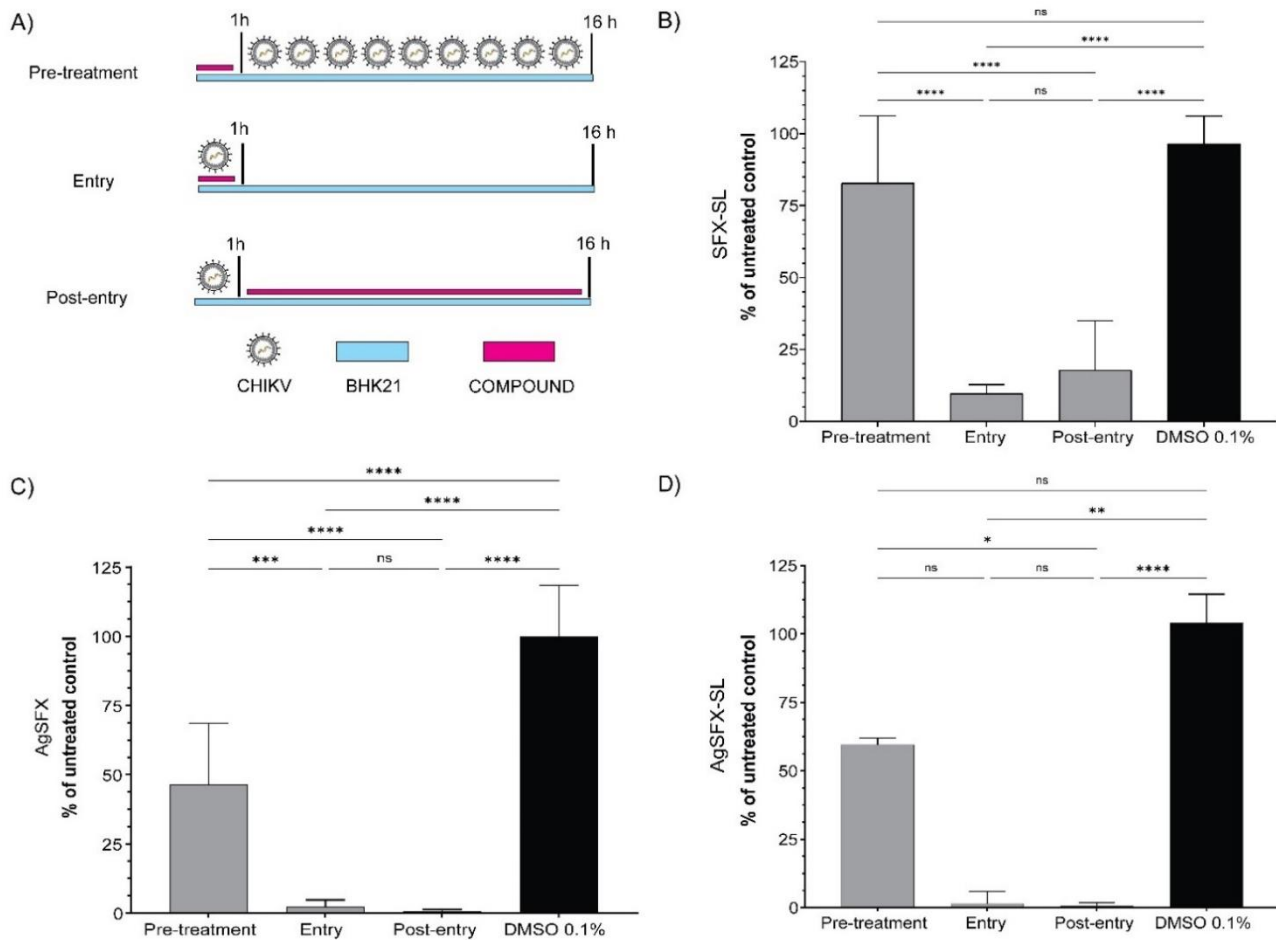


Figure 4. Antiviral activity of SFX and its derivatives on different stages of CHIKV replicative cycle. Scheme of time of drug-addition assay, where cells were infected and treated with compound at different times (A). Time-of-drug-addition assay of SFX-SL at 50 $\mu\text{mol.L}^{-1}$ (B), AgSFX at 2 $\mu\text{mol.L}^{-1}$ (C) and AgSFX-SL (D) at 2 $\mu\text{mol.L}^{-1}$. Statistical comparisons were performed using one-way ANOVA with multiple comparisons with the Tukey test in SFX-SL and AgSFX treatments (B and C, which mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate), and Kruskal-Wallis' test with Dunn's multiple comparisons test in AgSFX-SL treatment (D, which medians and interquartile range are shown). (***) $p < 0.001$, (****) $p < 0.0001$. All images were generated using GraphPad Prism 8 and CorelDrawGraphics Suite 2020. AgSFX Ag(I) coordinated with sulfadoxine, AgSFX-SL Ag(I) coordinated with sulfadoxine salicylaldehyde Schiff base, CHIKV *Alphavirus chikungunya*, SD standard deviation, SFX sulfadoxine, SFX-SL sulfadoxine salicylaldehyde Schiff base.

Interestingly, under SFX-SL treatment, distinct steps of viral replication were inhibited (one-way ANOVA, $F_{3, 26} = 55.55$, $p < 0.0001$). As revealed by Tukey's multiple comparisons test, SFX-SL did not protect cells against CHIKV infection (17% inhibition, $p = 0.2917$ vs. DMSO control), however, it strongly impaired viral entry and replication, decreasing CHIKV replication in 91% ($p < 0.0001$, vs. DMSO control) and 82% ($p < 0.0001$, vs. DMSO control), respectively. Further, it also revealed that among the treatment types, no difference was identified between entry and post-entry (p

= 0.8116), but both were significantly different from pre-treatment ($p < 0.0001$ for pre-treatment vs. entry and pre-treatment vs. post-entry) (**Figure 4B**).

Alternatively, AgSFX at $2 \mu\text{mol.L}^{-1}$ impaired all investigated steps of CHIKV replication (one-way ANOVA, $F_{3, 26} = 64.91$, $p < 0.0001$). When compared using Tukey's multiple comparisons test, AgSFX protected cells from infection by 54% ($p < 0.0001$ vs. DMSO control), and inhibited entry and post-entry replication by 98% ($p < 0.0001$ vs. DMSO control), and 99% ($p < 0.0001$ vs. DMSO control), respectively (**Figure 4C**). Furthermore, the same profile was observed when data from entry and post-entry were compared, where no differences were observed ($p = 0.9983$), however, both values were different from those observed in the pre-treatment group ($p = 0.0001$ and $p < 0.0001$, respectively) (**Figure 4C**).

Similarly to SFX-SL, the AgSFX-SL at $2 \mu\text{mol.L}^{-1}$ significantly impaired entry and post-entry steps of CHIKV replication ($H = 21.21$, $N_1 = 9$, $N_2 = 6$, $N_3 = 6$, $N_4 = 8$, $p < 0.0001$, Kruskal–Wallis test). When compared using Dunn's multiple comparisons test, AgSFX-SL inhibited 98% of CHIKV entry to the host cells ($p = 0.0011$ vs. DMSO control), and 91% of post-entry viral replication ($p < 0.0001$ vs. DMSO control) (**Figure 4D**). This complex also inhibited viral replication at a pre-treatment step in 51% ($p = 0.3141$ vs. DMSO control), however, differences were not significant ($p = 0.3141$ vs. DMSO control). (**Figure 4D**). As for the treatments, pre-treatment vs post-entry was statistically different ($p = 0.0388$), while no statistical differences were observed between entry vs post-entry and entry vs pre-treatment ($p = 0.2393$ and $p > 0.9999$, respectively) (**Figure 4D**).

SFX-SL, AgSFX, and AgSFX-SL promote molecular changes in CHIKV

The strong activity shown on the entry assays by these compounds raised a hypothesis about their effects on CHIKV particles. To address this question, we performed an infrared spectra analysis of each compound separately, or in association with the CHIKV-*nanoluc*, focusing on the infrared fingerprint region $1800\text{--}800 \text{ cm}^{-1}$ that could indicate functional groups in proteins, lipids, carbohydrates, and RNA (**Figure 5**). Interestingly, the CHIKV infrared profile shifted in the presence of all complexes compared with their parental controls. The vibrational modes suggested 3 functional group interactions that were present in all associations of CHIKV-*nanoluc* and SFX-SL, AgSFX, or AgSFX-SL (**Table 2**). The vibrational mode at 1653 cm^{-1} assigned to amide I was present in the sample containing CHIKV-*nanoluc* only, however, in the presence of AgSFX, the vibrational mode was completely absent. Additionally, a drastic reduction of this vibrational mode was also seen in SFX-SL and AgSFX-SL (**Figures 5A, 5D, 5G**). Further, the vibrational mode at 1544 cm^{-1} correspondent to an amide II, also present in the CHIKV-*nanoluc* sample, was absent after incubation of CHIKV virions with AgSFX or AgSFX-SL, and strongly reduced in the presence of SFX-SL (**Figures 5B, 5E, 5H**).

A profile change was also identified at the vibrational mode 1050 cm^{-1} , which was not detected in the presence of all compounds, suggesting a C-O-C stretching, corresponding to nucleic acids (**Figures 5C, 5F, 5I**).

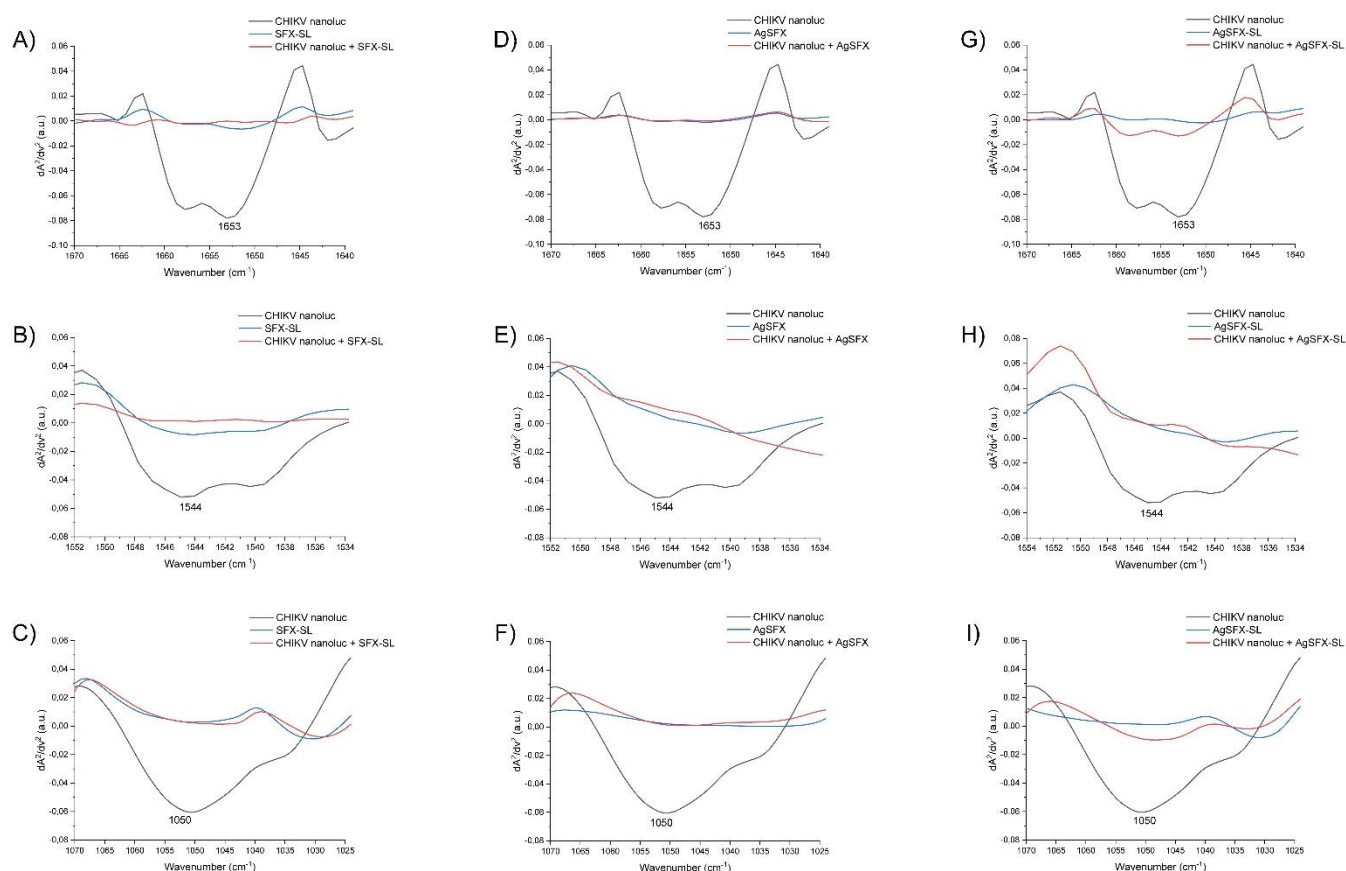


Figure 5. ATR-FTIR analysis indicates interactions between SFX derivatives and CHIKV virions. The representative infrared average spectrum analysis of SFX-SL (A-C), AgSFX (D-F), and AgSFX-SL (G-I) with CHIKV, in which the virus profile is shown by the black line, metal complexes profile by the blue line, and each metal complex in the presence of CHIKV by the red line, employing a FTIR methodology, in wavenumber ranging from 1800 to 800 cm^{-1} . The spectra were created based on original data plotted in the Origin Pro 9.0 (OriginLab, Northampton, MA, USA) software and adjusted using Savitzky-Golay algorithm with polynomial order 2 and 20 points of the window. The vibrational modes suggested 3 functional group interactions that were present in all associations of CHIKV-nanoluc and the SFX complexes. AgSFX Ag(I) coordinated with sulfadoxine, AgSFX-SL Ag(I) coordinated with sulfadoxine salicylaldehyde Schiff base, ATR attenuated total reflectance, CHIKV *Alphavirus chikungunya*, FTIR fourier transform infrared, SFX sulfadoxine, SFX-SL sulfadoxine salicylaldehyde Schiff base.

Table 2. Assignments for vibrational modes indicating interaction between CHIKV virion and SFX derivatives.

Peak (cm^{-1})	Assignment	Reference
1653	Amide I	[47]
1544	Amide II	[48]
1050	C-O-C	[49]

Molecular docking approach with CHIKV main cell receptor

Combining the experimental evidence from the previous assays, we used a molecular docking approach to evaluate the binding of SFX, AgSFX, SFX-SL, and AgSFX-SL to the human matrix remodeling associated 8 (MXRA8) cellular receptor, which is recognized by the CHIKV E protein [34]. MXRA8 has two Ig-like domains with unique structural topologies that bind to two protomers of the E spike on the surface of the virion (**Figure 6A and 6B**) [35]. This structure was docked with each complex or SFX, and an overlap of the best-ranked poses using GOLD and the ChemPLP scoring function showed that all compounds bound to the D1 domain at the human MXRA8 receptor (**Figure 6C**). The higher docking scores showed that SFX-SL favored the frontal region, AgSFX-SL the D1 cytoplasmic pocket, while SFX and AgSFX SFX molecules favored binding to the anterior region of D1 (**Figure 6D-G and Table 3**). Therefore, considering that both regions must be free to form the trimer shown in **Figure 6A**, all compounds could potentially disturb the MXRA8/CHIKV E recognition and interaction.

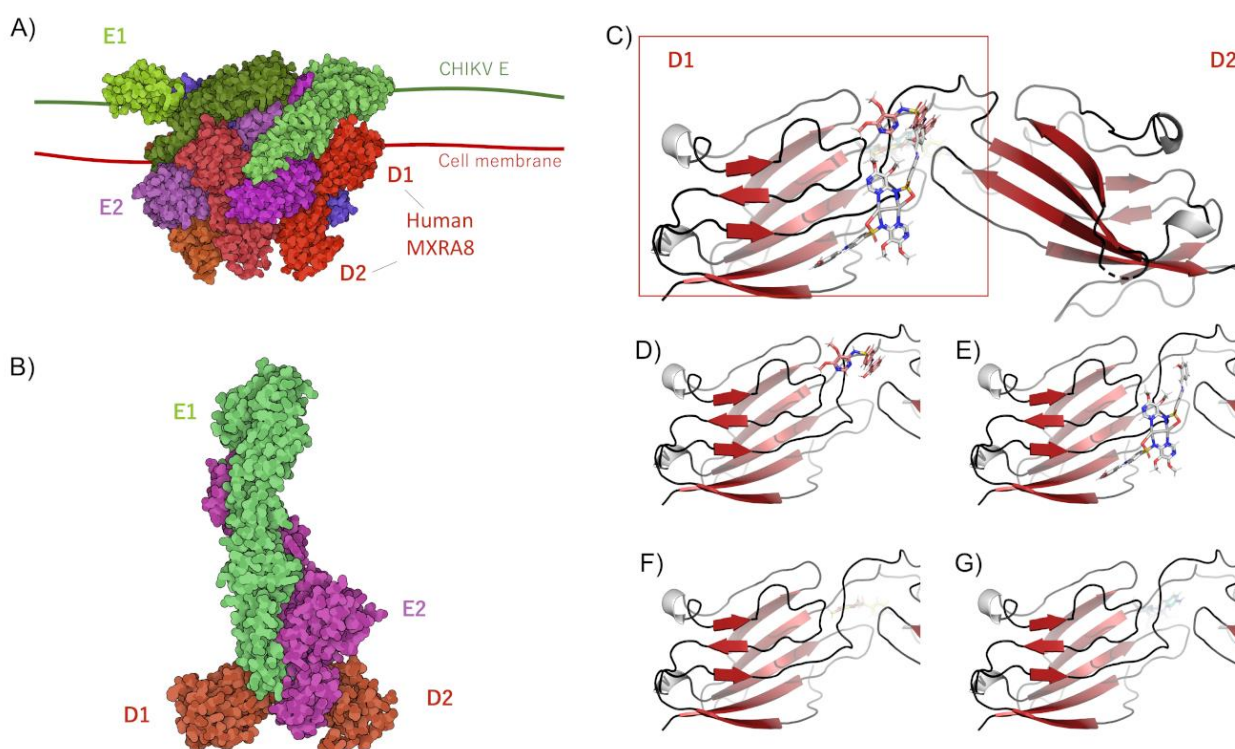


Figure 6. In silico interactions between CHIKV and MXRA8 in the presence of SFX derivatives. Schematic representation of the CHIKV E protein (trimer spike, E1 domain color-coded in green; E2 in purple) interacting with the human entry receptor MXRA8 (color-coded in red) (A). Interaction of the envelope protein E with the human receptor MXRA8, with domain D1 and D2 assigned. The complex comes for the PDB entry 6JO8. The envelope protein binds to the region between D1 and D2 (B). An overview of the best-ranked docking poses for all compounds against the human receptor MXRA8 (C). SFX-SL (D), AgSFX-SL (E), SFX (F), and AgSFX (G). Docking was carried out with GOLD software, and the poses were ranked using PLP fitness. Images A and B were prepared with

Illustrate [46], while C to G with Pymol. AgSFX Ag(I) coordinated with sulfadoxine, AgSFX-SL Ag(I) coordinated with sulfadoxine salicylaldehyde Schiff base, CHIKV *Alphavirus chikungunya*, E envelope glycoprotein, MXRA8 matrix remodelling associated 8 receptor, PDB protein data bank, SFX sulfadoxine, SFX-SL sulfadoxine salicylaldehyde Schiff base.

Table 3. Summary of the ChemPLP fitness for the best ranked posed obtained for the binding of SFX-SL, AgSFX-SL, SFX, and AgSFX to the human Matrix Remodeling Associated 8 (Mxra8) cellular receptor. Higher values indicate a tighter binding.

Compound	ChemPLP fitness
SFX-SL	59.2119
AgSFX-SL	64.7992
SFX	42.0969
AgSFX	41.5793

DISCUSSION

Data presented here demonstrated that the coordination of SFX with Schiff base and/or Ag(I) resulted in enhanced antiviral activity against CHIKV replication when compared with the free ligand SFX, being the inhibitory rates of these derivatives up to 3 times higher. In agreement with our data, the coordination of Ag(I) to mafenide, an FDA-approved drug, resulted in increased antiviral activity against CHIKV-*nanoluc*, while the free ligand mafenide was not able to significantly impair viral replication [36]. In addition, Wang and colleagues described the coordination of hydroxtaminoguanidines (SB-HAG) with the Schiff base and demonstrated that this complex presented enhanced antiviral activity, increasing the inhibition 376 times against mouse hepatitis virus (MHV) [37]. The same profile was found here, in which SFX-SL was 2.6x more active than its free ligand SFX.

Aiming to evaluate the antiviral potency of these SFX derivatives, a dose-response assay was carried out. SFX-SL was demonstrated to be well tolerated by cells and was able to inhibit CHIKV replication with EC₅₀ of 42.1 μ M. More interestingly, the presence of Ag(I) strongly reduced the EC₅₀ of the compounds (~ 2 μ M), but it did result in a higher cytotoxicity in BHK-21 cells (lower CC₅₀). This could be expected, since metal coordination significantly changes the properties of molecules, such as lipophilicity, absorption, and metabolism, thus cytotoxicity levels [38]. Radko and colleagues showed that the coordination of Ag(I) to metronidazole and 4-hydroxymethylpyridine resulted in complexes with greater cytotoxicity than their free ligands when evaluated in Balb/c 3T3 cells (murine fibroblast) [39]. However, is important to emphasize that the coordination with Ag(I) also decreased the EC₅₀, resulting in a lower dose to inhibit viral. In this context, further structure modification could

improve their cytotoxic profile, making the structures presented here a good scaffold for further chemical design. Nonetheless, it also makes these compounds attractive for antiviral drug discovery.

The effects of these SFX-based compounds were also investigated in the context of the viral replicative cycle, aiming to better understand their action in the early and late stages of virus replication. SFX-SL and AgSFX-SL strongly inhibited CHIKV entry and post-entry stages, with no statistically significant activity in protecting cells against infection. Alternatively, AgSFX presented moderate but significant activity protecting cells from infection, and strongly impaired CHIKV entry and replication. Ag(I) ions and nanoparticles are known to inhibit viral infection by direct interaction with the viral particle or cell receptors to disrupt viral entry [40,41]. It corroborates with our data in which viral entry was strongly inhibited by all SFX derivatives, but an increased antiviral effect was shown for the Ag(I) complexes AgSFX and AgSFX-SL. It could be hypothesized that the presence of Ag(I) promotes an antiviral effect through direct interactions and/or stabilization of these SFX derivatives with viral particles, consequently blocking virus entry to the host cells. This is further highlighted by the ATR-FTIR results obtained here that demonstrated the interaction of SFX-SL, AgSFX, and AgSFX-SL with constituents of CHIKV virions, shown by the attenuation of the IR bands assigned to amides I and II (wavelength 1653 cm^{-1} and 1544 cm^{-1}). In this context, it is possible to suggest that the lack or the attenuation of those signals is related to the interaction of SFX complexes with CHIKV envelope glycoproteins, and therefore, impairing virus attachment in a binding site suggested by our data from molecular docking analysis. What is more interesting, the mode of action here described was similar to an Ag(I) - N heterocyclic carbene that inhibited the *Human immunodeficiency virus* (HIV) by interacting with the envelope proteins of the virion, thus interfering in the virus-cell membrane interactions [42].

In another matter, our results also demonstrated that SFX-SL, AgSFX, and AgSFX-SL inhibited post-entry stages of CHIKV infection. Previous reports showed that SFX inhibits the dihydropteroate synthetase (dhps) enzyme, which directly impacts the folate pathway [43]. This pathway is involved in the *de novo* synthesis of purines and pyrimidines, and its disruption affects DNA and RNA synthesis, essential for cellular processes and virus replication [44]. Since CHIKV depends on the synthesis of new RNA to produce new virions, we can suggest that the presence of the SFX may be one of the main factors responsible for the halted CHIKV replication levels at the replication stage. Remarkably, in a study aiming to promote the repurposing of drugs for the treatment of COVID-19, the authors found SFX as a SARS-CoV-2 inhibitor *in vitro*, observing a considerable drop in the number of RNA copies in a general infection assay using caco-2 cells, but lacked the elucidation or suggestion of the mechanism of action in SARS-CoV-2 replication [45]. Interestingly, our ATR-FTIR analysis demonstrated an interaction of the SFX derivatives with CHIKV at the

vibrational mode 1050 cm^{-1} (C-O-C), suggesting an effect on viral RNA and therefore, potentially affecting CHIKV replication. However, the assay was performed free of cells, which suggests that the compounds could be impacting the virion RNA, impairing the initial stage of viral replication, when the viral genome is used as a template for viral RNA production (antigenomic RNA and viral mRNA), further contributing to its strong activity. Nonetheless, further investigation of the mechanism of antiviral action of SFX-based compounds needs assessment.

CONCLUSION

The data presented here demonstrated that the synthesis of the SFX derivatives SFX-SL, AgSFX, and AgSFX-SL represents an innovative approach to the development of novel antiviral drug candidates against CHIKV. Ag-SFX and AgSFX-SL demonstrated an active role in different stages of the CHIKV replicative cycle, mainly inhibiting entry and post-entry stages. Assays aiming to address mechanistic approaches and *in vivo* analysis need to fully describe the mode of action exerted by SFX molecules, however, our results present these molecules as potential antiviral candidates to treat CHIKF, also proposing the drug repurposing as an important tool to improve and accelerate solutions against diseases caused by emerging viruses. To the best of our knowledge, this is the first description of the antiviral activity of SFX and its derivatives, which could contribute to the development of future coordinating compounds against CHIKV infection and other emerging viruses.

Authors contributions

Daniel Oliveira Silva Martins: Conceptualization, Data curation, Investigation, Formal Analysis Methodology, Visualization, Writing – original draft, Writing – review & editing.

Uriel Enrique Aquino Ruiz: Formal Analysis, Investigation, Methodology, Writing – review & editing.

Igor Andrade Santos: Formal Analysis, Investigation, Methodology, Writing – review & editing.

Igor Santos Oliveira: Formal Analysis, Investigation, Methodology, Writing – review & editing.

Marco Guevara-Vega: Formal Analysis, Investigation, Methodology, Writing – review & editing.

Raphael Enoque Ferraz de Paiva: Formal Analysis, Investigation, Methodology, Writing – review & editing.

Camilla Abbehausen: Resources, Supervision, Writing – review & editing.

Robinson Sabino-Silva: Formal Analysis, Resources, Writing – review & editing.

Pedro Paulo Corbi: Resources, Supervision, Writing – review & editing.

Ana Carolina Gomes Jardim: Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data supporting the findings of this study are mainly available within the paper and its Supplementary Information. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

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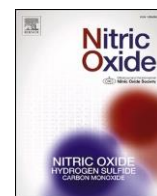
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3. CONSIDERAÇÕES FINAIS

Considerando os resultados obtidos, este estudo sugere que a adição do íon de Ag(I) e ou SL à molécula antiparasitária SFX, potencializou a atividade antiviral contra o vírus CHIKV in vitro. Os derivados de SFX resultantes da coordenação com Ag e/ou SL apresentaram atividade antiviral em diferentes etapas do ciclo replicativo, representando uma abordagem nova e inovadora no desenvolvimento de candidatos à fármacos contra o CHIKV. Estudos adicionais são necessários para a melhor compreensão do mecanismo de ação antiviral destes compostos.

APÊNDICES: ARTIGOS PUBLICADOS

Neste capítulo foram adicionados todos os trabalhos científicos publicados em periódicos no período de 2020-2024. Algumas alterações de formatação foram realizadas para melhor apresentação no formato de tese.



Influence of diimine bidentate ligand in the nitrosyl and nitro terpyridine ruthenium complex on the HSA/DNA interaction and antiviral activity

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ABSTRACT

Nitric oxide (NO) acts in different physiological processes, such as blood pressure control, antiparasitic activities, neurotransmission, and antitumor action. Among the exogenous NO donors, ruthenium nitrosyl/nitro complexes are potential candidates for prodrugs, due to their physicochemical properties, such as thermal and physiological pH stability. In this work, we proposed the synthesis and physical characterization of the new nitro terpyridine. ruthenium (II) complexes of the type $[\text{Ru}^{\text{II}}(\text{L})(\text{NO}_2)(\text{tpy})]\text{PF}_6$ where tpy = 2,2':6',2''-terpyridine; L = 3,4-dia-minobenzoic acid (bdq) or *o*-phenylenediamine (bd) and evaluation of influence of diimine bidentate ligand NH₂-NHq-R (R = H or COOH) in the HSA/DNA interaction as well as antiviral activity. The interactions between HSA and new nitro complexes $[\text{Ru}^{\text{II}}(\text{L})(\text{NO}_2)(\text{tpy})]^+$ were evaluated. The K_a values for the HSA- $[\text{Ru}^{\text{II}}(\text{bdq})(\text{NO}_2)(\text{tpy})]^+$ is 10 times bigger than HSA- $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO}_2)(\text{tpy})]^+$. The sites of interaction between HSA and the complexes via synchronous fluorescence suppression indicate that the $[\text{Ru}^{\text{II}}(\text{bdq})(\text{NO}_2)(\text{tpy})]^+$ is found close to the Trp-241 residue, while the $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO}_2)(\text{tpy})]^+$ complex is close to Tyr residues. The interaction with fishperm *fs*-DNA using direct spectrophotometric titration (K_b) and ethidium bromide replacement (K_{sv} and K_{app}) showed weak interaction in the system *fs*-DNA- $[\text{Ru}^{\text{II}}(\text{bdq})(\text{NO}_2)(\text{tpy})]^+$. Furthermore, *fs*-DNA- $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO}_2)(\text{tpy})]^+$ and *fs*-DNA- $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO})(\text{tpy})]^{3+}$ system showed higher intercalation constant. Circular dichroism spectra for *fs*-DNA- $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO}_2)(\text{tpy})]^+$ and *fs*-DNA- $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO})(\text{tpy})]^{3+}$, suggest semi-intercalative accompanied by major groove binding interaction modes. The $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO}_2)(\text{tpy})]^+$ and $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO})(\text{tpy})]^{3+}$ inhibit replication of Zika and Chikungunya viruses based in the nitric oxide release under S-nitro-sylation reaction with cysteine viral.

Introduction

Nitric oxide (NO) is an important signaling molecule in modulating vascular, neuronal, inflammatory, and immune responses [1]. The effect of NO on viral infections was first reported in the early 1990s on ectromelia, vaccinia, vesicular stomatitis [2], herpes viruses [3], and later on for HIV [4] and influenza [5]. During the world pandemic, some works, described NO as antiviral against SARS-CoV [6] and SARS-CoV-2/COVID-19 [7], demonstrating that NO appears to be effective in the clearance of both DNA and RNA viruses [8]. In antiviral mechanisms, NO

can have several pathways of action, mostly at the inflammatory response [9,10], which is based on type I interferon (IFN) host defense, through activation of toll-like receptors (TLRs). TLRs initiate an intracellular signaling cascade to upregulate NO synthase (NOS) and subsequent NO production [9]. NO is highly reactive with oxygen, superoxide, and hydrogen peroxide to generate reactive nitrogen species (RNS), such as nitrite (NO_2^-). RNS developed from NO induced deamination of adenine and guanine bases and oxidative

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damage against the viral genome [8]. Klingström et al. showed that NO-generated compounds can strongly inhibit Hantavirus replication in Vero E6 cells, by inducing NO production, via IFN antiviral effects [11]. Additionally, NO can inhibit viral enzymes through nitrosylation of viral proteins; can damage viral DNA via oxidative stress, and modulate viral-encoded transcription factors [4,8]. Akaberi et al. showed that NO-donor compounds have a dose dependent inhibitory effect on SARS-CoV-2 replication, via S-nitrosylation of the 3CL protease active site cysteine (Cys) [12]. Cys nitrosylation induces the formation of a disulfide bridge, impairing DNA binding [13].

In some circumstances, the endogenous levels of NO are not sufficient to achieve antiviral effects, the administration of additional NO may have a positive therapeutic effect [8]. For example, some antiviral components such as acetylsalicylic acid [14], chebulagic acid [15], and punicalagin [16] have been shown to suppress iNOS activity via MAPK kinase signaling pathways, which can downregulate the NO production, further indicating the need for exogenous NO therapeutics. An alternative is the study of NO donor compounds as antiviral, a scaffold to target possible specific viral disease states.

Among the NO donor compounds, the ruthenium complexes containing $\{Ru^{II}-NO^+ \text{ or } Ru^{II}-NO_2\}$ bonds have been successfully employed as NO delivery agents by accessing the reduction potential or biological reduction of nitrosyl ligand (NO^+) [17–19] or by light stimulation [20–33].

Ruthenium compounds containing nitrogen oxide as one of their ligands generally exist as hexacoordinated species like $[RuL_5(NO_x)]^{n+}$. The influence of co-ligands “L” species is remarkable and will drive all the properties that are directly related to the nitro and nitrosyl ligands [17]. The effects of the co-ligands “L” in the ruthenium complexes as NO donor on arterial pressure control were evaluated by the “L” π -receptor character in the series $cis-[Ru^{II}(L)(NO_2)(bpy)_2]^{2+}$ and $[Ru(L)(NO^+)(tpy)]^{3+}$ [34]. The main chemical characteristic of these complexes is its ability to release NO in the presence of the vascular tissue occasioned by biological reduction. da Silva et al. has been investigating polypyridine ruthenium complex as NO delivery, like $cis-[Ru^{II}(L_a)(NO^+)(bpy)_2]^{n+}$ (L_a Cl, pyridine (py), 4-acetylpyridine (4-acpy), pyrazine (pz), or 4-picoline (4-pic)) and $[Ru^{II}(L_b)(NO)(tpy)]^{3+}$ (L_b bdq (3,4-diaminebenzoic acid) and bd (*o*-phenylenediamine)) as photoactivatable ruthenium-based prodrugs and undergoes one-electron reduction centered on the NO ligand site [34]. The vasodilatation mechanism involved $[Ru^{II}(bdq)(NO^+)(tpy)]^{3+}$ complex was described by Bendhack et al., the nitrosyl ruthenium complex induced aorta vasodilatation in physiology medium, containing phenylephrine or norepinephrine (known reducing agents) or prostaglandin F 2α , the $[Ru^{II}(bdq)(NO^+)(tpy)]^{3+}$ complex showed the same vasodilatation profile [35]. Other hand, Antoniali et al. reported that the aqua complex ($[Ru^{II}(bd)(H_2O)(tpy)]^{2+}$), did not promote relaxation which suggests that NO activates the mechanisms involved in smooth muscle cell relaxation [36].

The photochemical reaction of $[Ru^{II}(bdq)(NO)(tpy)]^{3+}$ nitrosyl

ruthenium compounds can produce NO, depending on the wavelength of

the irradiated light. The quantum yields observed for NO release from $[Ru^{II}(bdq)(NO)(tpy)]^{3+}$ complex, were obtained by 355 nm and 532 nm light irradiation (0.47–0.03) and (0.0065–0.001) mol Einstein $^{-1}$, respectively [27]. The photolysis at 355 nm is due to light irradiation on the MLCT band occasioned by $d\pi(Ru^{II}) \rightarrow \pi^*(NO)$ transition, in addition of NO, the $[Ru(bdq)(H_2O)(tpy)]^{n+}$ complex should be expected as one of the photoproducts.

In general, the polypyridines ruthenium complexes coordinated to nitrosyl ligand, presents low chemical stability in physiological pH considering the interconversion of nitrosyl to nitrite coordinated to ruthenium (II). Therefore, the possibility to employ nitro ruthenium complexes species as a source of NO were considered. da Silva et al. published the photochemical studies about nitro ruthenium complexes

$cis-[Ru^{II}(L_c)(NO_2)(bpy)_2]^{+}$ (L_c = pyridine (py), 4-picoline (4-pic) and

pyrazine (pz)) and $[Ru^{II}(bpy)(NO_2)(tpy)]^{+}$ in physiological medium under 355 nm light irradiation. The NO released was detected by NO sensor and the other photoproduct identified as aqua ruthenium complex [28].

Considering the promising results of ruthenium complexes as NO donor, in this work, we proposed the study of terpyridine ruthenium complexes as a NO donor from nitrosyl (NO^+) and new nitro (NO_2) terpyridine ruthenium complexes like as $[Ru^{II}(L)(NO)(tpy)](PF_6)_3$ and $[Ru^{II}(L)(NO_2)(tpy)]PF_6$, where tpy 2,2':6',2''-terpyridine, L 3,4-diaminobenzoic acid (bdq) or *o*-phenylenediamine (bd) (Fig. 1) as new therapeutics against Chikungunya and Zika virus replication *in vitro*.

Aiming to probe the potential biological targets of these compounds, we have also investigated how nitro and nitrosyl ruthenium complexes interact with DNA and serum human albumin (HSA). Recently, our research group has reported on the chemical features of nitrosyl terpyridine complex and HSA interaction [37]. The $[Ru^{II}(L)(NO)(tpy)](PF_6)_3$, L $NH_2NHq-COOH$ (bdq) or NH_2NHq-H (bd), showed the constant binding value and interaction sites with albumin are dependents of the COOH substituent group in the diimine bidentate ligand.

Experimental approaches

Chemicals and reagents

$RuCl_3 \cdot nH_2O$, 2,2':6',2''-terpyridine (tpy), 3,4-diaminobenzoic acid (bdq), *o*-phenylenediamine (bd), L-cysteine (L-cys), human serum albumin (HSA), ethidium bromide (EB) and *Fish Sperm* DNA (*fs*-DNA) were purchased as high purity reagents from Aldrich Chemicals. Tris (tris(hydroxymethyl)aminomethane) was purchased from Chemicals. All chemicals were of analytical grade and were used without further purification.

The average molecular weight value of 66,500 g mol $^{-1}$ was used in the preparation of HSA protein solutions. Before each experiment, all solutions were freshly prepared in PBS buffer solution pH 7.4. *fs*-DNA was dissolved in Tris buffer solution (50 mM) and NaCl (5 mM) at pH 7.4. Deionized water was used in the preparation of the buffer solution.

Apparatus

The ultraviolet-visible (UV-vis) spectra were recorded on a Cary 5000 Agilent®. The fluorescence emission spectra were recorded on an Agilent® Cary Eclipse fluorescence spectrophotometer, using a quartz cell with a 1.0 cm optical path, employing an Agilent® Peltier thermostatic cuvette holder. IR spectra were recorded in a spectrometer FT-IR

Frontier Single Range e MIR from Perkin Elmer coupled with an attenuated total reflectance (ATR) apparatus with diamond cell, in the 4000–200 cm $^{-1}$ range. JASCO-J-810 CD spectropolarimeter using a 10 mm optical length quartz cuvette. Elemental analyses were performed with a PerkinElmer®, Series II 2400 microanalyzer. MALDI-TOF-MS measurements were performed with an Autoflex III MALDI-TOF-MS Bruker equipped with an Nd:YAG laser (330–360, 495–540, 808, 990–1080 nm) laser. The matrix (α -cyano-4-hydroxycinnamic acid) solution was

freshly prepared in acetonitrile (ACN) and trifluoroacetic acid (TFA) 0.1 % at a concentration of about 1 mg mL $^{-1}$ and complex solution at about 2 mg mL $^{-1}$ in acetonitrile. The sample solution and matrix solution were applied (1:1) to a stainless steel MALDI target plate. ^1H NMR spectra were obtained in DMSO- d_6 using a Bruker AscendTM 400 Avance III HD spectrometer operating at 400 MHz at 25 °C. ^1H NMR signals were referenced to the residual solvent peak, δ 2.50 ppm (DMSO). Conductivity analysis obtained from Tecnon® model mCA-150. The octanol/water partition coefficient ($\log K_{ow}$) was determined using the shake-flask method [38]. The NO measurements were performed by using the ISO-NO Nometer® and DUO-18 acquisition board.

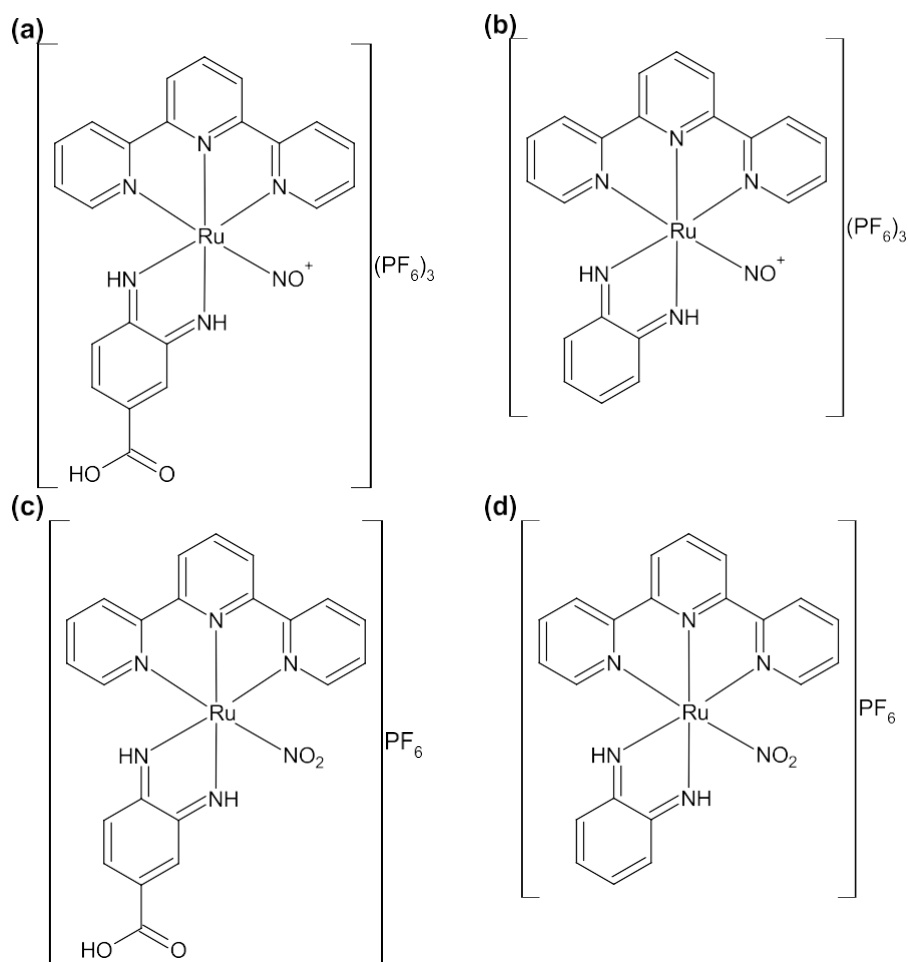


Fig. 1. Chemical structures of the $[\text{Ru}^{\text{II}}(\text{L})(\text{NO})(\text{tpy})](\text{PF}_6)_3$ **RubdqNO** (a) **RubdNO** (b) complexes, $[\text{Ru}^{\text{II}}(\text{L})(\text{NO}_2)(\text{tpy})]\text{PF}_6$ **RubdqNO₂** (c) **RubdNO₂** (d) complexes.

Synthesis of the nitrosyl and nitrite ruthenium complex

The nitrosyl ruthenium complex, $[\text{Ru}^{\text{II}}(\text{bdq})(\text{NO}^+)(\text{tpy})](\text{PF}_6)_3$ (**RubdqNO**) and $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO}^+)(\text{tpy})](\text{PF}_6)_3$ (**RubdNO**) were prepared according to methods previously described [27]. The complexes were characterized by UV-vis and infrared spectroscopy, and then compared to the published results [27].

The new nitro ruthenium complexes were prepared by synthesis with an excess of aqueous sodium nitrite solution as described below.

$[\text{Ru}^{\text{II}}(\text{bdq})(\text{NO}_2)(\text{tpy})]\text{PF}_6$ (RubdqNO₂**) complex:** in a 50 mL flask, 30 mL of water was added. Then, 0.0385 g of the complex $[\text{Ru}^{\text{II}}(\text{bdq})(\text{Cl})(\text{tpy})]\text{PF}_6$ (0.058 mmol) and 0.0186 g of sodium nitrite (NaNO_2 0.27 mmol) were added. The color of the solution changed from purple to red. The solution was kept under heating (temperature 85 °C) for 2 h. After this time, 1 g of ammonium hexafluorophosphate (NH_4PF_6) was added to provide the counter-ion. The formation of a red precipitate was observed. Subsequently, the solution remained under refrigeration for 48 h (temperature 5 °C). The red precipitate obtained was filtered, washed with ethyl ether, and dried under a vacuum. Yield: 0.0210 g (54 %). Anal. Calc. for $[\text{C}_{22}\text{H}_{21}\text{F}_6\text{N}_6\text{O}_6\text{PRu}]$ ($M = 711.49 \text{ g mol}^{-1}$): C, 37.14; H, 2.98; N, 11.81. Found: C, 36.94; H, 2.36; N, 10.74. UV-visible $\lambda(\log \epsilon)$ (NaOH 0.1 M) = 271 (4.12); 281 (4.12); 310 (4.02); 326 (3.96); 512 (4.01). IR (ATR) $\nu_{\text{ass}}(\text{NO}_2^-)$ 1356 cm^{-1} and $\nu_s(\text{NO}_2^-)$ 1281 cm^{-1} . MALDI TOF-MS (m/z) calculated for $\text{C}_{22}\text{H}_{17}\text{F}_6\text{N}_6\text{O}_4\text{PRu}$ [$M - 2\text{H}$] $^+$ 673.98, found 673.14. Λ_m : 167 $\Omega \text{ cm}^2 \text{ mol}^{-1}$ (ACN).

$[\text{Ru}^{\text{II}}(\text{bd})(\text{NO}_2)(\text{tpy})]\text{PF}_6$ (RubdNO₂**) complex:** This compound was obtained in a similar way to that described in the previous item, changing only the precursor complex. For this 0.1284 g of the $[\text{Ru}^{\text{II}}(\text{bd})$

$(\text{Cl})(\text{tpy})]\text{PF}_6$ (0.21 mmol) and 0.0720 g of sodium nitrite (1.04 mmol) were used. Yield: 0.0739 g (57 %). Anal. Calc. for $[\text{C}_{21}\text{H}_{19}\text{F}_6\text{N}_6\text{O}_3\text{PRu}]$ ($M = 649.45 \text{ g mol}^{-1}$): C, 38.84; H, 2.95; N, 12.94. Found: C, 38.09; H, 2.34; N, 13.26. UV-visible $\lambda(\log \epsilon)$ (NaOH 0.1 M) = 270 (4.00); 280 (3.96); 308 (4.00); 326 (3.88); 504 (3.80). IR (ATR) $\nu_{\text{ass}}(\text{NO}_2^-)$ 1377 cm^{-1} and $\nu_s(\text{NO}_2^-)$ 1281 cm^{-1} . MALDI TOF-MS (m/z) calculated for $\text{C}_{21}\text{H}_{17}\text{F}_6\text{N}_6\text{O}_2\text{PRu}$ [$M - 2\text{H}$] $^+$ 629.99, found 629.19. Λ_m : 131 $\Omega \text{ cm}^2 \text{ mol}^{-1}$ (ACN).

HPLC analysis

High-performance liquid chromatography (HPLC) analyses were obtained using Waters® equipment (Palo Alto, CA, USA); with a binary solvent pumping system with diode detector model 2998 PDA. An Agilent® Eclipse XDB-C18 (150 4.6 mm I.D.) column consisting of 5 μm particles was used. To obtain the data, the Empower3® software was used. The mobile phase consisted of a phosphate buffer solution in the presence of 1 % trifluoroacetic acid (pH 7.4). The mobile phase used was a mixture of 1 % trifluoroacetic acid phosphate buffer solution (pH 7.4) and methanol (85:15) (v/v) [37]. Mobile phase elution was isocratic and flow was maintained at 0.8 mL min^{-1} . The samples were dissolved in their mobile phase and 20 μL were injected.

Partition coefficient (log P)

The partition coefficient was determined by the shake-flask methodology as described previously with some modifications [38,39]. Briefly, the complex was dissolved in aqueous solution and the initial

concentration was measured spectrophotometrically (ca. 100 μM). This solution was mixed with an equal volume of n-octanol and shaken for 24 h using an automated shaker. After that time, the solution remained at rest for 1 h. Aliquots from the aqueous phase were extracted and analyzed by UV-vis spectroscopy to determine the amount of the metal complex in this solution. The concentration in the n-octanol layer was calculated by the difference of the concentration found in the aqueous layer and expressed as log P, where: $\log P_{o/w} = \log ([\text{Ru}]_{\text{octanol}}/[\text{Ru}]_{\text{water}})$.

UV-vis and fluorescence spectroscopy analysis for HSA interaction

The exact concentration of HSA was determined spectrophotometrically using the molar extinction coefficient of $35,700 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm [40].

During a typical fluorescence measurement, 3.0 mL of HSA solution (1 μM) was first added to a 1.0 cm quartz cell and the fluorescence spectrum was recorded. Then, the ruthenium complexes (0–16.4 μM) solution aliquots [37] were incubated in the presence of the complex for 24 h before each acquisition. The wavelength 280 nm was used for sample excitation (tryptophan and tyrosine excitation) [41]. The fluorescence spectrophotometer was with the excitation and emission slit widths of 5 and 20 nm, respectively. The fluorescence emission spectra were measured at 298, 303, and 308 K. All intrinsic quenching fluorescence experiments were performed in duplicate. To compensate for the inner filter effect, fluorescence intensity values were corrected for the absorption of each studied ruthenium complex at the excitation and emission wavelength using Equation 1 [42]:

$$F_{\text{Cor}} = F_{\text{Obs}} \times \frac{2.3 d A_{\text{exc}}}{1 - 10^{-d A_{\text{exc}}}} \times 10^g A_{\text{em}} \times \frac{2.3 s A_{\text{em}}}{1 - 10^{-s A_{\text{em}}}}$$

where F_{Cor} and F_{Obs} are the corrected and observed fluorescence intensities, respectively; A_{exc} and A_{em} are the absorbances at the excitation (in this case, at 280 nm) and emission (in this case, at 335 nm) wavelengths, respectively; d is the optical path of the cuvette (1000 cm); g is the distance between the edge of the excitation beam and the edge of the cuvette (0.40 cm); and s is the excitation beam thickness (0.10 cm).

Synchronous fluorescence spectroscopy

Synchronous fluorescence spectra of HSA with various concentrations of complexes were obtained ($\Delta\lambda = 15 \text{ nm}$ and $\Delta\lambda = 60 \text{ nm}$) [37] with the excitation and emission slit widths of 5 and 20 nm, respectively.

Time-resolved fluorescence spectra

Lifetime measurements were recorded on Tsunami – Spectra Physics, sapphire titanium laser, and pulsed laser Millennia X Spectra Physics, the selected wavelength of excitation was 281 nm at two incubation temperatures (298 and 308 K). Into a solution of HSA (1 μM), it was added successive aliquots of complex solution, both prepared in PBS. The detected signal as pulse excitation is called IRF (instrument response function). The number of counts found in the peak of the curves was higher than 5000. The quality of the fit was judged by the reduced χ^2 value, which takes a value around 1 or slightly larger for a high-quality fit. The average fluorescence lifetime ($\langle\tau\rangle$) was calculated using the following equation 2 [43]:

$$\langle\tau\rangle = \alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3$$

The resulting curves were fit by an exponential adjustment of the Origin 8.0® program.

Interaction with DNA: spectrophotometric titration in the UV-vis region

The interaction of complexes with sperm fish *fs*-DNA was verified through direct spectrophotometric titration in the UV-vis region as described in the literature [44]. To this end, 50 μM solutions of nitro and nitrosyl ruthenium complexes were titrated with 9 μL aliquots of a 4.0 mM *fs*-DNA solution, both solutions prepared with 50 mM Tris buffer, pH 7.4, 5 mM NaCl. Spectra were obtained 5 min after mixing the solutions. The data were treated according to the Benesi-Hildebrand equation 3 [45]:

$$\frac{A_0}{A_0 - \epsilon_{\text{HG}} - \epsilon_{\text{G}}} = \frac{1}{K_b [\text{ADN}]}$$

where A_0 is the absorbance in the studied wavelength without *fs*-DNA, A is the absorbance in the studied wavelength after serial additions of *fs*-DNA aliquots, ϵ_{G} is the molar absorptivity in the studied wavelength, ϵ_{HG} is the molar absorptivity in the studied wavelength for the complex-DNA adduct and K_b is the binding constant, given by the ratio of linear and angular coefficients. The DNA integrity test is based on the ratio of absorbances at 260 and 280 nm (A_{260}/A_{280}), so that, if this ratio is greater than 1.8, DNA maintains its structure with a double helix and there are no free proteins in the solution [46].

Interaction with DNA: ethidium bromide displacement

A mixture of 50 μM *fs*-DNA and ethidium bromide (EB, 5 μM) in 50 mM Tris buffer, pH 7.4, 5 mM NaCl solution remained under stirring for 1 h at 25 °C and protected from light. After this period, the *fs*-DNA-EB

solutions were titrated with nitrosyl and nitro ruthenium complexes.

The concentrations of complexes ranged from 3 μM to 70 μM . The interaction was monitored by fluorescence emission spectroscopy ($\lambda_{\text{exc}} = 526 \text{ nm}$, $\lambda_{\text{em}} = 605 \text{ nm}$) from 545 nm to 700 nm at 37 °C. The apparent binding constant (K_{app}) was determined according to the following equation 4 [45]:

$$K_{\text{EB}}[\text{EB}] = K_{\text{app}} [\text{complex}]$$

where $K_{\text{EB}} = 1.0 \cdot 10^7 \text{ M}^{-1}$, $[\text{EB}] = 5 \mu\text{M}$, K_{app} is the apparent binding constant, and $[\text{complex}]$ is the complex concentration that quenched 50 % of *fs*-DNA-EB adduct fluorescence.

Interaction with DNA by circular dichroism (CD)

CD spectra of *fs*-DNA and *fs*-DNA-complexes were recorded in a range from 230 to 350 nm, with 0.5 nm resolution, in Tris buffer 50 mM, pH 7.4 at 25 °C, using a 10 mm optical length quartz cuvette. CD spectra were obtained by using a scan speed of 100 nm min^{-1} and a response time of 0.5 s. Presented spectra were an average of four successive scans. All *fs*-DNA-complexes solutions were incubated for 1 h before measurements.

Biological studies

The nitrosyl and nitrite terpyridine ruthenium complex (**RubdNO**, **RubdqNO**, **RubdNO₂**, and **RubdqNO₂**) were dissolved in H₂O Milli-Q sterile to the concentration of 1 mM. The compounds were diluted in the medium immediately before the assays.

Viruses

The CHIKV expressing *nanoluciferase* reporter (CHIKV-*nanoluc*) used for the antiviral assays is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype) and was produced, rescued, and titrated as previously described [47]. The wild-type ZIKV isolate from a clinical sample of a patient in Brazil (ZIKVPE243) [48] was amplified and titrated as previously described [49].

Cell culture

Vero E6 (isolated from the kidney of an African green monkey; ATCC CRL-1587) and BHK-21 cells (fibroblasts derived from Syrian golden hamster kidney; ATCC CCL-10) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), supplemented with 100 U mL⁻¹ of penicillin (Hyclone Laboratories), 100 mg mL⁻¹ of streptomycin (Hyclone Laboratories), 1 % dilution of stock of non-essential amino acids (Hyclone Laboratories) and 5 % of fetal bovine serum (FBS, Hyclone Laboratories) in a humidified 5 % CO₂ incubator at 37 °C. Sub genomic replicon (SGR) harboring cell lines (BHK-21-RepZIKV_IR-ES_Neo and BHK-CHIKV-NCT) [50,51] were maintained under the same conditions, except for the addition of G418 (Sigma-Aldrich) at 500 µg mL⁻¹ and 5 µg mL⁻¹, respectively.

Cell viability assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich) assay. BHK21-RepZIKV_IRES-Neo and BHK-CHIKV-NCT cells were cultured in 96-well plate and treated with different concentrations of each compound for 72 h at 37 °C with 5 % of CO₂. 72h post treatment, media containing compound was removed from and MTT at 1 mg mL⁻¹ solution was added to each well, incubated for 30 min, and replaced with 100 µL of DMSO to solubilize the formazan crystals. The absorbance was measured at 560 nm on a Glomax microplate reader (Promega). Cell viability was calculated according to the equation (T/C) 100 %, in which T and C represented the optical density of the treated well and control groups, respectively. H₂O was used as a non-treated control. To calculate the cytotoxic concentration of 50 % (CC₅₀), BHK-21 were plated into 48 well plates at a density of 5 × 10⁴ cells per well for CHIKV assays, while for ZIKV assays Vero E6 cells were plated into 96 well plates at 5 × 10³ cells per well. All cells were incubated overnight at 37 °C with 5 % CO₂. Medium containing serial dilutions of each complex ranging from 1.5 to 200 µM were added to cells and incubated for 16h and 72h for CHIKV and ZIKV, respectively. After this, the MTT assay was carried out as described above. The cytotoxic concentration of 50 % (CC₅₀) was calculated using GraphPad Prism 8.0 employing a non-linear regression.

Antiviral assays

To assess the antiviral activity of **RubdNO**, **RubdqNO**, **RubdNO₂**, and **RubdqNO₂** against CHIKV, BHK-21 cells were seeded at a density of 5 × 10⁴ cells per well into 48 well plates 24 h prior to assays. Cells were infected with CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 in the presence of each compound in two-fold serial dilutions ranging from 1.5 to 200 µM. Samples were harvested using *Renilla* luciferase lysis buffer (Promega®) at 16 h post-infection (h.p.i.), and virus replication levels were quantified by measuring *nanoluciferase* activity using the *Renilla* luciferase Assay System (Promega®). For ZIKV antiviral assays, Vero E6 cells were seeded at a density of 5 × 10³ cells per well into 96-well plates 24 h before the infection. ZIKV_{PE243} at a MOI of 0.01 was used to infect cells in the presence of each compound following the same range of concentrations. 72 h.p.i., cells were fixed with paraformaldehyde 4 %, washed with PBS, and an immunofluorescence assay was performed as previously described [49]. FFU under treatment with each compound or untreated control was counted. The effective concentration of 50 % inhibition (EC₅₀) was calculated using GraphPad Prism 8.0 software. The values of CC₅₀ and EC₅₀ were used to calculate the selectivity index (SI = CC₅₀/EC₅₀) [47, 52].

RNA replication assay using BHK21-RepZIKV_IRES-Neo and BHK21-CHIKV-NCT cells

BHK21-RepZIKV_IRES-Neo and BHK21-CHIKV-NCT cells were seeded at a density of 1 × 10⁴ cells per well into 96 well plates for 24h

and treated with the highest non-cytotoxic concentration of each

compound and maintained at 37 °C with 5 % of CO₂. After 72h, the impact on viral RNA replication was measured by quantification of luminescence levels using the *Renilla* luciferase Assay System (Promega®). The inhibition was calculated according to the equation (T/C) 100 %, where T and C represent the mean of the treated group and the mean of the last concentration, respectively.

Statistical analysis.

Individual experiments were performed in quadruplicate and all assays were performed a minimum of three times to confirm the reproducibility of the results. GraphPad Prism 8.0 software was used to assess statistical differences in means of readings using Student's unpaired *t*-test or Mann-Whitney tests. P values < 0.01 were considered statistically significant. The EC₅₀ and CC₅₀ were calculated using a non-linear regression considering log(inhibitor) vs response, with variable slope (four parameters). Data were expressed as mean ± SD and were submitted to a normality and lognormality test.

Results and discussion

Terpyridine ruthenium complex characterization

The new nitro ruthenium complexes were subjected to elemental analysis, spectroscopic, and molar conductivity characterization.

Conductance measurements in acetonitrile indicated that the nitro complexes are 1:1 electrolytes (complex **RubdqNO₂** = 167 Ω cm² mol⁻¹ and complex **RubdNO₂** = 131 Ω cm² mol⁻¹ at 25 °C), indicating the presence of PF₆⁻ counter ion in the compounds [53].

The **RubdqNO₂** and **RubdNO₂** complexes were obtained from a reaction between chloride ruthenium complexes, [Ru^{II}(NH₃)(NH₂Q-R)(Cl)(tpy)]⁺, and NaNO₂ [27]. The nitro group in N-bound metal-nitro complexes typically displays symmetric (ν_s) and asymmetric (ν_{as}) stretches with the asymmetric stretch having the higher frequency [26, 54–56]. Through the FTIR spectra obtained for **RubdqNO₂** and **RubdNO₂** (Fig. 2) the NO₂-vibrations of the configuration Ru-NO₂ were

identified. The FTIR spectrum of **RubdqNO₂** displayed bands at 1356 cm⁻¹ for ν_{as}NO₂⁻ and 1245 cm⁻¹ for ν_sNO₂⁻. While for **RubdNO₂** the bands ν_{as}NO₂⁻ and ν_sNO₂⁻ appear 1377 cm⁻¹ and 1281 cm⁻¹, respectively. The ρ_wNO₂⁻ band for **RubdqNO₂** was founded 597 cm⁻¹, while for **RubdNO₂** at 618 cm⁻¹. The stabilization of oxidized form (quinone) from the 3,4-benzoquinone diamine acid and *o*-phenylenediamine ligand is confirmed by the imine (C=N) stretching frequency at 1603 cm⁻¹ and 1563 cm⁻¹. The carbonyl stretching frequency at 1702 cm⁻¹

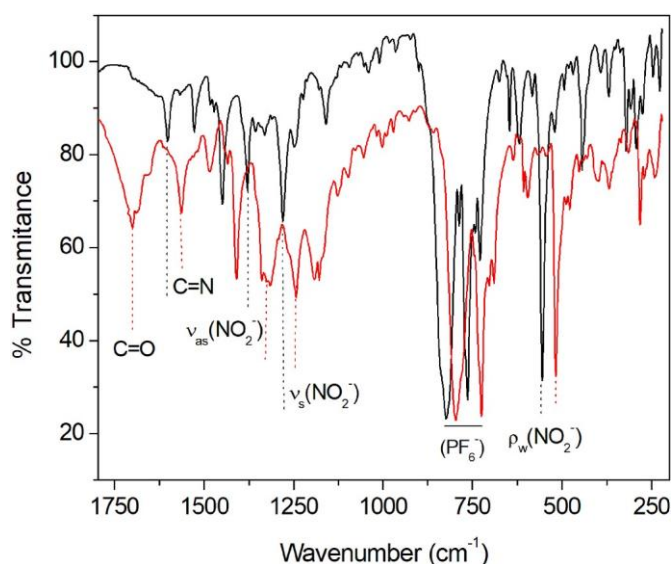


Fig. 2. Infrared spectra of **RubdNO₂** (red line) and **RubdqNO₂** (black line) using ATR accessory.

occurring only in **RubdqNO₂** is due to carboxyl substituent.

The electronic spectra for the **RubdqNO₂** and **RubdNO₂** complexes (Fig. 3), show absorptions in the UV region, between 270 and 320 nm, characteristic of $\pi \rightarrow \pi^*$ transitions of the IL type. The other band occurring in the visible region ~ 500 nm is characterized by the mix of the transitions $d\pi(\text{Ru}^{\text{II}}) \rightarrow \pi^*(\text{NO}_2^-)$ $d\pi(\text{Ru}^{\text{II}}) \rightarrow \pi^*(\text{tpy})$ $d\pi(\text{Ru}^{\text{II}}) \rightarrow \pi^*(\text{bdq}$ or **bd**) [27].

The chromatogram obtained for **RubdqNO₂** complexes shows two separated peaks (t_1 3.87 min and t_2 5.03 min) (Fig. 4a). As previously reported, the HPLC analysis for **RubdqNO** identified two separated peaks (t_1 12 min and t_2 16 min). Based on NMR spectroscopy and mass spectrometry, the authors concluded that the **RubdqNO** isolated after synthesis is composed by two position isomers [57]. The **RubdqNO₂** chromatogram profile was similar to **RubdqNO**, where the two peaks can be attributed as isomers justified by NH.NHq-COOH bidentate co-ligand position in the complexes structure [37,57]. In contrast, in the **RubdNO** complex, analyzed by HPLC [37], only one peak was identified. In accordance to the **RubdNO₂** chromatogram, where one peak was found at t_1 5.99 min (Fig. 4b).

The HPLC technique was proposed to evaluate the acid-base stability of the $[\text{Ru}^{\text{II}}\text{NO}_2^-]$ species for the complexes studied in this work during the time. Comparing the chromatogram profiles obtained at zero time and after 2 h of dissolution of the **RubdqNO₂** and **RubdNO₂** complexes in the mobile phase and the dark, no significant increase or decrease of the peaks was displayed, nor the appearance of any other peaks (Fig. 4 a and b). The complexes **RubdqNO₂** and **RubdNO₂** were also analyzed by MALDI-TOF mass spectrometry. The fragments peaks were observed at $m/z = 673.14$ (Fig. S1, Supplementary Material) $[\text{M} - 2\text{H}]^+$ molecular formula $\text{C}_{22}\text{H}_{15}\text{N}_6\text{O}_4\text{PF}_6\text{Ru}$ for **RubdqNO₂** and $m/z = 629.19$ (Fig. S2, Supplementary Material) correspond to $[\text{M} - 2\text{H}]^+$ molecular formula $\text{C}_{21}\text{H}_{15}\text{N}_6\text{O}_2\text{PF}_6\text{Ru}$ for **RubdNO₂**. The oxidation of the metal ion to Ru^{III} is required for the assignments of the peaks. The theoretical simulation of the mass spectra is consistent with the experimental results.

Finally, the complexes **RubdNO₂** (Fig. S3 in Supplementary Material) and **RubdqNO₂** (Fig. S4 in Supplementary Material) were further analyzed by ^1H NMR spectroscopy. Both ruthenium(II) diimine complexes synthesized in this work exhibited two characteristic broad singlet imine NH resonance at low field (ca. δ 14–15 ppm and δ 11–12 ppm) in ^1H NMR spectra from DMSO-*d*₆ solutions (Figs. S3 and S4 in the Supplementary Material) [58,59]. The resonances of the aromatic

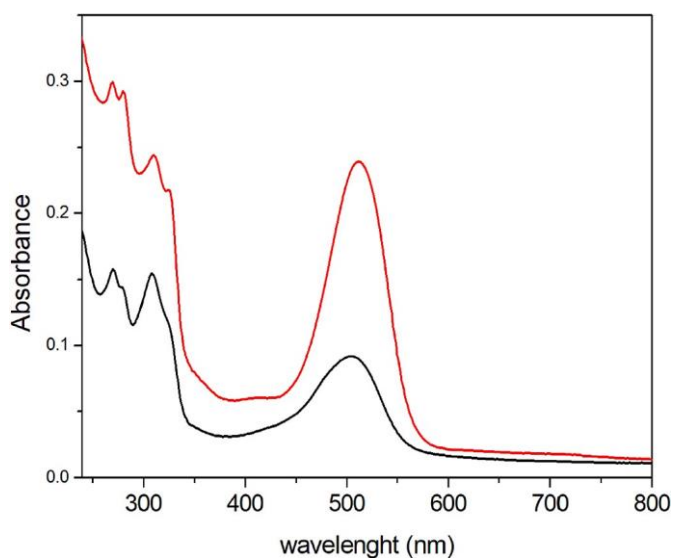
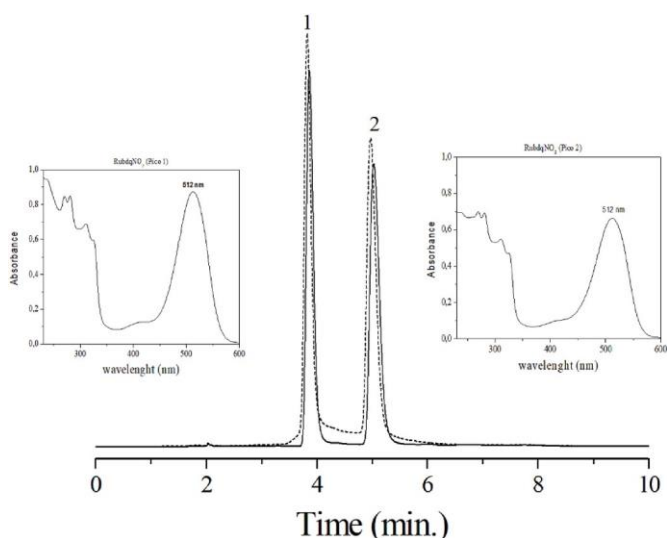
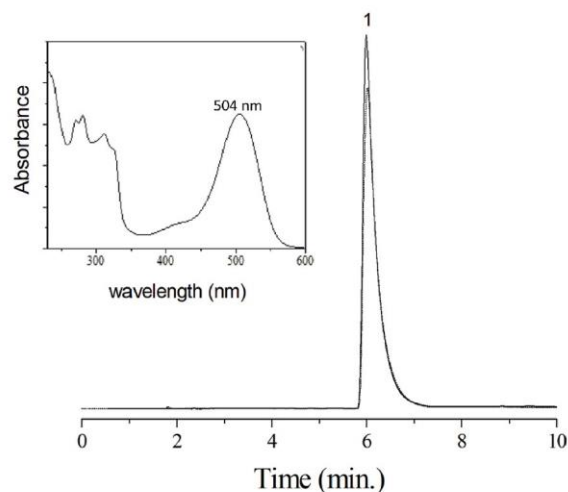


Fig. 3. UV vis spectra of **RubdNO₂** (15 μM black line) and **RubdqNO₂** (23 μM red line) dissolved in NaOH 0.1 M aqueous solution.



(a)



(b)

Fig. 4. Chromatogram of **RubdqNO₂** (a) and **RubdNO₂** (b). Mobile Phase Composition: 1 % TFA in phosphate buffer pH = 7.4: methanol (85:15 v/v). Time after complex dissolution: 0 h (black line) and 2 h (dash line). Insert UV visible spectra at each peak.

hydrogen atoms from the chelating ligands are located at a high field (δ 9 - 7 ppm). The ^1H NMR data provide additional confirmation of the identities of these species.

Lipophilicity measurement

Octanol-water partition coefficients ($\log P_{o/w}$) provide a measure of drug lipophilicity, which indicates the ability of a molecule to pass through cell membranes [39] and higher cellular uptake. The lipophilicity of nitrosyl and nitro terpyridine ruthenium complexes was determined by measuring the concentration ratio of the corresponding complex in the aqueous phase at the equilibrium state. After mixing with n-octanol and water, the complexes with **bd** bidentate ligand were

distributed mostly in the n-octanol phase, while the complexes **bdq** bidentate ligand were in the water phase (Table 1). The **RubdNO** and **RubdNO₂** log $P_{o/w}$, -0.29 and -0.11 , showing them to be more lipophilic in comparison to **RubdqNO** (-1.47) and **RubdqNO₂** (1.70). The presence of the **bd** ligand a chelating ligand that cannot make hydrogen bonds with water molecules, in the **RubdNO** and **RubdNO₂** complexes, make them more hydrophobic in comparison to the ruthenium complexes coordinated to the **bdq** chelating ligand. In the literature, Soliman et al. reported higher water solubility for Ru(II) complexes with 3,4-diaminobenzoic acid ligand [60]. Čanović et al., described for $[\text{Ru}^{\text{II}}(\text{bd})(\text{Cl})(\text{Cl-Ph-tpy})]^+$ complex a negative Log $P_{o/w} = 1.14$ [61]. The lipophilicity character of the polypyridines ruthenium complex cannot be discussed only in function of chelating ligands, but also the influence of monodentate ligands. In the studies about the interaction between $[\text{Ru}_3\text{O}(\text{CH}_3\text{COO})_6(3\text{-pic})_2(\text{NO})]^+$ and albumin, Nikolaou et al. affirmed that the nitrosyl ligand constitutes a lipophilic site [62].

Protein HSA interaction studies

UV vis spectra

The absorption spectrum in the UV/visible region for HSA in the absence of the ruthenium complexes presents an absorption band at 280 nm ($\epsilon_{280\text{nm}} = 35700 \text{ M}^{-1} \text{ cm}^{-1}$), attributed to the $\pi \rightarrow \pi^*$ transition from the aromatic amino acids tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe) [40]. On the other hand, the absorption spectra for HSA in the presence of different concentrations of the nitro ruthenium complexes show only absorption bands characteristic of the complexes (Fig. 5).

Fluorescence emission suppression

The changes in the maximum or shift in wavelength of intrinsic fluorescence emission from the tryptophan residue (Trp 214), located in the hydrophobic cavity of subdomain IIA, are indicative of the interaction of HSA [63]. Fluorescence suppression occurs in response to changes in conformation or changes in polarity of the protein micro-environment [64]. The **RubdqNO₂** and **RubdNO₂** complexes show the fluorescence suppression of HSA with increasing additions of the complexes, associated with a bathochromic shift of the maximum emission, of about 8 nm. This effect can be rationalized in terms of the binding of the nitro complexes with the protein, alternating the polarity in the microenvironment around Trp 214. Stern-Volmer constant (K_{SV}), obtained from Stern Volmer equation 5, values for nitro complexes are reported in Table 2 obtained from Stern-Volmer plot analysis (Fig. 6).

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q]$$

The K_{SV} values increased with increasing temperature in the presence of the nitro ruthenium complexes, indicating that the fluorescence suppression mechanism refers to the dynamic one. In the dynamic suppression mechanism, the increase in temperature results in a high diffusion process, and thus, a high number of molecular collisions will occur. The rate constant of the suppression process (k_q) which is equal to K_{SV} divided by τ_0 (assuming $\tau_0 = 10^{-8} \text{ s}$) was calculated (Table 2). The results revealed that the values of k_q were higher than the maximum

diffusion rate constant of the biomolecule ($2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), which indicated that the fluorescence suppression of HSA was initiated by complex formation between HSA and ruthenium complex, suggesting the static suppression contribution. The fluorescence lifetime for HSA in the presence of the complexes with increasing temperature decreases (Table S1. Supplementary Material) indicating of a dynamic mechanism. Based on the trends in k_q and τ values, we can propose that the interaction of nitro ruthenium complexes contributes to the mechanism of static and dynamic fluorescence suppression.

Binding parameters

The formation of the adduct between the complexes and HSA has a binding constant (K_a), which is influenced by temperature, the solubility of the species in water or lipids, and the nature of chemical interactions (electrostatic attraction, covalent bonding or van der Waals forces) between complex with the structure, and can be used to assess the affinity of the compound for the protein [65].

The binding constants (K_a), obtained from modified Stern Volmer equation 6, values obtained from plot $\log (F_0 - F)/F$ vs $\log [Q]$ (Fig. 6) (see Table 3) for the nitro ruthenium complexes tend to increase with higher temperature.

$$\log \left(\frac{F_0 - F}{F} \right) = \log K_a + n \log [Q]$$

The affinity constant between HSA and ruthenium complexes between 10^3 – 10^4 M^{-1} was found for nitrosyl and nitro ruthenium complexes indicating moderated-to-weak interactions. The ruthenium complex coordinated with the **bdq** ligand showed more strong interaction than the **bd** ligand. Recently, our group published the HSA interaction with nitrosyl terpyridine ruthenium complexes, **RubdqNO** and **RubdNO**, where were showed the higher K_a value for nitrosyl complex coordinated by **bdq** ligand. The theoretically calculated studies using the noncovalent interaction analysis showed that the hydroxyl group (OH) of the Ser-192 residue from HSA could interact with both the hydroxyl group (OH) and the carbonyl group (C=O) of the carboxyl group (COOH) [37].

Synchronized fluorescence suppression

The sites of interaction were verified through the alterations of the microenvironments of the tryptophan (Trp) and tyrosine (Tyr) residues were evaluated through the synchronized fluorescence spectra. When the variation of $\Delta\lambda$ 60 nm, the synchronized fluorescence predicts information about the tryptophan microenvironment. While the variation of $\Delta\lambda$ 15 nm, the information will be about the tyrosine micro-environment [37,66]. The nitro ruthenium complex coordinated to the **bd** co-ligand show a shift to the blue region (from 285 nm to 278 nm) of the maximum emission of the Tyr residue ($\Delta\lambda = 15 \text{ nm}$) (Fig. 7b). Considering the spectra synchronized with $\Delta\lambda$ 60 nm, only the nitro ruthenium complexes with **bdq** ligand showed a subtle shift towards the red region (from 280 to 281 nm) of the maximum emission of the Trp residue (Fig. 7d). The synchronized fluorescence behavior obtained from nitro ruthenium complex was similar to the nitrosyl ruthenium complex reported previously [37].

These results suggest that the nitrosyl and nitro terpyridine ruthenium complexes with **bd** co-ligand when binding to the protein alter the microenvironment around Tyr making it more hydrophobic and less exposed to the solvent. While the **bdq** co-ligand changes the microenvironment around Trp, making it more hydrophilic and more exposed to the solvent [66].

Recently, Petrović et al. showed from molecular docking studies with HSA, that $[\text{Ru}^{\text{II}}(\text{bd})(\text{Cl})(\text{tpy})]^+$ complex has high HSA-binding affinity in subdomain-IIIA [67]. Similar results were obtained by our group where the $[\text{Ru}^{\text{II}}(\text{bd})(\text{NO})(\text{tpy})]^{3+}$ complex increased the hydrophobic characteristics in the microenvironment of Tyr-452 localized in the subdomain

Table 1
Lipophilicity (log $P_{o/w}$ values) of the nitrosyl and nitro terpyridine ruthenium (II) complexes.

Complex	Log $P_{o/w}$
RubdqNO₂	-1.47
RubdNO₂	-0.29
RubdqNO	-1.70
RubdNO	-0.11

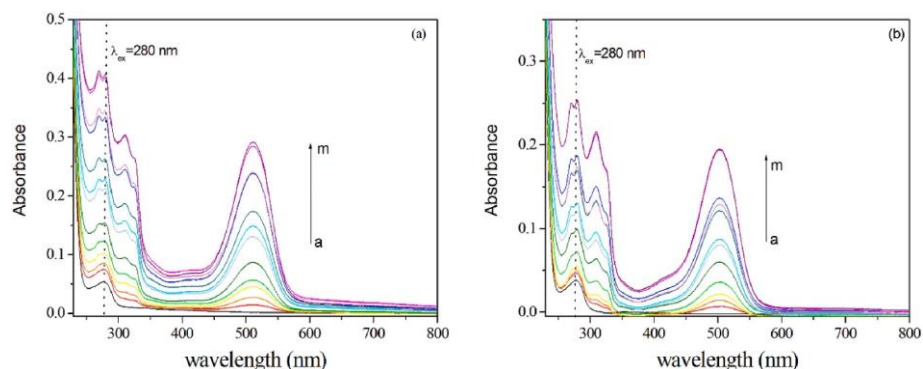


Fig. 5. Absorption spectra in the UV-visible region for HSA in the presence of **RubdqNO₂** (a) and **RubdNO₂** (b) complexes in PBS (pH = 7.4) at 308 K. [HSA] = 1.0 μM; [Complex] = 0; 1.0; 1.7; 2.3; 3.3; 5.0; 6.6; 8.3; 9.9; 11.5; 13.2; 14.8; 16.4 μM (a → m).

Table 2

Stern-Volmer quenching constants (K_{SV}) for system HSA-RubdqNO₂ and HSA-RubdNO₂ at different temperatures.

Adduct	T (K)	$K_{SV} (\times 10^4 \text{ M}^{-1})$	$k_q (\times 10^{12} \text{ M}^{-1} \text{ s}^{-1})$	R^2
HSA-RubdqNO ₂	298	1.48 ± 0.01	1.48	0.9889
		1.50 ± 0.26	1.50	0.9875
HSA-RubdNO	308	1.89 ± 0.26	1.89	0.9612
2	298	1.22 ± 0.02	1.22	0.9768
	303	1.56 ± 0.14	1.56	0.9749
	308	1.67 ± 0.12	1.67	0.9931

IIIA [37].

1.1. Interaction with DNA

Monitoring the changes in absorption and position of the maximum of the bands from the UV-vis spectrum of ruthenium complex titrated in

the presence of *fs*-DNA solution can indicate the mode of interaction

between the compound and the biomolecule (Fig. S5, Supplementary Material). The binding constant for the **RubdNO** complex is equal to K_b ($4.23 \pm 0.93 \times 10^4 \text{ M}^{-1}$) (Fig. 8 a and b). The value measured for **RubdNO** indicated a strong affinity to *fs*-DNA in comparison to $[\text{Ru}^{\text{II}}(\text{bd})(\text{Cl-Ph-tpy})(\text{Cl})]\text{Cl}$ ($K_b = (3.0 \pm 0.1 \times 10^4 \text{ M}^{-1})$ [61] and $[\text{Ru}^{\text{II}}(\text{bd})(\text{Cl})(\text{tpy})]\text{Cl}$ ($K_b = (2.3 \pm 0.2 \times 10^4 \text{ M}^{-1})$ [67]. For the **RubdqNO**, **RubdqNO₂**, and **RubdNO₂** complexes, were not observed alteration in

Data are expressed as mean $\pm$ SD of two measurements.

Table 3

Binding constant K_a , and the number of binding sites n for system HSA-RubdqNO₂ and HSA-RubdNO₂ at different temperatures.

System	T (K)	$K_a (\times 10^3 \text{ M}^{-1})$	n	R^2
HSA-RubdqNO ₂	298	6.54 ± 0.53	0.90	0.9978
	303	5.03 ± 0.65	0.93	0.9824
	308	11.52 ± 3.69	0.98	0.9743
HSA-RubdNO	298	0.44 ± 0.06	0.71	0.9699
	303	3.25 ± 0.02	0.84	0.9606
	308	4.42 ± 0.07	0.89	0.9885

Data are expressed as mean $\pm$ SD of two measurements.

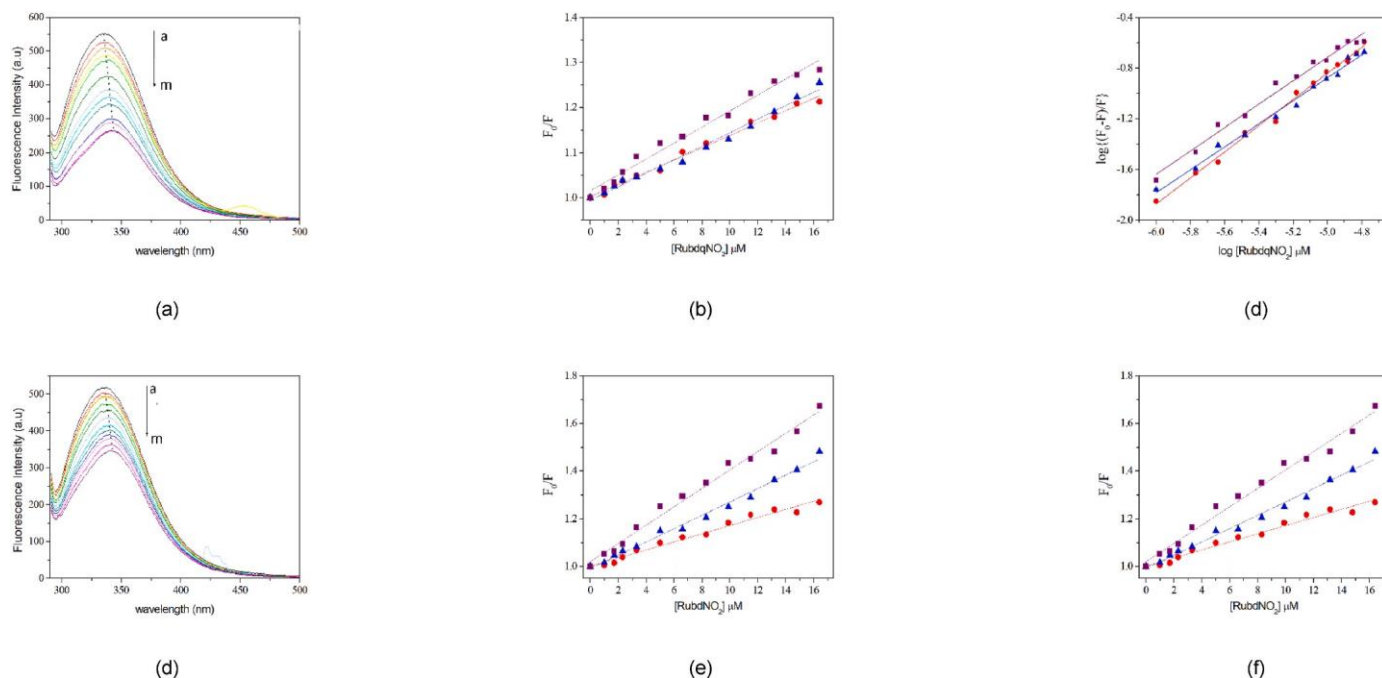


Fig. 6. Fluorescence suppression spectra, Stern-Volmer and modified Stern-Volmer plots for HSA in the presence of **RubdqNO₂** (a-c) and **RubdNO₂** (d-f) complexes in PBS (pH = 7.4) at 308 K. $\lambda_{\text{exc}} = 280 \text{ nm}$. Excitation/emission windows: 5/20 nm [HSA] = 1.0 μM (black line); [Complex] = 0; 1.0; 1.7; 2.3; 3.3; 5.0; 6.6; 8.3; 9.9; 11.5; 13.2; 14.8; 16.4 μM (a → m). ■ (308K); ▲ (303K) and ● (298K).

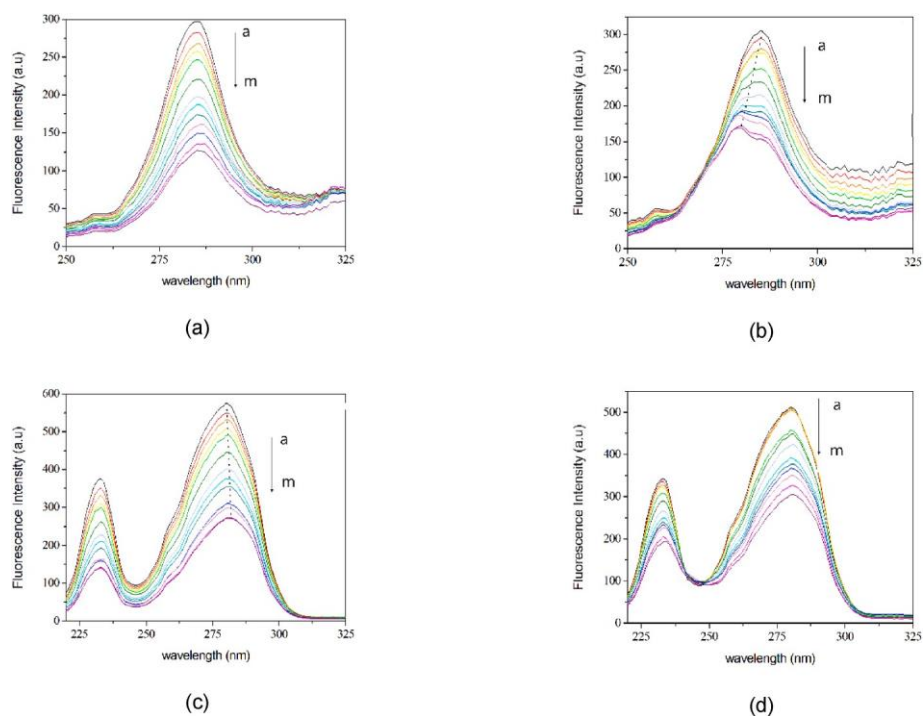


Fig. 7. Synchronous fluorescence suppression spectra ($\Delta\lambda = 15$ nm) (a–b) and ($\Delta\lambda = 60$ nm) (c–d) for HSA in the presence of the complexes **RubdqNO₂** (a and c) and **RubdNO₂** (b and d), in PBS (pH = 7.40) at 308 K for 24 h. Excitation/emission windows: 5/20 nm [HSA] = 1.0 μ M (—); [Complex] = 0; 1.0; 1.7; 2.3; 3.3; 5.0; 6.6; 8.3; 9.9; 11.5; 13.2; 14.8; 16.4 μ M (a→m).

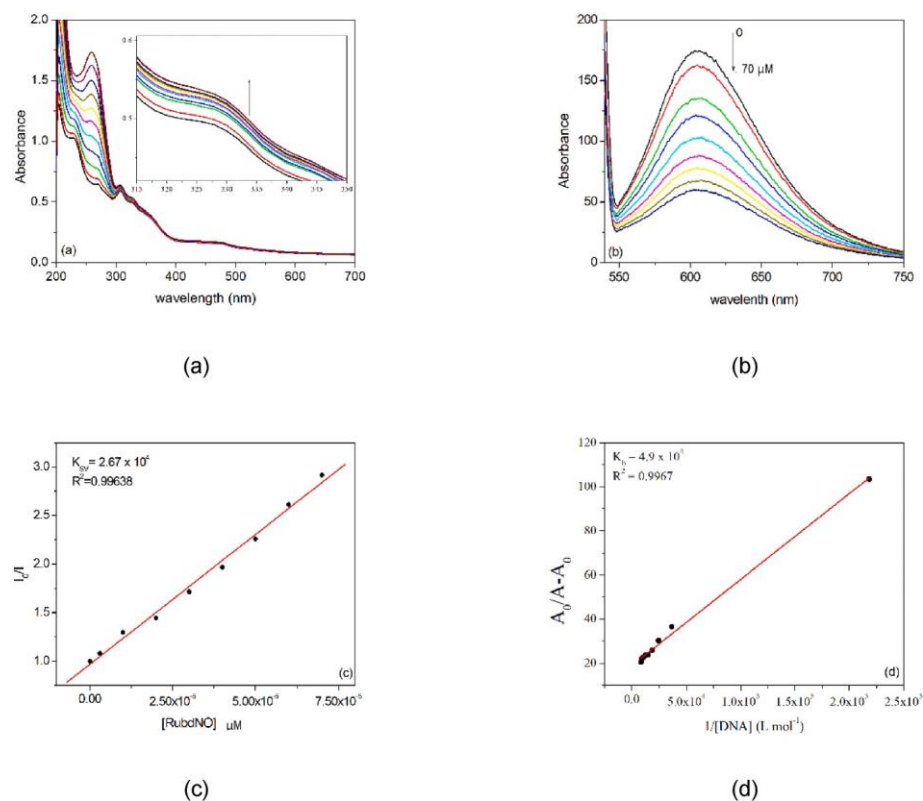


Fig. 8. UV-vis spectra variation of **RubdNO** complex (50 mM Tris buffer, 5 mM NaCl, pH = 7.4) with addition of the *fs*-DNA solution 3 mM (0–119.1 μ M) at 310 K (a). Fluorescence spectra of *fs*-DNA-EB (50 mM Tris buffer, 5 mM NaCl, pH = 7.4) in the presence of varying amounts of **RubdNO** complex (0–70 μ M) (b). Stern-Volmer plots of *fs*-DNA-EB for **RubdNO** complex (c). Benesi-Hildebrand plots for the spectral variation of complex **RubdNO** at 350 nm after titration with *fs*-DNA (d).

UV-vis spectra during *fs*-DNA addition.

Ethidium bromide (EB = 3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide) when intercalated with the DNA molecule presents fluorescence emission at 606 nm. The displacement of the EB by another DNA intercalator molecule decreases the fluorescence intensity. The magnitude and propensity of the binding are measured by calculating K_{SV} (Stern–Volmer constant), using the Stern–Volmer equation 5.

The K_{SV} values, for **RubdNO** (Fig. 8c), **RubdNO₂**, **RubdqNO**, and

RubdqNO₂ (Fig. S6 Supplementary Material) were found to be $(5.09 \pm$

$0.35) \times 10^4 \text{ M}^{-1}$, $(2.90 \pm 0.22) \times 10^4 \text{ M}^{-1}$, $(1.26 \pm 0.21) \times 10^4 \text{ M}^{-1}$ and $(6.58 \pm 0.54) \times 10^3 \text{ M}^{-1}$, respectively. The quenching constant (K_{SV}) values for EB displacement were observed in extent order: **RubdNO** > **RubdNO₂** > **RubdqNO** > **RubdqNO₂**. The low binding affinities for the **RubdqNO₂** have made them displace less amount of EB from DNA suggesting their partial intercalation behavior.

The *fs*-DNA + EB solution was titrated with nitrosyl and nitro complexes solution, and the comparison of the intercalation constant values for EB ($K_{app} \pm 10^7 \text{ M}^{-1}$) [45] allows the relative intercalating capacities to be assessed. The **RubdNO** ($K_{app} 1.25 \times 10^6 \text{ M}^{-1}$) and **RubdNO₂** ($K_{app} 1.67 \times 10^6 \text{ M}^{-1}$) are moderated intercalant agents ($K_{app} 1 \times 10^6 \text{ M}^{-1}$). While the **RubdqNO** ($K_{app} 7.1 \times 10^5 \text{ M}^{-1}$) exhibited two orders of magnitude lower than EB and can be considered weak intercalant agent. The **RubdqNO₂** does not reach 50 % of EB fluorescence quenching, which proposes its very poor intercalating capacity coherent to K_{SV} value. Probably, the **bd** diimine bidentate ligand improves a great planarity area for the complexes and higher hydrophobicity [68,69], than **RubdNO** binds to DNA more strongly by intercalation mode.

The influence of the **RubdNO** and **RubdNO₂** complexes on the secondary structure of *fs*-DNA was verified by circular dichroism (CD) spectroscopy (Fig. 9). CD analysis provides rich information about DNA conformation. It can monitor the region between 230 and 310 nm, in which it is possible to see a strong positive signal around 275 nm (B-DNA) due to base stacking and a negative signal around 250 nm (Z-DNA) due to polynucleotide helicity [45,70].

Increased amounts of **RubdNO** to a *fs*-DNA solution showed a decrease in the B-band and no change in the Z-band. This behavior is indicative of groove binding. The **RubdNO₂** complex promotes a reduction of the intensity of both λ 245 and 275 nm bands at the ratio [complex/*fs*-DNA] 1.66. In addition, a red shift of the positive and negative bands with a hypochromic effect on the second one can be observed. These results have already been described for copper-diimine complexes [70] and primarily suggest groove binding nature accompanied by the intercalation mode.

1.2. Antiviral activity

Antiviral studies of nitrosyl (**RubdNO** and **RubdqNO**) and nitro

(**RubdNO₂** and **RubdqNO₂**) ruthenium complexes were realized using the Vero E6 cells infected with ZIKV_{PE243} at a MOI of 0.01 (Fig. 10) and BHK-21 cells infected with CHIKV-*nanoluc* at a MOI of 0.1 (Fig. 11). The ruthenium complexes were used to treat both cell lineages with concentrations ranging from 1.5 to 200 μM , in a two-fold serial dilution. After 72h, cells were fixed, and infection by ZIKV was analyzed by immunofluorescence assay [49]. For CHIKV, after 16h, cells were lysed and a *Renilla*-luciferase assay was performed [71]. Simultaneously, the

cytotoxicity of the complexes was also evaluated through the

dose-response assay. The effective concentration of 50 % (EC_{50}) and cytotoxic concentration of 50 % (CC_{50}) were calculated employing a non-linear regression. The values of CC_{50} and EC_{50} were used to calculate the selectivity index ($SI = CC_{50}/EC_{50}$).

As a result, for ZIKV, **RubdNO** had an EC_{50} of 13.9 μM and a CC_{50} of 169.1 μM (Fig. 10A–Table 4), while **RubdqNO** had an EC_{50} of 39.5 μM and a CC_{50} of 128.7 μM (Fig. 10B–Table 4). The complexes **RubdNO₂** and **RubdqNO₂**, on the other hand, had an EC_{50} of 12.4 μM and 56.9 μM , respectively, and a CC_{50} of 179.2 μM and 180.5 μM (Fig. 10C and D, Table 4). For CHIKV, **RubdNO** and **RubdqNO** had an EC_{50} of 14 μM and 71.4 μM , and a CC_{50} of 343.7 μM and 41 μM (Fig. 11A and B, Table 4). Further, **RubdNO₂** and **RubdqNO₂** showed an EC_{50} of 79.6 μM and 44.3 μM and a CC_{50} of 11.4 μM and 23.7 μM , respectively (Fig. 11C and D, Table 4).

Complexes **RubdNO** and **RubdNO₂** were the ones with the highest SI of 12.2 and 14.5, respectively, for ZIKV. Similarly, the same complexes presented the highest SI for CHIKV, with 24.8 and 6.9, respectively (Table 4).

To investigate the action mechanism of nitrosyl and nitro terpyridine ruthenium complexes in virus RNA replication, BHK-CHIKV-NCT, and BHK-21-RepZIKV-IRES-Neo cells were used. These stable cell lines contain replicative CHIKV and ZIKV replicons which express the viral nonstructural proteins, the *Renilla* luciferase for the BHK-21-RepZIKV-IRES-Neo cells, and *Renilla* luciferase and Enhanced Green Fluorescent Protein (EGFP) for the BHK-CHIKV-NCT cells [50,51]. The measurement of these reporters allows the assessment of each compound effect on replication as well as on the production and translation of sub genomic RNAs. For antiviral assays, BHK-CHIKV-NCT and BHK-21-RepZIKV-IRES-Neo cells were treated with the highest non-cytotoxic concentration of each compound. The *Renilla* luciferase activity levels were measured 72 h post-infection as previously described [50,51]. The viability assay was performed simultaneously. H₂O was used as untreated control.

The treatment of BHK-CHIKV-NCT cells with 10 μM of complexes **RubdNO** and **RubdNO₂** inhibited 81.4 % and 39 % of CHIKV replication, respectively (Fig. 12A). The compounds **RubdqNO** and **RubdqNO₂** (10 μM) inhibited CHIKV replication in 27 % and 30.1 %, respectively. Further, at BHK-21-RepZIKV-IRES-Neo cells the *Renilla* luciferase expression was decreased by 72 % for **RubdNO** and 84.2 % for

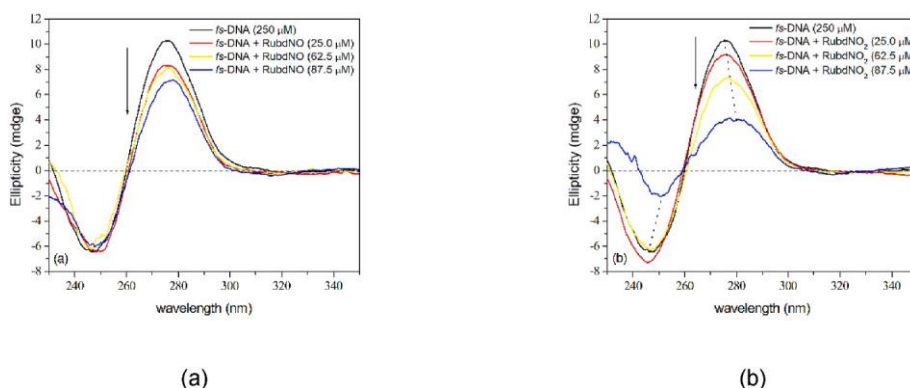


Fig. 9. CD spectra of *fs*-DNA in the absence and the presence of increasing amounts of **RubdNO** (a) and **RubdNO₂** (b) complexes. [*fs*-DNA] = 250 μM and [Complexes] = 25.0 μM , 62.5 μM and 87.5 μM . Time of incubation before each analysis: 1 h.

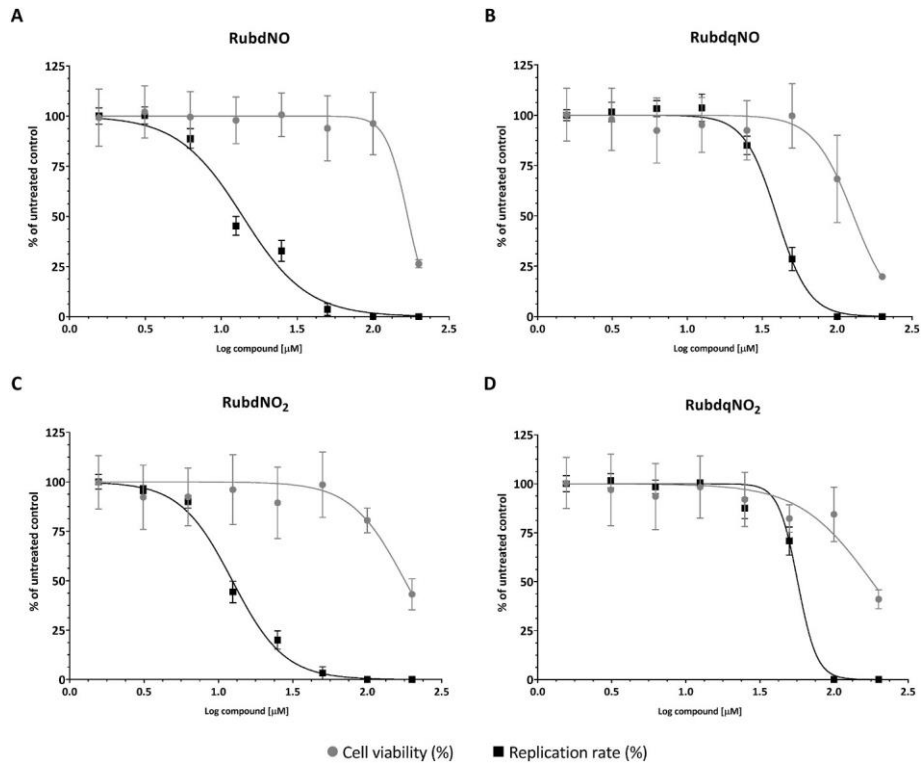


Fig. 10. Effect of RubdNO, RubdNO₂, RubdqNO, and RubdqNO₂ activity on ZIKV replicative cycle. (A) RubdNO, (B) RubdqNO, (C) RubdNO₂, (D) RubdqNO₂. Vero E6 cells were treated with complexes at concentrations ranging from 1.5 to 200 μM , and the effective concentration of 50 % (EC₅₀) and cytotoxic concentration of 50 % (CC₅₀) were determined. ZIKV_{PE243} replication was measured by FFU mL⁻¹ (indicated by ■) and cellular viability was measured using an MTT assay (indicated by ●). Mean values of three independent experiments each measured in triplicate including the standard deviation is shown.

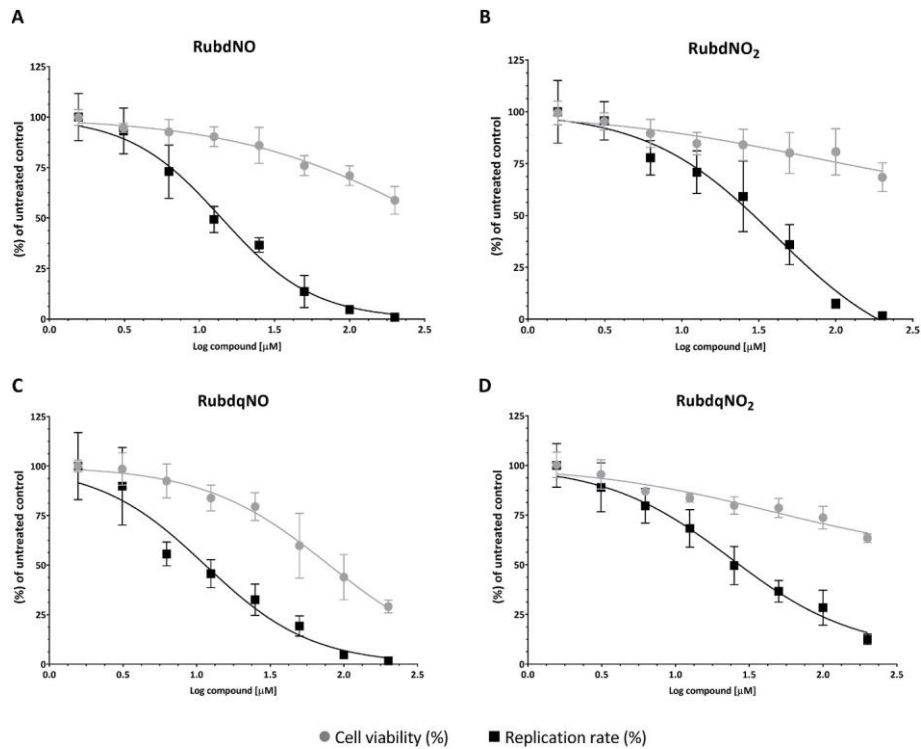


Fig. 11. CHIKV inhibition by RubdNO, RubdNO₂, RubdqNO, and RubdqNO₂ complexes. (A) RubdNO, (B) RubdNO₂, (C) RubdqNO, (D) RubdqNO₂. BHK-21 cells were treated with complexes at concentrations ranging from 1.5 to 200 μM , and the effective concentration of 50 % (EC₅₀) and cytotoxic concentration of 50 % (CC₅₀) were determined. The *nanoluciferase* activity levels were measured 16 h.p.i. for CHIKV (indicated by ■) and cellular viability was measured using an MTT assay (indicated by ●). Mean values of three independent experiments each measured in triplicate including the standard deviation is shown.

Table 4

ZIKV and CHIKV antiviral activity of the nitrosyl and nitro terpyridine ruthenium complex expressed as Selectivity index (SI) = ratio between cytotoxicity and antiviral activity (CC_{50}/EC_{50}).

Compound	ZIKV			CHIKV		
	CC_{50} (μ M)	EC_{50} (μ M)	SI	CC_{50} (μ M)	EC_{50} (μ M)	SI
RubdNO	169.1	13.9	12.2	343.7	14	24.8
RubdqNO	128.7	39.5	3.3	71.4	41	1.7
RubdNO₂	179.2	12.4	14.5	79.6	11.4	6.9
RubdqNO₂	180.5	56.9	3.2	44.3	23.7	1.8

RubdNO₂, both at 50 μ M and 10 μ M (Fig. 12B). The molecules **RubdqNO** and **RubdqNO₂** (50 μ M) on the other hand, decreased the

luminescence levels by 32.9 % and 46 %, respectively.

The antiviral activity from nitrosyl and nitro terpyridine ruthenium complexes was occasioned by the replication inhibition of RNA virus

probably promoted by nitric oxide released from the terpyridine ruthenium complex [72]. Recently, Akaberi et al. [12], demonstrated that NO released from SNAP (nitric oxide donor S-nitro-

so-N-acetylpenicillamine) can inhibit the replication of SARS-CoV-2 in Vero E6 and identified the SARS-CoV-2 main protease as a target for NO. The potential mechanism of the viral cysteine could be a possible target of S-nitrosylation resulting in inhibition of the protease activity and consequent reduction in viral replication [5]. In this perspective, we found that some nitrosyl and nitro ruthenium complexes act as NO donor agents under chemical reduction by reductors species such as phenylephrine [18,27] and thiols (L-cysteine and glutathione) [73]. Franco [74] reported that some nitrosyl metal complexes, act as NO donors via simple nucleophilic addition of a thiolate ion (RS^-) on the nitrosyl ligand (NO^+) coordinated to ruthenium complexes and subsequent decomposition of S-nitrosothiol in NO^0 (Scheme 1). Considering these data from the literature [73,75], we evaluated the behavior of the complexes in the presence of L-cysteine (Figs. 13 and 14). Fig. 13a and b shows the spectral alteration from **RubdNO** and

RubdqNO in PBS pH 7.4 with the addition of L-cysteine. The decreasing of the TCML ($Ru-NO^+$) band close to ~350 nm is indicative of NO release [27]. The NO release from **RubdqNO** is confirmed by the NO sensor as observed in the NO detected by chronoamperogram (Fig. 13c). The nitro ruthenium complex, **RubdNO₂**, and **RubdqNO₂**, were evaluated in the NO release in the presence of L-cysteine. The UV vis spectral profile as well as the chronoamperogram of NO release were obtained during the reaction between **RubdNO₂** and L-cysteine are described in Fig. 14 a and b. The UV-vis spectra for **RubdNO₂**, show a displacement for the MLCT band to lower energy during 60 min (504 $\rightarrow$ 510 nm). After 24h this band appears at 515 nm. This spectral alteration was accompanied by

NO sensor for the first time which showed the NO release. The mechanism of transformation of NO_2^- to NO at various redox-

active transition metals such as Fe/Co/Ni/Cu has been relatively well explored in the presence of a diverse array of exogenous reductants [76]. Warren et al. proposed the reaction mechanism between thiol (RSH) and $[Zn^{II}]$ -nitrite, presumably through an acid-base exchange route to yield $[Zn^{II}]-SR$ and HNO_2 . Subsequently, HNO_2 is proposed to nitrosate RSH , affording S-nitrosothiol ($RSNO$) [77]. The nonheme Fe^{II} complexes co-

ordinated to the dinitro react with RSH affording disulfide ($RSSR$) and NO [78]. Based on our experimental results and in the literature, data cited above, we proposed a speculative mechanism (Scheme 2) for NO release from nitro ruthenium complex in presence of L-cysteine. We suggest NO formation from $[Ru^{II}(NH-NHq-R)(NO_2)(tpy)]^{n+}$ complexes occurs through two pathways. Initially, the acid-base equilibrium relative to $\{Ru^{II}-NO^+\} \rightleftharpoons \{Ru^{II}-NO_2\}^+$ occurs by nucleophilic attack of hydroxy ion, which depends on pH [34]. The co-ligands L **bpy** provide $[Ru^{II}(L)(NO^+)(tpy)]^{3+}$ species with stability at pH < 5.0 [28]. Otherwise the co-ligands L **bd** or **bdq** confer stability to the complex at pH 7.4 [27]. The equilibrium between $\{Ru^{II}-NO^+\} \rightleftharpoons \{Ru^{II}-NO_2\}^+$ can shift by the consumption of $\{Ru^{II}-NO^+\}$ due to L-cysteine reduction. The NO release can be described similarly in Scheme 1. In the second mechanism, based in recent work described by da Silva

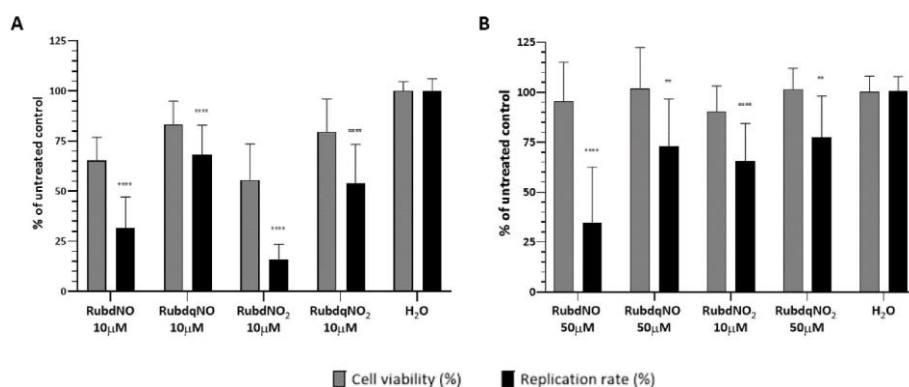
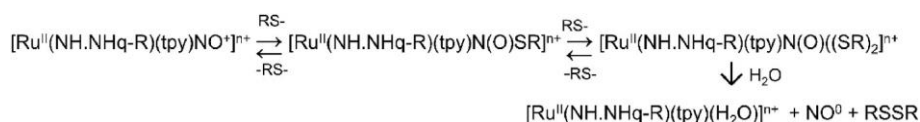


Fig. 12. Effect of **RubdNO**, **RubdNO₂**, **RubdqNO**, and **RubdqNO₂** complexes on BHK-CHIKV-NCT and BHK21-RepZIKV-IRES-Neo cells. (A) BHK-CHIKV-NCT and (B) BHK21-RepZIKV-IRES-Neo cells were treated with each compound at the highest non-cytotoxic concentration. After 72h, cells were lysed, and a *Renilla*-luciferase assay was performed to assess luciferase expression levels. Cell viability was accessed treating cells with the compounds at the specific concentrations. After treatment, the medium with the compound was removed and replaced with medium with MTT at 1 mg mL⁻¹ and incubated for 30 min. After incubation, the medium was removed, and the crystals solubilized with DMSO. Surviving cells were measured by absorbance (560 nm). H₂O was used as an untreated control. Mean values of three independent experiments each measured in quadruplicate including the standard deviation is shown. P values < 0.05 were considered significant. (**) P < 0.01, (***) P < 0.001 and (****) P < 0.0001.



Scheme 1. Mechanism of reaction between $[Ru^{II}(NH.NHq-R)(NO^+)(tpy)]^{n+}$ and L-cysteine (RS^-).

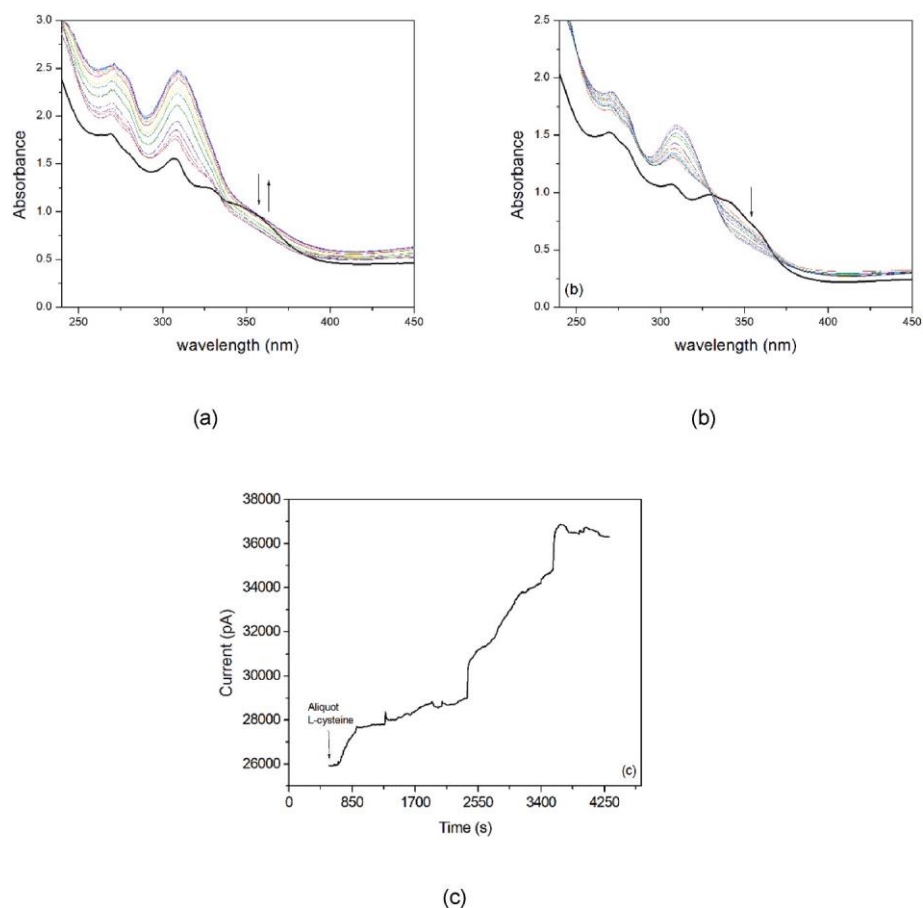


Fig. 13. UV-vis spectral changes for the reaction of **RubdNO** (a) and **RubdqNO** (b) with L-cysteine. [Complex] = 50 μ M, [L-cysteine] = 3.0 mM, pH = 7.4 (PBS solution), 25 $^{\circ}$ C. Chronoamperogram of NO release from **RubdqNO** in presence of L-cysteine (c).

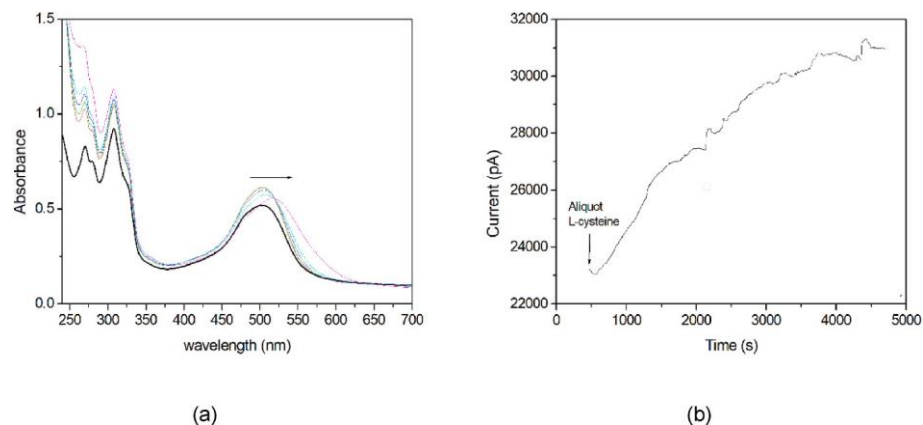
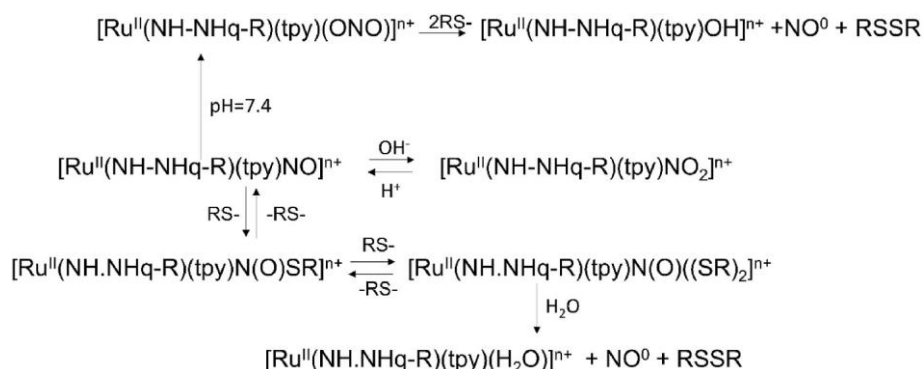


Fig. 14. UV-vis spectral changes (a) and chronoamperogram of NO release (b) for the reaction of **RubdNO₂** with L-cysteine. [Complex] = 10 μ M, [L-cysteine] = 3.0 mM, pH = 7.4 (PBS buffer solution), 25 $^{\circ}$ C.

et al., the isomerization of nitro ligand coordinated to polypyridine complex $cis-[(bpy)_2(4-pic)Ru-(NO_2)]^+$ in nitro-isomeric structure $cis-[(bpy)_2(4-pic)Ru-(ONO)]^+$ occurring during the nucleophilic attack of the hydroxide ion of $cis-[(bpy)_2(4-pic)Ru-(NO)]^{3+}$ [79]. Silva et al. isolated the $cis-[(bpy)_2(4-pic)Ru-(ONO)]^+$ in phosphate buffer solution pH 7.4.

We hypothesize that can be occurring during the acid - base equilibrium between $\{Ru^{II}-NO^+\} \rightleftharpoons \{Ru^{II}-NO_2\}^+$, there is the formation of $[(tpy)(L)Ru^{II}-ONO]^+$ isomeric specie. Probably, this isomerization, presents a slow kinetics reaction, since in the HPLC analysis (after 2h in

aqueous solution pH = 7.4) the other peaks were not identified. This argumentation was idealized from the UV-vis spectral observation, where after 24h, the MLCT band was displacement 515 nm (Fig. 14a). The hydroxo complex $[Ru^{II}(tpy)(bpy)(OH)]^+$ shows MLCT band at 510 nm [80], close region for $[(tpy)(L)Ru^{II}-OH]^+$ obtained from $[(tpy)(L)Ru^{II}-H_2O]^{2+}$ at pH 12 (Fig. S7 Supplementary Material). The $[(tpy)(L)Ru^{II}-OH]^+$ species can be produced from the nucleophilic attack by the thiol on $[(tpy)(L)Ru^{II}-ONO]^+$. In general, the antiviral action mechanism described from metal-lodrugs involving the inhibition of viral protein, or RNA viral inhibition



Scheme 2. Mechanisms of reaction between $[\text{Ru}^{\text{II}}(\text{NH-NHq-R})(\text{NO}_2)(\text{tpy})]^{n+}$ and L-cysteine (RS^-).

[81–83]. Furthermore, physical chemistry properties from metallodrugs as lipophilicity and metal ion charge influence the interaction of the virus with metal complexes [52,84].

The nitrosyl and nitro ruthenium complex has been more explored in the literature as exogenous NO donor in vasodilatation [17], antitumor [85] and antimicrobial activity [86]. We believe that the synergistic effects between greater lipophilicity and NO release under L-cysteine reduction from the **RubdNO** and **RubdNO₂** complexes described in this work are unprecedented, as far as we know, and can contribute to expanding the scope of applications of nitro and nitrosyl ruthenium complexes.

Conclusion

In this study two new ruthenium complexes with nitro substituent, **RubdNO₂** and **RubdqNO₂**, were synthesized. Their structures were confirmed through elemental analysis, mass spectrometry (MALDI-TOF), ¹H NMR, infrared and UV-vis spectroscopy. The **RubdqNO₂** complex is formed by 2 position isomers, as indicated by HPLC analysis, which was not observed with the **RubdNO₂** complex. The values of the partition coefficients of the **RubdqNO** and **RubdqNO₂** complexes indicate that they are more hydrophilic than the **RubdNO** and **RubdNO₂** complexes. Furthermore, they showed greater interaction with HSA, probably because the -COOH group of the **bdq** ligand can interact more efficiently with this protein. The **RubdNO** complex showed strong *fs*-DNA interaction, with moderated intercalation constant for **RubdqNO** and **RubdNO₂**. The most lipophilic complexes, **RubdNO** and **RubdNO₂**, has higher antiviral activity (higher SI values). The nitro and nitrosyl terpyridine ruthenium complexes proposed in this work are NO donors in the presence of the L-cysteine, contributing to the proposal of the S-nitrosylation mechanism of Cys in the viral replication.

CRediT authorship contribution statement

Naiara Cristina Bessas: Data curation, Formal analysis, Investigation. **Evelyn Christine de Souza Arantes:** Data curation, Formal analysis. **Natasha Marques Cassani:** Data curation, Formal analysis, Visualization. **Uriel Enrique Aquino Ruiz:** Data curation, Formal analysis, Visualization. **Igor Andrade Santos:** Data curation, Formal analysis, Visualization. **Daniel Oliveira Silva Martins:** Data curation, Formal analysis, Visualization. **Ana Laura Costa Oliveira:** Data curation, Formal analysis, Visualization. **Giovanna André Antoniucci:** Data curation, Formal analysis, Visualization. **Arthur Henrique Cavalcante de Oliveira:** Methodology, Visualization, Writing – review & editing. **Gilson DeFreitas-Silva:** Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Ana Carolina Gomes Jardim:** Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Renata Galvão de Lima:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Writing –

original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.niox.2024.04.006>.

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MINI REVIEW



An update on the development of antiviral against Mayaro virus: from molecules to potential viral targets

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Abstract

Mayaro virus (MAYV), first isolated in 1954 in Trinidad and Tobago islands, is the causative agent of Mayaro fever, a disease characterized by fever, rashes, headaches, myalgia, and arthralgia. The infection can progress to a chronic condition in over 50% of cases, with persistent arthralgia, which can lead to the disability of the infected individuals. MAYV is mainly transmitted through the bite of the female *Haemagogus* spp. mosquito genus. However, studies demonstrate that *Aedes aegypti* is also a vector, contributing to the spread of MAYV beyond endemic areas, given the vast geographical distribution of the mosquito. Besides, the similarity of antigenic sites with other *Alphavirus* complicates the diagnoses of MAYV, contributing to underreporting of the disease. Nowadays, there are no antiviral drugs available to treat infected patients, being the clinical management based on analgesics and non-steroidal anti-inflammatory drugs. In this context, this review aims to summarize compounds that have demonstrated antiviral activity against MAYV in vitro, as well as discuss the potentiality of viral proteins as targets for the development of antiviral drugs against MAYV. Finally, through rationalization of the data presented herein, we wish to encourage further research encompassing these compounds as potential anti-MAYV drug candidates.

Keywords Mayaro fever · Mayaro virus · Antiviral · Natural and synthetic compounds · Viral targets

Introduction

The Mayaro virus (MAYV) belongs to the *Togaviridae* family and *Alphavirus* genus, which is composed of a positive single-stranded RNA of 11,5 kb in length, covered by an icosahedral capsid and viral envelope (Fig. 1) (Diagne et al. 2020). It was first isolated in 1954 in Trinidad and

Tobago islands from the blood of five febrile rural workers (Anderson et al. 1957). Since then, sporadic outbreaks have been identified in countries with tropical forests, including Brazil, Bolivia, Suriname, Peru, Equator, Venezuela, and recently Haiti (Aguilar-Luis et al. 2020). In addition, cases in North America (Taylor et al. 2005) and Europe (Theilacker et al. 2013) were reported in citizens who traveled to South America.

MAYV belongs to the Semliki Complex, a subgroup of the *Alphavirus* genus that includes Bebaru virus, Chikungunya virus (CHIKV), Getah virus, Una Forest virus (UNAV), O'nyong-nyong virus, among others. Viruses from this complex are classified into the same serological group, sharing common antigenic sites, resulting in cross-reactivity in conventional serological tests, complicating the accurate diagnosis of MAYV infections and the underreporting of the disease (Acosta-Ampudia et al. 2018).

MAYV infection is the causative agent of the Mayaro Fever, a disease characterized by acute fever, rashes, headache, retro-orbital pain, nausea, diarrhea, myalgia, and arthralgia (Acosta-Ampudia et al. 2018) that remain for months or years in over 50% of cases (Mackay and Arden

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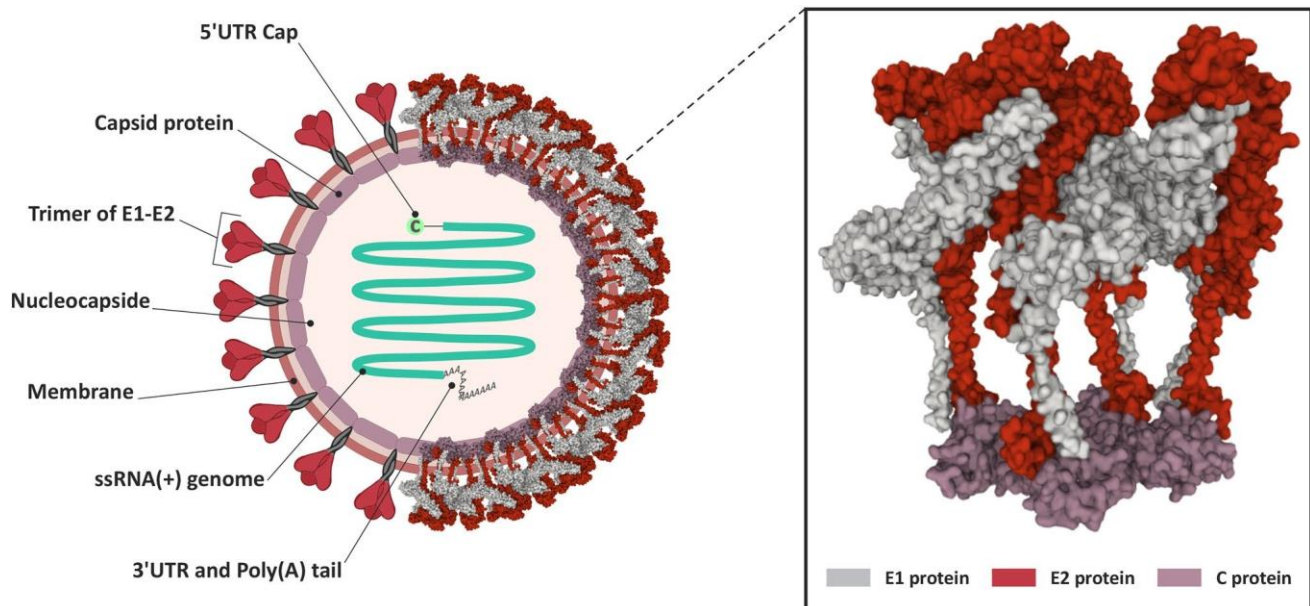


Fig. 1 Schematic structure of MAYV virion. Electron microscopy data show that the viral particle is about 70 nm in diameter. The virion is composed of a positive single-stranded RNA with an icosahedral capsid formed by the capsid protein (C), covered by an envelope membrane inserted of the E1, E2, and E3 glycoproteins. The E3

protein is not shown since it detaches from the E2 protein at the viral surface during maturation (Ribeiro-Filho et al. 2021). Viral structure was based on Cryo-EM structure of mature MAYV (PDB ID: 7KO8) (Ribeiro-Filho et al. 2021)

2016; Li et al. 2019), culminating in physical disability (Ferreira et al. 2018). In severe cases, Mayaro fever can cause intermittent fever, neurological complications, myocarditis, and ultimately death (Acosta-Ampudia et al. 2018).

MAYV is transmitted through the bite of the *Haemagogus* spp. mosquito, usually from infected non-human primates, birds, rodents, and small mammals to susceptible humans (Esposito and Fonseca 2017). The vector competence of *Aedes aegypti* mosquitoes in the transmission of MAYV is also known, and considering the wide geographic distribution of the *Aedes* genus, there is a concern about virus spread beyond endemic areas (Long et al. 2011; Choi et al. 2019).

The MAYV replication begins with virus entry into the host cells via specific receptors on the cell surface, such as MXRA8, triggering clathrin-mediated endocytosis or, alternatively, through a caveolin-coated pit (Carvalho et al. 2017; Zhang et al. 2018). Through pH changes in the endosome, the interaction between the endosome membrane and the viral envelope leads to the viral RNA release into the cytoplasm. MAYV RNA is composed of two open reading frames (ORFs). The first ORF is translated in the non-structural viral proteins (nsP) nsP1, nsP2, nsP3, and nsP4, which form the replicative complex (RC), responsible to produce a negative strand from the viral genome, that serves as templates for the synthesis of new positive-sense strands, besides the sub-genomic 26S RNA (Diagne et al. 2020). The other ORF is transcribed from the negative strand, and

translated into a polyprotein which is cleaved to the structural proteins C, E1, E2, E3, and 6K (viroporin). Additionally, a frameshift event occurs during translation of the 6K gene, yielding the production of the transframe (TF) protein, comprised of a C-terminal extension of the 6K protein in the +1 ORF. TF protein has been associated with *alphavirus* budding efficiency and/or assembly (Firth et al. 2008; Snyder et al. 2013). The replication process is followed by the assembling of the viral components and virus release through budding in mammalian cells membrane (Jose et al. 2017; Brown et al. 2018) (Fig. 2), or through an alternative exocytosis pathway in insect cells (Acosta-Ampudia et al. 2018).

Despite the chronic condition of a high number of MAYV infections, there is no licensed antiviral against Mayaro fever. The treatment is palliative and consists in managing the symptoms using analgesics and/or non-steroidal anti-inflammatory drugs (Sun and Wu 2019). Therefore, research data have been produced on the potential of natural and synthetic molecules against MAYV. Here we summarize the natural and synthetic compounds previously described to possess antiviral activity against MAYV (Table 1). Thus, we critically compare molecules that could be further investigated in vivo and suggest protein targets to be further evaluated as anti-MAYV molecules. Finally, we aim to encourage further research encompassing these compounds as potential anti-MAYV drug candidates to treat infected patients.

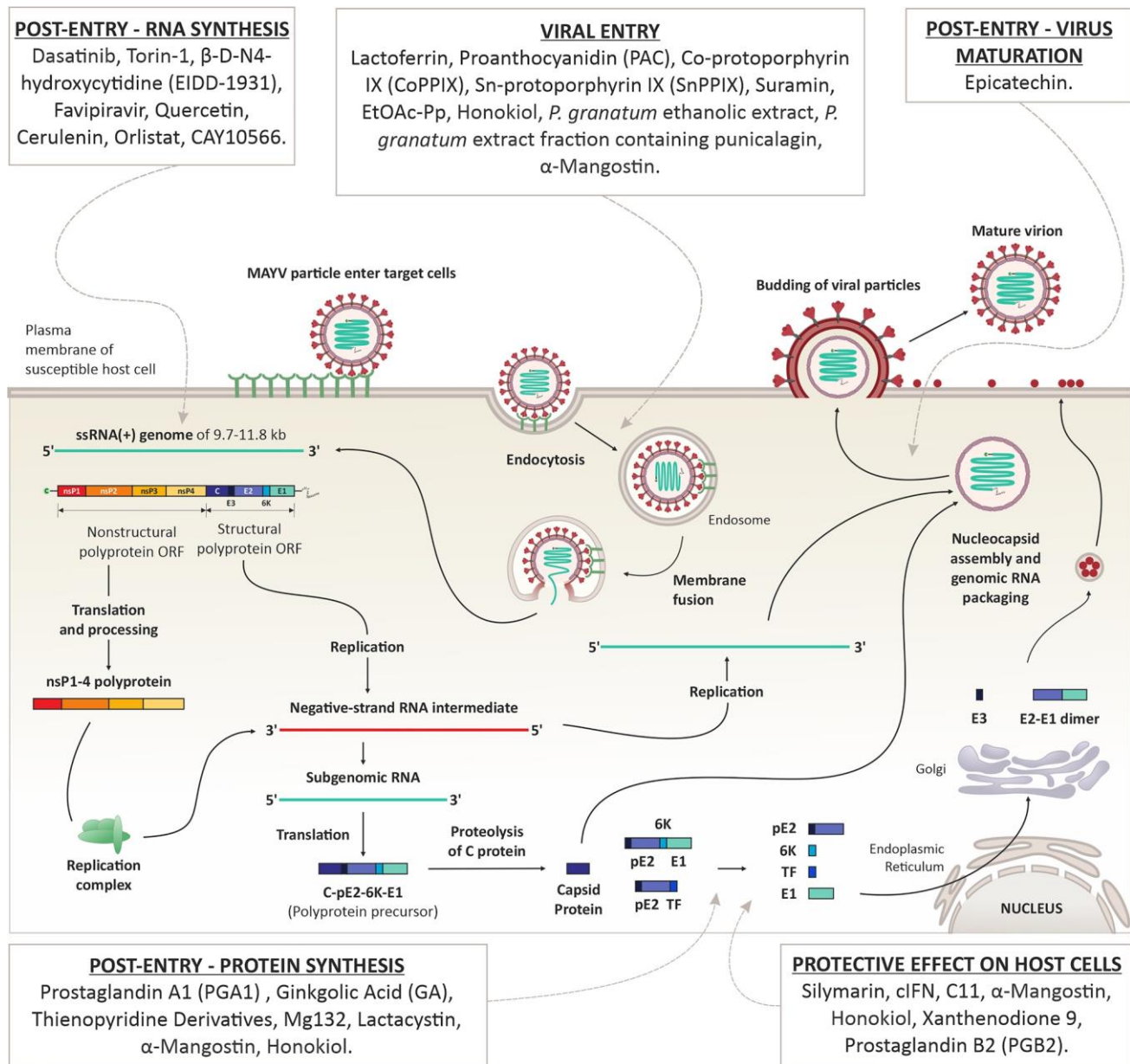


Fig. 2 Schematic representation of MAYV replicative cycle. The replicative cycle and genomic structure of MAYV. *E* envelope, *nsP* non-structural protein, *UTR* untranslated region, *nt* nucleotides. MAYV attaches to the host cells through the interaction between the host receptors and glycoproteins. Endocytosis occurs through a clathrin-coated pit or, alternatively, a caveolin-coated pit; virus internalization starts after interaction with host cell receptors; the low pH in the endosome leads to structural modifications in the viral envelope that reveal the E1. The latter mediates cell membrane-virus fusion, and later, endosomal cell membrane-virus fusion. Viral uncoating results in the release of the viral genome, and the replication stage occurs (translation and transcription). From the RNA of the virus, nsP precursors are generated; the replication complex is obtained from nsP proteins. This complex thus allows the synthesis of a minus-strand RNA, and it is used as the template to generate both genomic and

subgenomic RNAs. Polyprotein precursor is cleaved by an autoproteolytic serine protease; and genomic RNA involved in nucleocapsid core assembly and genomic RNA packaging. Maturation of glycoproteins pE2 and E1 occurs. In the Golgi, processed glycoproteins are associated. Then they are transported into the cell membrane. At the cell membrane, the pE2 is split into E2 and E3; there is also the association of the viral RNA with the capsid C and the recruitment of E1 allows viral assembly. Last, particles of MAYV associated with the core are released outside of the host cell through the membrane. Compounds with antiviral activity against MAYV are indicated in each step of the virus replication cycle: (i) Viral Entry; (ii) Post-Entry (Maturation, RNA and Protein Synthesis); and (iii) Protective Effect on Host Cells. Viral cycle was based on the work of Diagne and colleagues (Diagne et al. 2020)

Table 1 Compounds with antiviral activity against MAYV

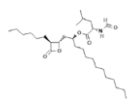
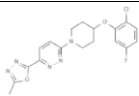
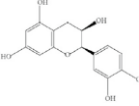
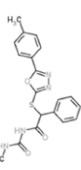
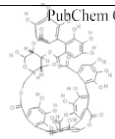
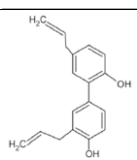
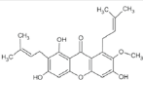
Compound*	Chemical Structure	Structure Reference	Antiviral activity Reference	Inhibition	Mechanism	EC ₅₀	CC ₅₀	Cell lineage
Prostaglandin A1 (PGA1)		PubChem CID: 5281912 (Kim et al. 2021)	(Ishimaru et al. 1998; Burlandy and Rebello 2001; Caldas et al. 2018)	Post-entry	Reduction in the synthesis of E1, E2, C, and in the precursors of virus proteins	3 µg/mL	ND	Vero
						1 µg/mL	ND	Hep-2
						3 µg/mL	ND	Vero
Prostaglandin B2 (PGB2)		PubChem CID: 5280881 (Kim et al. 2021)	(Ishimaru et al. 1998)	Host cells	ND	8 µg/mL	ND	Vero
Lactoferrin		PubChem CID: 126456119 (Kim et al. 2021)	(Carvalho et al. 2014)	Viral Entry	Prevents the viral entrance	ND	ND	Vero
Proanthocyanidin (PAC)		(Ferraz et al. 2019)	(Ferraz et al. 2019)	Viral Entry	Interacts with proanthocyanins on the envelope glycoproteins, preventing viral entry	37.9 µM	> 1640 µM	Vero
Ginkgolide Acid (GA)		PubChem CID: 5281858 (Kim et al. 2021)	(Campos et al. 2020)	Post-entry	Inhibits the initial stages of the viral cycle, and suppresses the expression of E1 and E2 viral proteins	ND	ND	Vero, HeLa
Silymarin		PubChem CID: 5213 (Kim et al. 2021)	(Camini et al. 2018)	Host cells	Decreases virus-induced oxidative stress on host cells associated with the	3.58 µg/ml	103.5 µg/mL	HepG2
Thienopyridine Derivative 104	 101: R=H 102: R=CH ₃ 103: R=CH ₃ 104: R=CH ₃ 105: R=CH ₃ 106: R=CH ₃ 107: R=CH ₃ 108: R=CH ₃ 109: R=CH ₃ 110: R=CH ₃ 111: R=CH ₃ 112: R=CH ₃	(Amorim et al. 2017)	(Amorim et al. 2017)	Post-entry	Decreases viral protein synthesis	20.0 µM	2500 µM	Vero
Co-protoporphyrin IX (CoPPIX)		PubChem CID: 3000479 (Kim et al. 2021)	(Neris et al. 2018)	Viral Entry	Impairs viral entry and adsorption	5.94 µM - without light 7.48 µM - with light	762.3 µM - without light 1018 µM - with light	BHK-21, Vero
Sn-protoporphyrin IX (SnPPIX)		PubChem CID: 3000478 (Kim et al. 2021)	(Neris et al. 2018)	Viral Entry	Impairs viral entry and adsorption Generates ROS	5.99 µM - without light 0.09 µM - with light	816 µM - without light 20.7 µM - with light	BHK-21, Vero
Dasatinib		PubChem CID: 3062316 (Kim et al. 2021)	(Broeckel et al. 2019)	Post-entry	Blocks the subgenomic RNA translation, impairing viral replication	ND	ND	ND

Natural compounds as inhibitors of the replicative cycle of MAYV

Some of the first natural molecules tested for their potential to inhibit MAYV replication in vitro were

Prostaglandin A1 (PGA1) and Prostaglandin B2 (PGB2) (Ishimaru et al. 1998). PGA1, a natural molecule that possesses a hormone-like lipid structure, is produced by cells in the presence of injury and/or inflammation (Caldas et al. 2018). Alternatively, PGB2 is a lipid mediator

Table 1 (continued)

Orlistat		PubChem CID: 3034010 (Kim et al. 2021)	(Bakhache et al. 2019)	Post entry	Decreases genome replication due to manipulation of FASN enzymatic activity.	ND	ND	HEK293T
CAY10566		PubChem CID: 16732433 (Kim et al. 2021)	(Bakhache et al. 2019)	Post entry	Decreases genome replication due to manipulation of SCD1 enzymatic activity.	ND	ND	HEK293T
Epicatechin		(Hosseinimehr et al. 2013)	Ferreira et al. (2018)	Post entry	Binds to mature virions released into the extracellular environment	0.247 μ M	> 1.723 μ M	Vero
C11		(Gall et al. 2018)	(Gall et al. 2018)	Host cells	Acts as an agonist of the STING Pathway	ND	ND	THF, MM6
α-punicalagin (<i>P. granatum</i> extract fraction containing punicalagin)		PubChem CID:16149716 (Kim et al. 2021)	(Salles et al. 2021)	Viral Entry	ND	523.1	62.5 μ g/ml	Vero μ g/ml
<i>P. granatum</i> ethanolic extract	NA	NA	(Salles et al. 2021)	Viral Entry	Virucidal	590.8 μ g/ml	12.3 μ g/ml	Vero
Honokiol		(Valdés-Torres et al. 2022)	(Valdés-Torres et al. 2022)	Host cells	Downmodulates the expression of E1 and nsP1 proteins decreases viral RNA and increases type I interferon cellular response	ND	ND	Vero-E6, HeLa
α-Mangostin		(Valdés-Torres et al. 2022)	(Valdés-Torres et al. 2022)	Host cells, Viral Entry and Post-entry	Downmodulates the expression of E1 and nsP1 proteins decreases viral RNA and increases type I interferon cellular response	ND	ND	Vero-E6, HeLa

ND not described, EC_{50} effective concentration of 50%, CC_{50} cytotoxic concentration of 50%, NA not applicable

*A list of abbreviations was provided as supplementary material

that acts as an auxiliary signal to T lymphocyte activation (Ishimaru et al. 1998; Raulin 2002). The antiviral effects of PGA1 and PGB2 against MAYV were investigated by using the plaque reduction assay in infected green monkey kidney cells (Vero cells) (Ishimaru et al. 1998). Cells were infected with MAYV at a concentration of 1 plaque-forming unit (pfu) per cell for 1 h, followed by the administration of PGs at concentrations ranging from 0 to 10 μ g/ml for 1 h. The EC_{50} was observed with doses of 3 μ g/ml for PGA1 and 8 μ g/ml for PGB2. A higher antiviral effect was observed when compounds were added up to 2 h post-infection (h.p.i.) (Ishimaru et al. 1998). PGA1 was more effective than PGB2, being able to inhibit the production of glycoproteins E1 and E2 by over 50% at

10 μ g/ml, resulting in a decrease of infectious particles (Ishimaru et al. 1998).

Later, Burlandy and Rebello corroborated the anti-MAYV activity of PGA1 that reduced 99% of MAYV replication in vitro at 10 μ g/mL through a reduction plaque assay (Burlandy and Rebello 2001). Moreover, to suggest which stage of the replicative cycle was affected, a time-of-drug addition assay was performed. The results showed that PGA1 possesses the strongest effect when added to the cells simultaneously to the virus. The authors also identified a reduction in the synthesis of E1, E2, and C, and in the precursors of virus proteins, which suggests the effect in the early stages of viral replication. Additionally, an increase in the heat shock protein 70 (HSP70) was detected, and since this molecule is

responsible for protein folding, disaggregation, and degradation to ensure proper cell function (Holbrook et al. 1992), it is possible to suggest a mode of action for PGA1 (Burlandy and Rebello 2001). Caldas and coworkers also assessed the effect of PGA1 on MAYV replication and the relevance of HSP70 in this process (Caldas et al. 2018). Human cervix carcinoma cells (Hep-2 cells) were infected with MAYV with a multiplicity of infection (MOI) of 1 for 1 h and treated with different concentrations of the compound. According to the authors, the PGA1 was not cytotoxic, presented an EC_{50} of 1 $\mu\text{g/mL}$, and inhibited up to 95% of viral production at 6 $\mu\text{g/mL}$. In agreement with the previous studies, PGA1 impaired p110, p62, E1/E2, and C synthesis, as well as a significantly increased HSP70 expression (Ishimaru et al. 1998; Caldas et al. 2018). The HSP70 increase seems to be related to the PGA1 antiviral effect since the inhibition of viral replication was only observed in PGA1 concentrations that induced the production of this HSP. In this context, the authors proposed that PGA1 may act in different stages of viral replication due to the different strategies presented by the molecule.

Alternatively, Carvalho and colleagues proposed the natural macromolecule Lactoferrin, a globular glycoprotein, as a candidate for treatment against MAYV (Carvalho et al. 2014). This molecule is produced by exocrine glands, such as mammary and lacrimal glands (Kell et al. 2020) and has been used as apolactoferrin, its apo form found in bovine whey (bLf). To assess the effect of Lactoferrin against MAYV, Vero cells were infected with viral particles labeled with DiD (1,1'-diiododecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt), a lipophilic carbocyanine marker that expresses red fluorescence, and then incubated with bLf at concentrations ranging from 0.2 to 1.0 mg/mL. bLf inhibited viral replication in a dose-dependent manner, with 1 mg/mL being able to inhibit 85% of viral infection. Furthermore, a time-of-drug addition assay was also carried out to investigate which stage of the replication cycle was affected (Carvalho et al. 2014). The bLf mainly acted on virus entry, suggesting that this molecule may interact with the sulfate groups of the glycosaminoglycans layer (GAGs) of the host cells, preventing the attachment of the virus. However, since the specific MAYV receptor at the time of the study was unknown (Carvalho et al. 2014), further studies involving the Mrxa8 are needed to corroborate this mechanism of action.

Other potential antiviral candidates tested against MAYV were the flavonoids 4'-O-methylepigallocatechin (MEP) and the proanthocyanidin [(-)-epicatechin- (4 β $\rightarrow$ 8)-(-)-4'-methylepigallocatechin] (PAC), an MEP dimerized with the epicatechin, isolated from *Maytenus imbricata* roots (Ferraz et al. 2019). The viability of MEP and PAC was evaluated through an MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide)] assay

using Vero cells, and both demonstrated no cytotoxic effect, with CC_{50} over 1640 μM . To assess the antiviral effect, cells were infected with MAYV and treated with concentrations of MEP and PAC. No activity was observed against MAYV with MEP, even at the highest tested concentration (1000 μM). However, the treatment of MAYV-infected cells with PAC resulted in EC_{50} of 37.9 μM and a SI (selectivity index) higher than 43. The treatment at different stages of the virus cycle demonstrated the virucidal activity of PAC, which was confirmed by a dialysis membrane assay. The authors proposed that the mechanism of action may be related to the interaction of proanthocyanins with the envelope glycoproteins, decreasing the infectivity of viral particles (Ferraz et al. 2019). The antiviral effect of the flavonoid Quercetin, as well as the EtOAc, and n-BuOH fractions, isolated from *Bauhinia longifolia*, was investigated by employing the viral yield inhibition assay. Vero cells with MAYV (MOI of 0.1) for 1 h, and then treated for 24 h with the substances (0–100 $\mu\text{g/mL}$). Subsequently, the culture supernatants were recovered and titrated to calculate the extracellular infectious viral particles using the plaque reduction assay. Among the tested compounds, quercetin and the fractions of EtOAc, and n-BuOH had an EC_{50} of 10 μM , 5 μM , and 3 μM , respectively. EtOAc had the highest SI (623), followed by n-BuOH (208) and quercetin (94). The mechanisms of action of these substances still need to be elucidated. It has been suggested that quercetin may act inhibiting viral RNA polymerase (dos Santos et al. 2014). The antiviral activity of Quercetin against MAYV was also studied by Bakhache and coworkers, in addition to the compounds Cerulenin, Orlistat, and CAY10566 (Bakhache et al. 2019). These molecules are known to interfere with fatty acid synthase (FASN) or stearoyl-CoA desaturase (SCD1), cofactors of MAYV infection. To assess their antiviral activity, human embryonic epithelial kidney cells (HEK293T) were infected with the MAYV-Luc reporter virus in an MOI of 1, and Quercetin, Cerulenin, Orlistat, and CAY10566 were added 1.5 h before or 1.5 h after the cell infection at concentrations of 0–150 μM , 0–25 μM , 0–100 μM , and 0–7.5 μM , respectively. Luciferase activity was then monitored for 6, 16, and 24 h.p.i.. As an outcome, the inhibitors induced a dose-dependent inhibitory effect on MAYV, when added before or after the infection. Among the tested drugs, Cerulenin presented the highest antiviral activity, demonstrating an increased ability to inhibit MAYV infectivity when added before the viral infection. The authors also screened these molecules against CHIKV, which demonstrated to decrease CHIKV genome replication due to the interference with FASN or SCD1 enzymatic activity, proposing that these compounds act in a similar mechanism against MAYV.

Similarly, the antiviral activity of EtOAc, n-BuOH, and EtOAc-Pp, extracted from *Cassia australis*, against MAYV were evaluated (Spindola et al. 2014). The production of MAYV in Vero cells decreased by about 70% and 85% in the presence of EtOAc and n-BuOH at 25 µg/mL, respectively, while EtOAc-Pp at 10 µg/mL decreased 90% the viral progeny production. The authors proposed that the highest antiviral effect of EtOAc-Pp may be due to the condensed tannins, which are present only in this substance. Tannins are a class of polyphenols, characterized by having different molecular sizes and degrees of polymerization, which affect their pharmacokinetics and antiviral activity (Maugeri et al. 2022). Condensed tannins have previously demonstrated antiviral activity against other viruses, such as the respiratory syncytial virus (RSV), influenza A virus (FLU-A), and parainfluenza virus (PIV) (Bruyne et al. 1999). The authors suggested that the mechanism of action of EtOAc-Pp is related to the binding of tannins with viral envelope proteins, resulting in the inhibition of viral binding and penetration into host cells. What is more, the EtOH fruit extract from *Punica granatum* (Pomegranate), and its two main fractions, one containing the α and β anomers of Punicalagin and another the Pomegranate ethanol extract, were assessed by their anti-MAYV activity (Salles et al. 2021). To this, Vero cells were infected with MAYV at an MOI of 0.1 for 1 h, and then added of medium-containing substances at concentrations of 12.5, 25, 50, and 100 µg/mL. Virus was titrated 24 h.p.i. and titrated by the TCID₅₀. As a result, *P. granatum* ethanolic extract demonstrated strong antiviral activity against MAYV infection, with a CC₅₀ of 590.8 µg/mL and an EC₅₀ of 12.3 µg/mL. The fraction containing punicalagin as the main component also showed strong antiviral activity, with a CC₅₀ of 523.1 µg/mL and an EC₅₀ of 62.5 µg/mL. The authors performed a virucidal assay, incubating the virus with each substance at 200 µg/mL for 1 h at 37 °C. Pomegranate ethanol extract showed 98% virucidal activity against MAYV particles, and punicalagin showed no visible virucidal activity, being the results confirmed by immunofluorescence microscopy.

Another natural compound identified to possess anti-MAYV activity is Epicatechin, isolated from extracts of *Salacia crassifolia*, which belongs to a genus of plants known by its use against diabetes, and as an anti-inflammatory, in folk medicine (Ferreira et al. 2018). Ferreira and coworkers selected Epicatechin for in vitro analysis after performing in silico screening tests that suggested a potential binding interaction with MAYV C protein. To assess their in vitro antiviral activity, cells were pretreated for 30 min with dilutions of the compound (0.013 to 0.574 µmol/mL), and then infected with MAYV at an MOI of 0.1 or 5 for 48 h. Epicatechin presented EC₅₀ of 0.247 µmol/mL, and prevented approximately 100% of the cytopathic effect in MAYV-infected cells at 0.430 µmol/mL. A time-of-drug

addition assay was also performed, and when added after infection, epicatechin reduced virus production by two logs in the first 12 h. The authors suggested that the compound acts in the second cycle of infection, when mature virions are released into the extracellular environment, but did not exclude an intracellular action in the late stages of viral replication, considering the MOI used in the assay, concluding that epicatechin acts by binding to components of the viral particle, but not to cellular components.

Ginkgolic Acid (GA), a compound isolated from *Ginkgo biloba*, is widely used in traditional Chinese medicine (Campos et al. 2020), reported to possess biological activities, such as anti-tumor (Ma et al. 2015; Qiao et al. 2017), antibacterial (Hua et al. 2017), anti-parasitic (Chen et al. 2008), and antiviral (Lü et al. 2012). Campos and colleagues evaluated the effects of GA on CHIKV, MAYV, and UNAV replication. Through a plaque reduction assay using epithelial cervical cancer cells (HeLa cells), it was found that GA at 10 µM reduced the MAYV viral progeny in almost 2log₁₀ at 16 h.p.i.. To confirm these results, an immunofluorescence assay was performed, demonstrating that treated cells presented a decrease in viral antigens. Additionally, a time-of-drug-addition assay was performed by adding GA to the cells hourly after adsorption, and supernatants were collected 24 h.p.i. to measure viral progeny yields. As a result, the compound was able to mainly inhibit the initial stages of the viral cycle, which was confirmed by the suppression of E1 and E2 viral proteins in western blot (Campos et al. 2020). Despite the promising effects of GA against MAYV in vitro, the molecule demonstrated to be toxic in other cell lines and animal studies (Berg et al. 2015; Qian et al. 2017). Therefore, the authors suggest that further investigations are needed to confirm the safety of the treatment, and the anti-viral activity against MAYV.

Another promising inhibitor of MAYV is Silymarin, a compound extracted from *Silybum marianum*. This molecule is widely used for the treatment of liver disorders (Camini et al. 2018), and was described to have antiviral activity against the Hepatitis C virus (Wagoner et al. 2010) and CHIKV (Lani et al. 2015). Camini and coworkers assessed the effects of Silymarin using hepatocarcinoma cells (HepG2 cells), which resulted in CC₅₀ of 103.5 µg/mL and EC₅₀ of

3.58 µg/mL. Then, HepG2 cells were treated with 25 µg/mL of Silymarin and simultaneously infected with MAYV at an MOI of 5. Supernatants were collected 48 h.p.i. and used to infect naïve Vero cells. The results demonstrated a reduction of up to two logs in MAYV replication, inhibiting almost 100% of the cytopathic effect. The production of reactive species of oxygen (ROS) was measured by employing infected HepG2 cells with a fluorogenic marker, confirming that MAYV infection increased ROS generation in a time-dependent curve. Other oxidative stress biomarkers were quantified, such as malondialdehyde (MDA) and

carbonyl protein, in the presence of MAYV and Silymarin, which culminated in a drop of these biomarkers, suggesting a protective effect on host cells, associated with the antiviral activity (Camini et al. 2018).

Finally, Valdés-Torres and collaborators investigated the potential of Honokiol and α -Mangostin against MAYV (Valdés-Torres et al. 2022). Primary human dermal fibroblasts (HDFs), Vero-E6, and HeLa cells were infected with MAYV at an MOI of 1 for 1 h, and then treated with Honokiol (5 to 10 μ M) or α -Mangostin (1 to 5 μ M) for 24 h. Honokiol and α -Mangostin reduced MAYV-induced cytopathic effects in a dose-dependent manner and inhibited the production of viral progeny in pretreatment assay. MAYV-infected HDFs revealed that α -Mangostin at 1 μ M affected MAYV binding to the host cells, while Honokiol showed a modest effect at 10 μ M. The viral entry was partially disturbed by α -Mangostin, but not by Honokiol. Both compounds affected the post-entry stage of MAYV infection. The authors also evaluated the expression of Interferon (IFN) type I and specific genes stimulated by IFN in treated or untreated HeLa cells through quantitative RT-PCR. As an outcome, treatment with Honokiol and α -Mangostin promoted a significant increase in IFN type I and interferon-stimulated genes. In addition, the compounds decreased the expression of MAYV E1 and nsP1, and affected viral RNA replication. The authors pointed out that Honokiol and α -Mangostin are post-entry inhibitors impairing MAYV replication through different mechanisms of action, and may act as potential broad-spectrum antivirals, also inhibiting UNAV, CHIKV, and Zika virus (ZIKV).

Synthetic and semi-synthetic inhibitors of MAYV replicative cycle

Natural compounds may present limitations in terms of large-scale production and patentability, and therefore, the production of synthetic and semi-synthetic compounds is an alternative and attractive approach to the development of antiviral candidates (Ortholand and Ganesan 2004).

Among the synthetic molecules, the Thienopyridine derivatives were described as antimicrobial agents against bacteria (Aly et al. 2011), protozoa (Pinheiro 2012), viruses (Bernardino et al. 2004), and parasites (Rolim Bernardino et al. 2006). In this context, Amorim and coworkers conducted a study based on the antiviral activity of thieno[2,3-b]pyridine derivatives 101 to 113 on the MAYV replicative cycle in vitro, using Vero cells (Amorim et al. 2017). Among the non-cytotoxic compounds, molecule 104 was the most viable compound, presenting CC_{50} of 2500 μ M, EC_{50} of 20.0 μ M, and an SI of 125. To further assess the effect of molecule 104 on viral protein synthesis, Vero cells were infected with MAYV at an MOI of 0.05 and treated with

100 μ M of the compound in a time-of-drug addition assay. Through the quantification of the plaque-forming assay, it was observed that 104 presented a higher effect when added prior or during the virus infection. Therefore, a virucidal assay was performed by incubating the virus and the compound for 1 h prior to the infection, confirming the virucidal effect of 104 at concentrations higher than 25 μ M. The authors also showed a decrease in protein synthesis in the presence of 104, by labeling viral proteins with 35S-methionine, confirmed by transmission electron microscopy, which demonstrated a reduction in the number of intracytoplasmic nucleocapsids and mature virus particles in treated cells.

Porphyrins are organic molecules that can bear a metal ion in the center of their tetrapyrrolic structure (Assunção-Miranda et al. 2016). These molecules are capable of absorbing light and are widely used in photodynamic therapies. The porphyrins have also been shown to inactivate viral particles by targeting the viral envelope of Dengue virus (DENV) and Yellow Fever virus (YFV) (Neris et al. 2018). Neris and coworkers evaluated the antiviral potential of the heme porphyrins Co-protoporphyrin IX (CoPPIX) and Sn-protoporphyrin IX (SnPPIX) in the presence or absence of light against MAYV using Baby Hamster Kidney Fibroblast (BHK-21) or Vero cells (Neris et al. 2018). With no light stimulation, CoPPIX and SnPPIX presented a CC_{50} of 762.3 μ M and 816 μ M, respectively, while in the presence of light, the compounds demonstrated a CC_{50} of 1018 μ M and 20.7 μ M, respectively. Later, 10^7 PFU of viruses were pre-incubated with different concentrations of each compound in the absence of light for 1 h at 37 °C, and then used to infect the cells with an MOI of 0.1. CoPPIX and SnPPIX at 300 μ M were able to completely inactivate MAYV, with an EC_{50} of 5.94 μ M and 5.99 μ M, respectively. Additionally, light-stimulated for 10 min before incubation for 1 h in the dark resulted in EC_{50} of 0.09 μ M for SnPPIX and 7.48 μ M for CoPPIX. To further investigate the CoPPIX and SnPPIX inhibition, MAYV was labeled with DiD to follow fusion between virus and endosomal membranes during endocytosis. After treatment with CoPPIX or SnPPIX at 300 μ M with no light stimulation, and SnPPIX at 10 μ M with light stimulation, MAYV was unable to efficiently fuse with the endosomal membrane. The authors highlighted that the porphyrins are hydrophobic molecules, that might interact with viral envelope lipids, impairing the attachment and entry of the virus into the cells. Furthermore, the photoactivation of SnPPIX generates ROS, which can modify viral proteins in the envelope environment, producing an antiviral activity. The authors emphasized that a single intensity of light stimulation was performed and suggested that an increase in luminous intensity might increase SnPPIX virucidal efficiency (Neris et al. 2018).

Another promising compound is β -D-N4-hydroxycytidine (EIDD-1931), a ribonucleoside analogue which previously

demonstrated antiviral activity against RNA viruses, such as SARS-CoV-2, Equine Encephalitis Virus, and CHIKV (Yousefi et al. 2021; Langendries et al. 2021). EIDD-1931 can rapidly reach plasma, is efficiently distributed in mice organs, and also presents a high genetic barrier for the development of viral resistance (Painter et al. 2019; Yousefi et al. 2021). A study conducted by Langendries and coworkers screened compounds with previously reported antiviral activity against other arboviruses, such as EIDD-1931, Favipiravir, and Suramin, against MAYV (strain TC625, MOI of 0.01), using Vero cells (Langendries et al. 2021). The experiments demonstrated CC_{50} of $> 100 \mu\text{M}$, $> 2000 \mu\text{M}$, and $2837 \mu\text{M}$, and EC_{50} of $1.6 \mu\text{M}$, $124 \mu\text{M}$, and $79 \mu\text{M}$ for EIDD-1931, Suramin, and Favipiravir, respectively. To evaluate which stage of the replicative cycle was affected by these compounds, a time-of-drug-addition assay was carried out. Vero cells were incubated with $300 \mu\text{M}$ Favipiravir, $500 \mu\text{M}$ Suramin, and $25 \mu\text{M}$ EIDD-1931 2 h before infection, and 0, 2, 4, 6, and 8 h.p.i. at an MOI of 1. Through end-point titration at 10 h.p.i and qRT-PCR quantification, the authors demonstrated that EIDD-1931 impaired viral replication when added at 0, 2, and 4 h.p.i., with a moderated effect if added at 6 h.p.i. Differently, Suramin and Favipiravir reduced viral titers at 0 and 2 h.p.i., respectively. From the results and previous data in the literature, the authors suggested that EIDD-1931 and Favipiravir impair viral RNA synthesis, resulting in non-functional copied viruses due to a large number of mutations in the viral genome (Joshi et al. 2021; Langendries et al. 2021). It was also proposed that Suramin acts during the early steps of the MAYV replicative cycle, more specifically in the viral entry, in a similar mechanism demonstrated by studies with CHIKV (Albulescu et al. 2020) and SARS-CoV-2 (Salgado-Benvindo et al. 2020). Cyclic ketones are natural and synthetic compounds with antiviral (Sivropoulou et al. 1997; Chung 2017), antioxidant (Pombal et al. 2017), and antifungal (Pizzolitto et al. 2015) properties. The antiviral activity of 24 cyclic ketones was assessed by Fernandes and coworkers, by screening these compounds, using MAYV-infected Vero cells (MOI 1), treated with each compound at concentrations ranging from 1 to $100 \mu\text{M}$ for 24 h. Among them, 8 compounds showed viability $\geq 50\%$ and were selected for viral inhibition assay. A plaque-forming assay was performed, and among the 8 compounds, the Xanthenodione 9-(5-(4-chlorophenyl)furan-2-yl)-3,6-dimethyl-3,4,5,6,7,9-hexahydro-1H-xanthen-1,8(2)-dione inhibited the MAYV with EC_{50} of $21.5 \mu\text{M}$ and SI of 15.8. The authors also performed a time-of-drug addition assay and found that the compound inhibits viral replication in the prior and post-treatment assays (Fernandes et al. 2021).

The Proteasome inhibitors MG132 and Lactacystin are also antiviral candidates against MAYV (Llamas-González et al. 2019). MG132 acts as a reversible inhibitor of the

chymotryptic activity of the proteasome, and Lactacystin is an irreversible inhibitor capable of binding to the catalytic β subunits of the 20S proteasome (Llamas-González et al. 2019). The anti-MAYV activity of these compounds was evaluated by Llamas-González and colleagues. Vero-E6 or HeLa cells were treated with MG132 at $10 \mu\text{M}$ or Lactacystin at $25 \mu\text{M}$ for 1 h, and then infected with MAYV (MOI of 1 or 10) for 1 h, when the inoculum was replaced by fresh medium-containing compounds. Viruses were titrated by plaque-forming assay 24 h.p.i.. MG132 and Lactacystin low cytotoxicity and significant antiviral effect using either cell lines. The authors also performed immunofluorescence of MAYV-infected HeLa cells, which demonstrated a significant reduction in the percentage of MAYV-positive cells under treatment, resulting in $< 5\%$ for MG132 and $< 10\%$ for Lactacystin, versus $> 20\%$ in the untreated control group. Viral titers were lower when compounds were added in the early stages of infection, and the treatment with MG132 or Lactacystin affected the production of viral proteins nsP1 and E1, showed by the reduced expression of MAYV proteins under the treatment with these proteasome inhibitors.

Exploiting host factors, the Src family of protein tyrosine kinases (SFK) are involved in intracellular signaling pathways related to virus elimination (Broeckel et al. 2019).

However, during infection, the virus can interact with kinases favoring viral replication. Therefore, cell signaling inhibitors, including SFK inhibitors, have been tested for their antiviral activity against *Alphavirus* in human fibroblasts (Broeckel et al. 2019). Since the knowledge of the signaling profile of SFK during *Alphavirus* is limited, the authors carried out a range of experiments using CHIKV infection, as a base to identify the kinase pathways involved in this genus infection. The SFK-phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR signaling was demonstrated to be the main pathway during CHIKV infection. Then, the authors evaluate the effects of dasatinib and Torin, inhibitors of this pathway, on fibroblasts infected with MAYV. Through the viral titration of the supernatant collected from infected and treated cells, it was shown that MAYV was significantly reduced by these inhibitors. The authors demonstrated that the SFK inhibitors blocked the subgenomic RNA translation, resulting in the impairment of *Alphavirus* replication (Broeckel et al. 2019).

A non-natural interferon (cIFN) that was designed according to the analysis of the amino acid sequences present in several subtypes of IFN- α was investigated by its anti-MAYV activity by Grabarz et al. (2021). Vero cells were treated with increasing concentrations of cIFN for two hours and then infected with MAYV (MOI = 0.1) for 48 h. MAYV infection was inhibited by cIFN, with EC_{50} of 35.7 fg/mL , being possible to obtain about 80% and 100% cell confluency with concentrations of 81.4 fg/mL , and 162.8 fg/mL of the compound, respectively. In comparison,

untreated cells showed almost complete destruction of the cell monolayer 24 h.p.i. with MAYV. In this study, cIFN was also able to inhibit the cytopathic effect of other viruses, such as ZIKV, CHIKV, and SARS-CoV-2.

Another promising compound is N-(methylcarbamoyl)-2-[[5-(4-methylphenyl)-1,3,4-oxadiazol-2-yl]sulfanyl]-2-phenylacetamide (known as C11), a molecule that induces type I IFN-dependent activity in human cell lines (Gall et al. 2018). In this context, Gall and collaborators produced THF cells and MM6 cells with deletion of the stimulator of interferon genes (STING), such as the RIG-1 mitochondrial antiviral signaling (MAVS) and the TIR-domain-containing adapter-inducing interferon- β gene (TRIF), related to the activation of the immune response. To assess the antiviral activity of C11, these cells were treated for 2 h with C11, IFN- β , or DMSO at non-cytotoxic concentrations and then infected with MAYV at an MOI of 1. The results demonstrated that C11 had an effective concentration of 90% (EC_{90}) of 25.19 μ M against MAYV. STING-deleted cells showed no inhibition of virus replication in the presence of the compound. C11 was also evaluated against CHIKV, VEEV, and RRV viruses in human cells, resulting in reductions in the virus titer, but when evaluated using non-human cells (RAW264.7 monocytic cells), a lack of antiviral activity in the murine RAW264.7 cell line was noticed. The authors proposed that C11 acts by activating specifically the human type I IFN response through a STING-dependent process, leading to the generation of an IFNAR-mediated antiviral cellular state.

Molecular target to drug development against MAYV

Drug development can be a time-consuming process, which can result in unexpected outcomes (Adamson et al. 2021). Additionally, the lack of treatment against viral infections, mainly for neglected diseases, may jeopardize the control of the disease, as well as its consequences, such as the loss of human lives (Young et al. 2019). In this context, several strategies can be applied to accelerate the progress toward the development of novel molecules. Therefore, this section focuses on the MAYV proteins which could be exploited as a target for antiviral development, considering the factors:

(i) previously described compounds with antiviral activity against MAYV and/or other *Alphaviruses*, (ii) resolved structure of viral proteins deposited in databanks, and (iii) structural similarity and conservation among viruses.

The glycoprotein E1 possesses an ectodomain, subdivided into subdomains I, II, and III, and a transmembrane subdomain (TM). Similarly, the E2 ectodomain is composed of subdomains A, B, C, and D, and a TM portion (Filho et al. 2020). The ectodomains of E1 and E2 interact with

each other to form a portion of the spike structure outside the envelope: the glycoproteins E1 and E2 interact forming a dimeric unit (Fig. 3A) that is further associated with additional two dimers to form the spike (Fig. 3B–D). The TM portions of E1/E2 bind the spike to the lipid membrane, while E2 C-terminal connects the spike to the capsid protein, through a well-described TPY consensus motif (West et al. 2006; Ribeiro-Filho et al. 2021), forming an E1–E2–capsid unit (Fig. 3C, E). As observed for other *Alphaviruses*, MAYV possesses an asymmetric unit formed by the assembling of four E1–E2–capsid units resulting in 80 spikes (Filho et al. 2020). MAYV belongs to the arthritogenic class of viruses, and, therefore, it is possible to suggest that Mxra8 is the main receptor related to the MAYV entry into host cells (Fig. 3E) (Powell et al. 2020). In this context, considering that the glycoprotein complex interacts with Mxra8, mainly through E2, this interaction could be impaired by active molecules through interference or destabilization of E2 binding sites (Zhang et al. 2018; Filho et al. 2020). Molecules were previously described to bind to viral glycoproteins of other arthritogenic viruses, such as CHIKV, consequently inhibiting viral entry to the host cells. For instance, Oliveira and coworkers described the *in vitro* virucidal activity of a coordinated organic compound against CHIKV, and demonstrated that *in silico* analysis suggested interactions between the compound and CHIKV E2 domain (Oliveira et al. 2020). Additionally, studies encompassing the compounds PAC (Ferraz et al. 2019), bLf (Carvalho et al. 2014), CoPPIX, and SnPPIX (Neris et al. 2018), which are discussed here, also demonstrated a virucidal activity against MAYV infection. Even though these molecules were not rationally designed for specific targets, further studies could investigate whether their mode of action is related to these hint targets.

Another spot protein in MAYV replication is the nsp4, an RNA-dependent RNA polymerase (RdRp) essential to the viral replication (Rubach et al. 2009). Therefore, it is an attractive target to the development of antiviral drugs. To the best of our knowledge, the RdRp crystal structure of MAYV has not been determined and characterized yet, which might postpone drug design applied to this target. Regardless, the RdRp amino acid sequence is commonly conserved among viruses from the *Alphaviruses* genus, and especially between the Semliki Forest serocomplex members (Rupp et al. 2015). Therefore, the RdRp structure from the Ross River virus (RRV) (Fig. 4), another member of the *Alphavirus* genus might be employed as a template for drug development. Its structure is composed of fingers, the palm containing the GDD active site, and thumb domains (Rubach et al. 2009). Therefore, it is possible to hypothesize that compounds with antiviral activity described against the RdRp of other viruses could act against MAYV infection (Chen et al. 2017). Indeed, most of the described and approved drugs are

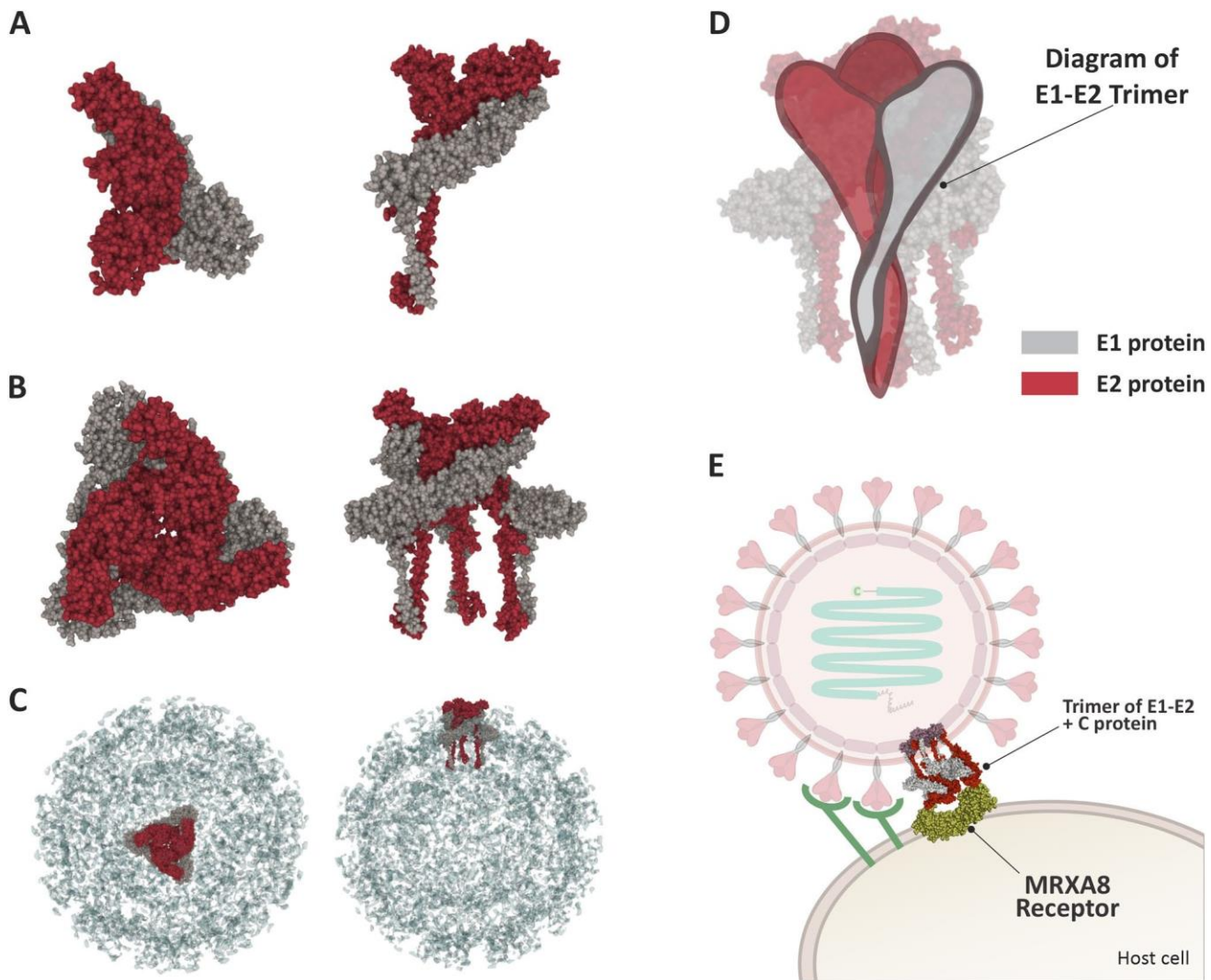


Fig. 3 Schematic structure of MAYV glycoprotein. The glycoproteins E1 and E2 interact forming a dimeric unit (A), that is further associated to two additional dimeric units to form a trimer (B). The localization of the trimers is demonstrated in (C), and a diagram of the E1–E2 trimer is shown in (D). The interaction between the glycoproteins

and the MRXA8 receptor in host cells is demonstrated in E. MAYV E1 and E2 structure were based on Cryo-EM structure of mature MAYV (PDB ID: 7KO8) (Ribeiro-Filho et al. 2021), and the receptor was based on human MXRA8 (PDB ID: 6JO8) (Powell et al. 2020)

nucleoside and non-nucleoside inhibitors that impair viral infections through RNA synthesis inhibition. Among them, Remdesivir, Azidothymidine (AZT), Sofosbuvir, and Lopinavir/Ritonavir, are licensed antivirals used to treat Ebola virus, Hepatitis C virus, and Human immunodeficiency virus infections (Tian et al. 2021).

The MAYV nsP3 is another protein that represents a target for antiviral development (Tsika et al. 2019). It is a modular protein with three domains: the N-terminal macro domain, a central zinc-binding domain, and the C-terminal hypervariable domain (Fig. 4) (Götte et al. 2018). Each domain plays a different role in viral replication and interferes with the virulence among Old and New World Alphaviruses, being necessary for RNA synthesis (Rupp et al. 2015). The MAYV

macro domain possesses four alpha-helices and six beta-strands (Malet et al. 2009; Melekis et al. 2015). On the other hand, the central zinc-binding domain plays an undefined, but essential role in minus-strand RNA synthesis, through an association with the macro domain, forming a ring-like structure to RNA binding (Shin et al. 2012). Alternatively, the hypervariable domain is a large protein that upholds several insertions and deletions, and is responsible for interactions with multiple host proteins during viral replication, probably favoring virulence (Davis et al. 1989; Dé et al. 2003; Uversky 2013). However, the macro domain is the most conserved site in the protein structure and could be exploited for drug development, particularly as a broad-spectrum antiviral. The macro domain site binds to ADP-ribose,

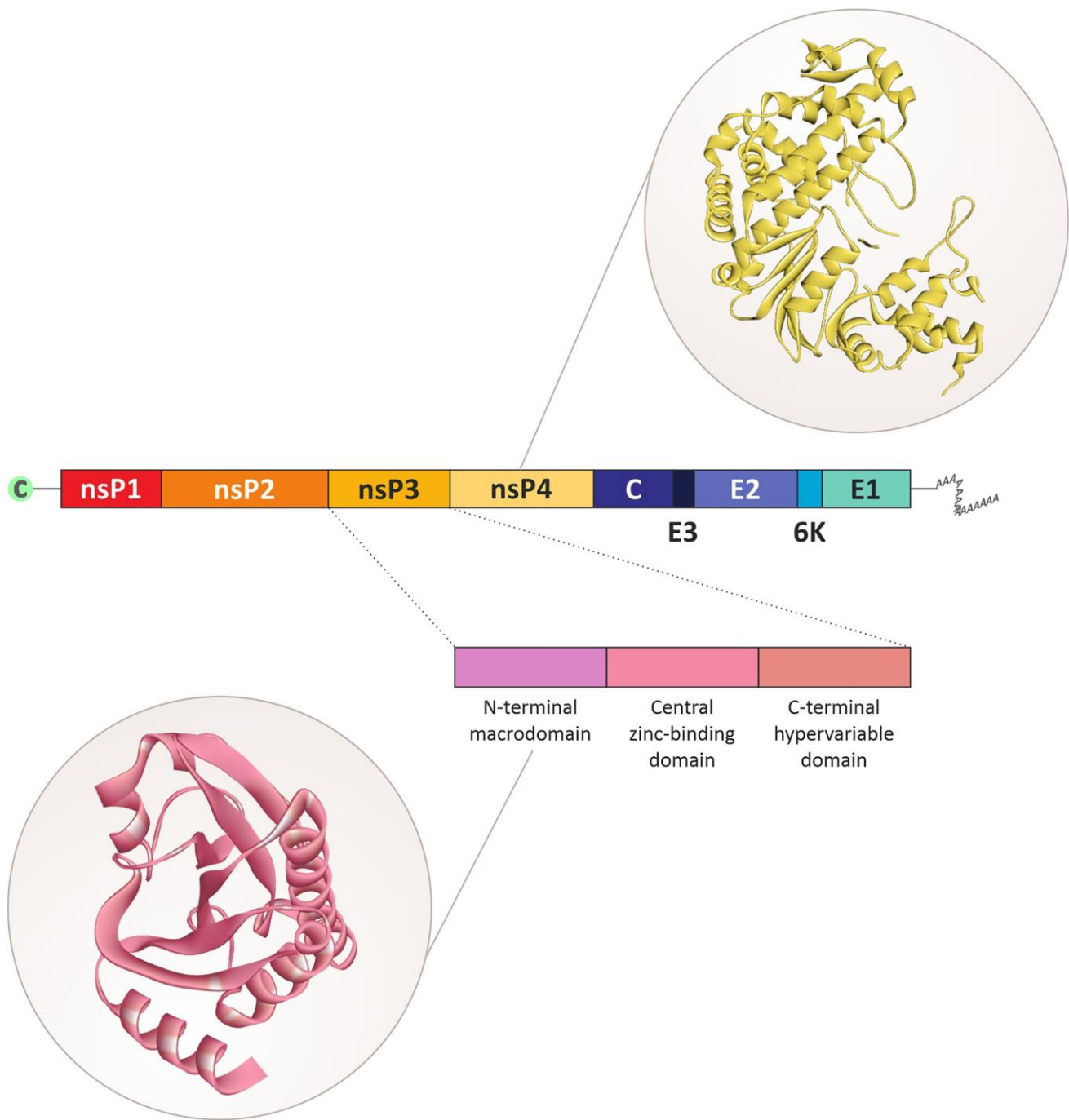


Fig. 4 Schematic structure of MAYV genome and the crystal structure of nsP3 macrodomain and nsP4. Nsp3 is a modular protein with three domains: the N-terminal macro domain, a central zinc-binding domain, and the C-terminal hypervariable domain, being the macro domain constituted by four alpha-helices and six beta-strands. The nsP4 protein acts as an RNA-dependent RNA polymerase (RdRp),

and its structure is composed of fingers, the palm containing the GDD active site, and thumb domains. Since RdRp crystal structure of MAYV has not been determined and characterized yet, the template used in the figure was obtained from Ross River virus RdRp (PDBID: 7F0S) (Tan et al. 2022). The nsP3 structure was based on MAYV macro domain structure (PDB ID: 5IQ5) (Tsika et al. 2019)

dephosphorylate ADP-ribose-1'-phosphate, and possesses a de-ADP-ribose hydrolase activity, which is essential to viral replication (McPherson et al. 2017). Several compounds were virtually screened employing the nsP3 macrodomain

of CHIKV (Nguyen et al. 2014; Shimizu et al. 2020; Subudhi et al. 2018), SARS-CoV-2 (Jung et al. 2020), and the Venezuelan equine encephalitis virus (VEEV) (Atasheva et al. 2014) as targets. Shimizu and collaborators selected

compounds that targeted the nsP3 macro domain of CHIKV by *in silico* analysis, and validated their antiviral activity performing *in vitro* assays (Shimizu et al. 2020). Therefore, it is possible to suggest the nsP3 macrodomain of MAYV as a relevant target for future antiviral discovery.

Furthermore, components of virus lipidic membrane are also potential targets yet to be explored. Among membrane components, the cholesterol is an important molecule related to *alphavirus* stability, infectivity, and assembly (Sousa et al. 2020). This molecule was recently associated with the successful infection in several viruses (Sun and Whittaker 2003; Huang et al. 2006), being also modulated to increase the infection efficiency (Zhang et al. 2019). Cholesterol can be found in increased proportions in MAYV viral particles isolated from vertebrate cells, being associated with the lateral organization of the viral envelope and binding to the host membrane (Sousa et al. 2020). Additionally, a decrease in *alphavirus* infectivity is observed when the arrangement of the viral envelope is disturbed, suggesting that the lateral membrane organization is important for the physical stability of the viral particle and, consequently, for virus-cell interactions (Kielian et al. 2010). Cholesterol is also associated with MAYV entry, being used as an alternative pathway for its entry through cholesterol-enriched caveolae-derived vesicles and release (Carvalho et al. 2017). Therefore, targeting cholesterol from the viral lipidic membrane represent an interesting approach for antiviral drug development. However, designing and identifying active compounds focusing on these components can be challenging, due to their similarity to the host cell membrane (Sousa et al. 2020). Interestingly, the compound 25-hydroxycholesterol (25HC), a reactive oxysterol catalyzed by cholesterol-25-hydroxylase, is a promising molecule that can interact with lipid on viral membrane, and demonstrated broad-spectrum antiviral activity, low toxicity, and ability to reduce viremia and protect embryonic mice against ZIKV *in vivo* (Li et al. 2017; Mao et al. 2022). In this context, molecules focused on interacting with cholesterol could be employed to drug development against MAYV.

Perspectives

MAYV infection can impact the quality of life of infected patients, due to the chronic condition that can result in progressive arthralgia and disabling disease. Besides that, the lack of approved antiviral treatment and vaccines against MAYV, associated with the presence of susceptible vectors in tropical and subtropical countries, can lead to future outbreaks.

In this context, this review summarized and discussed the literature concerning the compounds with anti-MAYV

activity, as well as presented possible viral proteins that could be further exploited as targets for drug development. To the best of our knowledge, only the studies described here reported antiviral activity against MAYV infection, demonstrating that further research on treatments against Mayaro fever is urgent. It is important to emphasize that the data presented here employed different methodologies, including cell lines, MOI for infection assays, and the design of the assays. However, these studies present relevant information on the antiviral activity of a range of molecules (natural and synthetic) against MAYV, and suggested possible mechanisms of action (MOA) related to each compound. The characterization of the MOA of these molecules represents an advance for further studies, providing information concerning the interactions among host cells, viruses, and compounds (Iorio et al. 2010; Salters-Pedneault 2014). Besides, the MOA of several compounds against MAYV are still unknown (Figueiredo et al. 2019). From the compounds discussed here, Lactoferrin, molecule 104 (thienopyridine derivative), and Silymarin presented significant antiviral activity against MAYV *in vitro*, with low or no cytotoxicity. The repurposing antiviral agent EIDD-1931 is also a promising treatment since it was able to completely inhibit MAYV replication *in vitro*. However, these compounds need to be further investigated *in vivo* against MAYV. Ginkgolic Acid (GA) was one of the most active compounds, and the only molecule tested *in vivo*, however demonstrating hepatotoxicity and nephrotoxicity effects, as well cytotoxicity in different cell lines. Qian and collaborators also observed that rats treated with either, high or low doses of GA, showed histopathological changes in the liver and kidneys. In addition, the serum levels of urea nitrogen and creatinine increased in the blood, and total protein and albumin decreased, indicating kidney dysfunction and liver damage (Qian et al. 2017). On the other hand, the genotoxicity evaluation of GA evidenced a low risk of genotoxicity *in vivo* (Berg et al. 2015). Therefore, more studies in human cells and animal models are necessary to analyze the safety of this compound.

Furthermore, we discussed and highlighted MAYV proteins that could represent relevant targets for drug development, acknowledging that focusing on host factors might produce cytotoxicity *in vitro*, and adverse effects *in vivo*. Here, we suggested the glycoproteins complex, the nsP4 (RdRp), and the nsP3 macro domain as targets. The lack of resolved structures of several MAYV proteins, such as nsP1, nsP2, C, and E, invalidate them as targets for drug development.

Finally, licensed drugs employed in the treatment of several diseases have the potential to be repurposed to target the viral molecules underlined here. In this sense, Langendries and colleagues identified EIDD-1931, favipiravir, and suramin, three licensed antivirals, with activity against MAYV (Langendries et al. 2021). Additionally, the

potential of Lactacystin (Llamas-González et al. 2019), Cerulenin, Orlistat (Bakhache et al. 2019), and Quercetin (dos Santos et al. 2014), as repurposing therapy against MAYV was also discussed. The compounds were active

with low EC₅₀ and low cytotoxicity, and showed potential to be applied as antiviral therapy in the treatment of infections caused by this pathogen, being a prospective target for further research. The use of approved drugs as repurposing therapy may favor patient access to drugs due to the potential to result in shorter clinical studies, since these molecules have their pharmacological profile and adverse effects already described (Santos et al. 2022). Bearing that in mind, it is an inconsistency that repurposing drugs against MAYV have been understudied. Therefore, we further emphasize and encourage researchers to screen licensed drugs against MAYV.

Conclusions

The spread of MAYV infections in tropical and subtropical regions represents a threat to developing countries. Additionally, the lack of epidemiological data on Mayaro fever might underestimate the importance of this disease and its consequences. There is an urgent need for studies to understand the biology of the virus, thus creating paths to combat future outbreaks. In this context, the compounds described to possess anti-MAYV activity, and the possible targets discussed herein, may contribute to the future development of antiviral drugs against MAYV.

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Data availability statement All data generated or analysed during this study are included in this published article (and its supplementary information files).

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that

could be construed as a potential direct or indirect conflict of interest.

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Effect of proteins isolated from Brazilian snakes on enterovirus A71 replication cycle: An approach against hand, foot and mouth disease

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Abstract

Enterovirus A71 (EVA71) belongs to the *Picornaviridae* family and is the main etiological agent of hand, foot, and mouth disease (HFMD). There is no approved antiviral against EVA71, and therefore the search for novel anti-EVA71 therapeutics is essential. In this context, the antiviral activity of proteins isolated from snake venoms has been reported against a range of viruses. Here, the proteins CM10 and CM14 isolated from *Bothrops moojeni*, and Crotamin and

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Declaration of competing interest

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

PLA2_{CB} isolated from *Crotalus durissus terrificus* were investigated for their antiviral activity against EVA71 infection. CM14 and Crotamin possessed a selective index (SI) of 170.8 and 120.4, respectively, while CM10 and PLA2_{CB} had an SI of 67.4 and 12.5, respectively. CM14 inhibited all steps of viral replication (protective effect: 76 %; virucidal: 99 %; and post-entry: 99 %). Similarly, Crotamin inhibited up to 99 % of three steps. In contrast, CM10 and PLA2_{CB} impaired one or two steps of EVA71 replication, respectively. Further dose-response assays using increasing titres of EVA71 were performed and CM14 and Crotamin retained functionality with high concentrations of EVA71 (up to 1000 TCID₅₀). These data demonstrate that proteins isolated from snake venom are potent inhibitors of EVA71 and could be used as scaffolds for future development of novel antivirals.

Keywords

Enterovirus A71; Antiviral; Brazilian snakes; Toxins; EVA71

Introduction

Enterovirus A71 (EVA71) is one of the major causes of hand, foot, and mouth disease (HFMD), characterized by fever and rash with blisters on the hands, feet, buttocks, and mouth [1]. EVA71 infection may also be associated with more severe cases of encephalitis, aseptic meningitis, and myocarditis, which could result in long-term sequelae and high mortality rates, especially in children [2–5]. EVA71 belongs to the *Enterovirus* genus from the *Picornaviridae* [6]. It is a non-enveloped virus, with an icosahedral capsid and a single-stranded positive RNA genome [7]. The viral RNA contains one open reading frame (ORF) that encodes a polyprotein cleaved into four structural proteins (VP1, VP2, VP3, and VP4) and seven non-structural proteins (P2-2A, 2B, 2C, P3-3A, 3B, 3C, and 3D) [8–11].

Transmission occurs mainly via fecal-oral or oropharyngeal secretions, being highly contagious. In addition, it can also contaminate surfaces and promote transmission by fomites [12]. Interestingly, the incidence increases at the warmest times of the year [13]. EVA71 replicates in gastrointestinal and respiratory epithelium cells, however, the viremia can result in infection of neurons and cardiomyocytes [14]. An infected individual can be asymptomatic, and in most cases the infection is self-limiting, however, symptomatic cases can progress to HFMD. Thus, the infection may reach the central nervous system and cause dysregulation of the autonomic nervous system, resulting in cardiopulmonary failure in more severe cases.

Among the causes of HFMD, EVA71 has become the most relevant because of its neurotropism, which can trigger diseases associated with the neurological system, such as aseptic meningitis, encephalitis, poliomyelitis syndrome, and acute flaccid paralysis [15,16]. In severe cases, EVA71 can affect the brainstem, causing sequelae and even death [17].

This virus has been responsible for outbreaks associated with neurological complications in children [18,19]. In recent years, epidemics in Southeast Asian countries generated concern about the potential of EVA71 dissemination in highly populated regions. In 2012, China registered >2 million cases and 567 deaths related to HFMD [20]. Since the emergence

of the coronavirus disease in 2019 (COVID-19), the application of non-pharmacological control measures (social distancing, masks, and hand sanitization) decreased the incidence of HFMD in 2020 [21]. However, new outbreaks in children were reported in 2022 due to the re-establishment of common activities [22].

Currently, there is no approved antiviral therapy against HFMD [23,24], and only one vaccine approved in China against HFMD subgenotype C4 [25–27]. In this context, the search for therapeutics against EVA71 is essential and could provide substantial health benefits. Proteins isolated from animal venoms have shown to be a fruitful source of molecules with several biological activities, such as anti-inflammatory, anti-cancer, and cardiovascular activity [28–30]. Among those, proteins isolated from Brazilian snake venoms, such as *Bothrops moojeni* and *Crotalus durissus terrificus* [31] have shown antiviral activity against hepatitis C virus (HCV) [32], dengue virus (DENV) and yellow fever virus (YFV) [33], measles virus [34], Chikungunya virus (CHIKV) [35], and ZIKV [36]. Therefore, the biological properties presented by the constituents of snake venoms represent promising sources for the identification and development of drugs against emergent viruses.

In this study, the antiviral activity of proteins CM10, CM14, Crotamin, and PLA2_{CB} isolated from Brazilian venom snakes *B. moojeni* and *C. durissus terrificus* were investigated against the EVA71 replication cycle.

Material and methods

Venoms and isolated proteins

The crude venom of *Bothrops moojeni* was purchased from serpentarium “Serpentário Proteínas Bioativas Ltda” of Batatais/SP, registered at the Ministry of the Environment, n° 471301. For *Crotalus durissus terrificus*, the crude venom was purchased from serpentarium “Centro de Extração de Toxinas Animais”, registered at the Ministry of the Environment, n° 3002678. Isolation and purification of the proteins Crotamin and PLA2_{CB} were carried out at the Laboratory of Toxinology of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (IBAMA authorization: 1/35/1998/000846-1), as previously described [37], using the Itzhaki and Gill method [38]. CM14 and CM10 were isolated from the crude venom of *Bothrops moojeni* by ion-exchange chromatography on CM-Sepharose (Pharmacia) followed by reverse phase chromatography as previously described [39,40]. The evaluation of the purification efficiency was monitored by 12 % SDS-PAGE and Coomassie blue R250 staining [41]. Lyophilized proteins were dissolved in PBS (Phosphate-Buffered Saline), filtered, aliquoted, and stored at –80 °C. Stocks were diluted in DMEM immediately prior to the experiments. PBS was used as untreated control. The source of each protein is shown in Table 1.

Cell culture

Vero E6 cells (kidney tissue derived from a normal adult African green monkey, ATCC E6) were grown in minimal Dulbecco’s Modified Eagle medium (DMEM; Sigma-Aldrich, USA) supplemented with 100 U/mL penicillin (Gibco Life Technologies, USA), 100 U/mL streptomycin (Gibco Life Technologies, USA), 1 % HEPES (Gibco Life Technologies USA), and 10 % fetal bovine serum (FBS; Gibco Life Technologies, USA) at 37 °C in a humidified incubator with 5 % CO₂.

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Virus rescue and titration

An EVA71 reverse genetics system was generated from the strain MS/7423/87 as previously described [42]. The plasmids were linearized with *XhoI* (ThermoFisher Scientific) and purified using Wizard® DNA Clean-Up System (Promega). The linearized DNA was transcribed using the HiScribe™ T7 High Yield RNA Synthesis Kit (NEB). The infectious RNA was electroporated into Vero cells (1×10^6 cells) at 250 μ F and 450 V. After 48 h, the supernatant was harvested and titrated as follows: Vero cells were seeded in 96 well plates at 1×10^4 cells/well 24 h prior to infection with EVA71 cell culture supernatants diluted in a 10-fold serial dilution in DMEM. After incubation for 48 h cells were fixed with 4 % paraformaldehyde (PFA) and stained with crystal violet 0.5 % for 30 min. Titres were determined using the 50 % tissue culture infectious dose (TCID₅₀) method and calculated by the Spearman & Kärber algorithm as described by Killington and Hierholzer [43]. All work was performed at biosafety level 2 under the authorization number SEI: 01245.006267/2022-14 CBQ: 163/02 from the CTNBio — National Technical Commission for Biosecurity from Brazil.

Cell viability assays

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich) method [35,44,45]. Vero cells were seeded in a 96 well plate at 1×10^4 cells/well and incubated overnight at 37 °C in a humidified 5 % CO₂ incubator. Medium-containing compounds at 50, 25, 5, and 1 μ g/mL was added to the cell culture and incubated for 72 h. The supernatant was removed and DMEM containing MTT at 1 mg/mL was added to each well, incubated for 30 min at 37 °C in a humidified 5 % CO₂ incubator, and replaced with 100 μ L of DMSO to solubilize the formazan crystals. PBS was used as untreated control. The absorbance was measured at 560 nm on Glomax Explorer (Promega). The cell viability was determined in percentage by the average of treated wells $\times$ 100/average of control wells. To determine the cytotoxic concentration of 50 % (CC₅₀), the proteins were diluted in 2 % DMEM in two-fold serial dilution series from 1.2 to 150 μ g/mL for CM14, CM10, and Crotamin, and from 0.4 to 50 μ g/mL for PLA2_{CB}, then added to the cells for cell viability assays, as described above.

Antiviral assays

To evaluate the anti-EVA71 activity of proteins isolated from snake venom, a suspension of Vero cells was plated in 96 well microplates at a concentration of 1×10^4 cells/well and incubated at 37 °C and 5 % CO₂ for 24 h. The compounds were diluted in a medium containing EVA71 at 100 TCID₅₀ at specific concentrations and then added to the cells for 72 h. Then, the supernatant was removed and 100 μ L of a solution containing MTT (Sigma-Aldrich) at 1 mg/mL of culture medium was added to the cells, which were incubated at 37 °C for 30 min. The MTT solution was removed, replaced by the same volume of DMSO, and the absorbance was measured at 560 nm on GloMax plate reader (Promega). PBS was used as an untreated control. The inhibition of EVA71 replication in cells by each protein was calculated using the following formula $[(\text{ODt}) \text{ EVA71} - (\text{ODc}) \text{ EVA71}] / [(\text{ODc}) \text{ cell} - (\text{ODc}) \text{ EVA71}] \times 100 (\%)$, where (ODt)EVA71 is the measured optical density of treatment on EVA71 infected cells; (ODc)EVA71 is the optical density measured for untreated control EVA71 infected cells; and (ODc)cell is the measured optical density of uninfected control cells [46]. –

Antiviral activity was presented as % inhibition of EVA71 replication. To determine the effective concentration of 50 % (EC_{50}), CM14, CM10, Crotamin, and PLA-2C_B were diluted in a culture medium containing EVA71 at 100 TCID₅₀, in a two-fold serial dilution with concentrations ranging from 1.2 to 150 µg/mL for CM14, CM10, and Crotamin, and from 0.4 to 50 µg/mL for PLA2C_B, and the antiviral assay was performed as described above.

Time of addition assays

The activity of the proteins isolated from snake venoms was evaluated at different stages of the EVA71 replicative cycle. To evaluate whether compounds presented a protective effect against EVA71 infection, Vero cells were treated with each compound for 1 h at 37 °C in a humidified 5 % CO₂ incubator prior to the infection. After incubation, cells were washed extensively to remove compounds and were infected with EVA71 for 1 h. The infectious supernatant was removed, additional washes were performed for virus removal and fresh media was added.

For the virucidal assay, the infectious supernatant was incubated with each protein for 1 h at 37 °C, prior to use for infection of Vero cells. Virus and protein were incubated with cells for 1 h at 37 °C. The inoculum was removed; cells were washed three times with PBS to completely remove the virus and proteins and replaced with fresh media.

To investigate the antiviral activity on EVA71 replication, infectious supernatant was added to Vero cells for 1 h, washed with PBS to remove non-endocytosed virus particles, and replaced by compound-containing media.

For all antiviral assays, the supernatant was collected 72 h post-infection (h.p.i.) and used to infect Vero cells in 96 well plates. Cells were washed with PBS after 1 h and incubated with fresh media for 72 h. The titres were determined by the TCID₅₀ method as described above.

Western Blot

Cells were lysed in Passive Lysis Buffer (PLB, Promega) with added protease inhibitors (Sigma-Aldrich). The protein concentration of samples was calculated using Pierce BCA Protein Assay Kit (ThermoFisher). 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 mouse anti-enterovirus VP0 (anti-VP0) monoclonal antibody at a 1:2000 dilution (mAb979, Millipore), secondary anti-mouse horseradish peroxidase-conjugated antibody (Sigma) and ECL substrate (Thermo scientific).

TCID₅₀ challenge in vitro assay

To investigate the inhibitory potential of the proteins in the presence of high amounts of EVA71, Vero cells were infected with increasing concentrations of virus from 1 TCID₅₀ to 10,000 TCID₅₀ in the presence of the proteins at the highest non-cytotoxic concentration (CM10, CM14, and Crotamin at 50 µg/mL, and PLA2C_B at 5 µg/mL). Then, the supernatant was removed 48 h.p.i. and 100 µL of a solution containing MTT (Sigma-Aldrich) at 1 mg/mL of culture medium was added to the cells, which were incubated at 37 °C for 30 min. Antiviral activity was determined as described above (antiviral assays section) [46] and was presented as % inhibition of EVA71 replication. PBS was used as an untreated control.

Statistical analysis

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 <0.05 (indicated by asterisks) were considered statistically significant. For the establishment of EC₅₀ and CC₅₀ values, the data were transformed into Log(X), where X is the concentration, and submitted into a non-linear regression with four parameters in variable slope. All analyses were performed using GraphPad Prism 8.

Results

Purification of the isolated proteins

The process of isolation and characterization of PLA2_{CB} and Crotamin from *Crotalus durissus terrificus* venom, used in this study, was previously described by Muller and colleagues [33,37]. The protein CM10 was isolated from *Bothrops moojeni*, and its purification and characterization were published by Amorin and coworkers [40]. The protein CM14 was purified from *Bothrops moojeni* venom using the same protocol previously described for CM10 [40], which represents a two-step chromatographic process. Briefly, the first step was performed on a CM sepharose cation-exchange column (Fig. 1), in which the peak corresponding to CM14 is indicated (Fig. 1). Then, the fraction of CM14 was subjected to C18 reversed-phase chromatography (Fig. 2A) resulting in a major fraction, revealing a single band (Fig. 2B). Through the biochemical characterization using SDS-PAGE, pI (data not shown) and N-terminal amino acid sequencing (data not shown) CM14 was identified as myotoxin II, according to [47].

CM10, CM14, Crotamin and PLA2CB strongly impair EVA71 with high selectivity index

In order to assess the inhibitory potential of these proteins on the EVA71 replicative cycle, a dose-response assay was performed by treating cells with a two-fold serial dilution ranging from 1.2 to 150 µg/mL for the compounds CM10, CM14, and Crotamin, and from 0.4 to 50 µg/mL for PLA2_{CB}, in the presence or absence of 100 TCID₅₀ of EVA71. The EC₅₀, CC₅₀ and calculated selective index (SI) for the proteins are shown in Fig. 3A–D and Table 1. From this data and based on previously performed cytotoxicity assays (Supplementary Fig. 1), non-toxic concentrations of each protein were selected for further antiviral assays (Table 2): CM10, CM14, and Crotamin were used at 50 µg/mL and PLA2_{CB} at 5 µg/mL.

CM10, CM14, Crotamin and PLA2CB inhibit multiple stages of the EVA71 life cycle

Time of drug addition assays were employed to identify the activity of each protein in distinct steps of the EVA71 replication cycle. Firstly, Vero cells were pre-treated with each protein for 1 h, washed with PBS to remove the compounds, and followed by infection with EVA71 for 1 h. The supernatant was replaced by fresh media after further PBS washes, and cells were incubated for 72 h (Fig. 4A). The results demonstrated that CM14 and Crotamin reduced virus titres by 76 % and 62 % (*p* < 0.001), respectively (Fig. 4B). In contrast, pre-treatment with CM10 and PLA2_{CB} did not protect cells against EVA71 infection (Fig. 4B).

To evaluate the virucidal activity, an inoculum containing infectious supernatant and each protein was incubated for 1 h, then added to Vero cells for further 1 h. The inoculum was removed, cells were washed with PBS, and replaced with fresh media for 72 h (Fig. 4C). Crotamin and CM14 inhibited 99 % of virus infectivity (*p* < 0.001), while CM10 significantly reduced virus infectivity to 88 % (Fig. 4D). However, PLA2_{CB} did not inhibit virus infectivity (Fig. 4D).

The effect of the proteins on EVA71 post-entry steps was also investigated. To perform this, Vero cells were infected with EVA71 for 1 h, washed with PBS to remove unbound virus particles, and replaced with compound-containing media (Fig. 5A). CM10 and CM14 inhibited 99 % and 100 % of viral replication, respectively (Fig. 5B). Similarly, Crotamin and PLA2_{CB} reduced EVA71 replication by 97 % and 89 %, respectively (Fig. 5B). Effects on EVA71 replication were also evaluated by detecting the EVA71 VP0 protein by western blot, confirming that replication was also reduced in the presence of CM10, CM14, Crotamin, and PLA2_{CB} (Fig. 5C). The results of the time of drug-addition assays are summarised in Table 2.

CM10, CM14 and Crotamin inhibit high titres of EVA71

To further describe the effect of the proteins on the EVA71 replicative cycle, we aimed to analyze the capacity of each compound in inhibiting increasing concentrations of EVA71 [48,49]. To this, cells were infected with virus at 1, 10, 100, 1000, and 10,000 TCID₅₀ in the presence of CM10, CM14, and Crotamin at 50 µg/mL or PLA2_{CB} at 5 µg/mL. The results showed that none of the proteins were able to inhibit EVA71 at 10,000 TCID₅₀ (Fig. 6A–D). However, CM14 inhibited EVA71 infection at 1000 TCID₅₀ by 84.2 % and was able to completely impair viral replication at 100 TCID₅₀ or lower titres (Fig. 6B).

Alternatively, CM10 inhibited 51.1 % of the infection at 1000 TCID₅₀, and 67.8 % or 93 % at 100 TCID₅₀, or 10 TCID₅₀, respectively (Fig. 6A). Crotamin inhibited EVA71 infection at 100 TCID₅₀ (75.3 %) but not at higher doses (Fig. 6C). Lastly, PLA2_{CB} was not able to completely inhibit EVA71 infection, however, presented a similar inhibition (≈35–45 %) of infection up to 1000 TCID₅₀ (Fig. 6D).

Discussion

Currently, there is no specific antiviral therapy to treat EVA71 infection, a virus associated with HFMD [50]. It is considered an endemic virus in Pacific Asia, where there are concerns about the potential of dissemination in highly populated areas [51], and it poses a threat to public health due to the viral neural tropism [52]. Therefore, the search for active compounds against EVA71 is essential.

Proteins isolated from snake venoms demonstrated antiviral activity against viruses from different families such as *Flaviviridae* [32], *Para-myxoviridae* [53], *Retroviridae* [54], *Bunyaviridae* [53], and *Togaviridae* [33,55]. However, to the best of our knowledge this is the first description of proteins isolated from snake venoms possessing antiviral activity against the *Picornaviridae* family.

The protein PLA2_{CB} investigated in this study was isolated from *C. durissus terrificus* and previously demonstrated antiviral activity against HCV [56] and Chikungunya virus (CHIKV) [55], RNA enveloped viruses belonging to the *Flaviviridae* and *Togaviridae* families, respectively. Against HCV and CHIKV, PLA2_{CB} was able to protect host cells from virus infection, reduced virus replication, and presented virucidal activity, differing from what was presented here against EVA71. In addition, a PLA2 isolated from *Vipera*

nikolskii exhibited potent antiviral effect against SARS-CoV-2, presenting low cytotoxicity and decreasing virus titres by acting on virion lipidic membrane [57]. Muller and co-workers demonstrated that PLA2_{CB} was not able to inhibit the non-enveloped virus Coxsackie B5 virus (also from *Picornaviridae* family) [33,37], which corroborates with our results, and since PLA2_{CB} is a phospholipase with activity relying on membranes, we suggest that the modest SI, as well as the lack of virucidal and/or protective activity, could be related to the absence of an envelope on EVA71.

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Interestingly, the PLA2_{CB} effect was observed in the post entry steps of EVA71 replication. Considering that the EVA71 proteins 2B, 2C, and 3A play roles in the formation of a lipid environment for EVA71 replication, termed membranous replication organelles [58], which are essential for virus genome replication, one possible mechanism of action of PLA2_{CB} on EVA71 replication could be the disruption of these replicative structures. However, this hypothesis needs to be further investigated.

In addition, the results presented herein showed for the first time the antiviral activity of CM10 and CM14 (both from *B. moojeni*), and Crotamin (*C. durissus terrificus*) against EVA71. Crotamin is a low molecular weight cationic polypeptide [59] that is able to penetrate cell membranes [60], and was described to possess antimicrobial [61] and antifungal activities [62]. Muller and colleagues investigated the activity of Crotamin against both DENV and YFV both enveloped viruses that perturb lipid metabolism, however, this molecule did not show any significant effect against these viruses [37]. What is more interesting is that Crotamin strongly inhibited EVA71, with activity in several steps of viral replication. Crotamin is capable of inhibiting voltage-gated potassium channels, resulting in depolarization of the cell membrane, and inducing the entry of Ca²⁺ [63] and activating Ca²⁺ dependent cell signalling cascades. This molecule was also reported to be able to interact with lipid bilayers and penetrate different cell lines [64]. In addition, Crotamin was shown to disrupt endosomes and lysosomal vesicles in mammalian cells [65,66], and since EVA71 replication is dependent on the lipid environment, as well as vesicle trafficking in the cytoplasm [58], it is possible that this protein is impairing EVA71 replication by interfering with cytoplasmic membrane trafficking.

What is more, both CM10 and CM14 proteins inhibited EVA71 replication and impaired viral infection through a virucidal activity. In addition, CM14 also protected cells against EVA71 infection, being active against all stages of virus replication investigated in this study. CM10 is a glycoprotein that is also known as a moojase, possessing serine protease activity [40]. In the literature, CM10 (moojase) has not been reported to possess therapeutic potential against infectious diseases, being characterized only as a thrombin-like protein, and resulting in interactions with the coagulation cascades in mammals [40]. However, CM14 is a Lys49 phospholipase A2 homologue, also known as MjTx-II, and does not possess catalytic activity [67]. This protein exhibited antimicrobial activity against *Escherichia coli* and *Candida albicans*, as well as antiparasitic activity against *Schistosoma mansoni* and *Leishmania* spp. [68]. The mechanism of action of CM14 was associated with its ability to destabilize membranes [69,70], and in this context, we note that EVA71 hijacks the autophagy process during infection [71]. It is possible therefore that the antiviral activity of CM14 against EVA71 replication was due to an effect on cytoplasmic membranes. Even though no antiviral activity was previously reported for these two proteins from *B. moojeni*, the capacity of CM14 to interfere with several infectious pathogens demonstrates the potential of these molecules to be further exploited for antiviral development against HFMD.

Although EVA71 lacks an envelope, the virus presents a depression in the capsid termed the canyon, an important region where cellular receptors bind to the virus particle [72]. The canyon harbours a hydrophobic lipid pocket factor that is involved in regulating particle stability [73,74]. The release of a lipid pocket factor from the capsid is required for the conformational changes, which ultimately lead to viral infectivity. Based on the data presented here, CM10, CM14, and Crotamin demonstrated a significant virucidal activity and we suggest that these proteins could be acting by: 1) stabilising the particles and preventing uncoating (thus infection); 2) destabilising the particles and prematurely inducing uncoating; or 3) interacting at places on the capsid required for cell interaction or receptor engagement, thus preventing receptor binding. However, more studies are needed for a better understanding of how these compounds are acting on EVA71.

Altogether, the results demonstrated that all the evaluated proteins isolated from snake venom inhibited the EVA71 replicative cycle. Most interestingly, CM14 and Crotamin were able to significantly inhibit all stages of virus infection here investigated, with high SI. This study is the first report of proteins isolated from Brazilian snake venoms with antiviral activity against EVA71. In addition, the molecules presented here emphasize the employment of natural sources to identify antiviral candidates, as well as identify scaffold molecules for drug design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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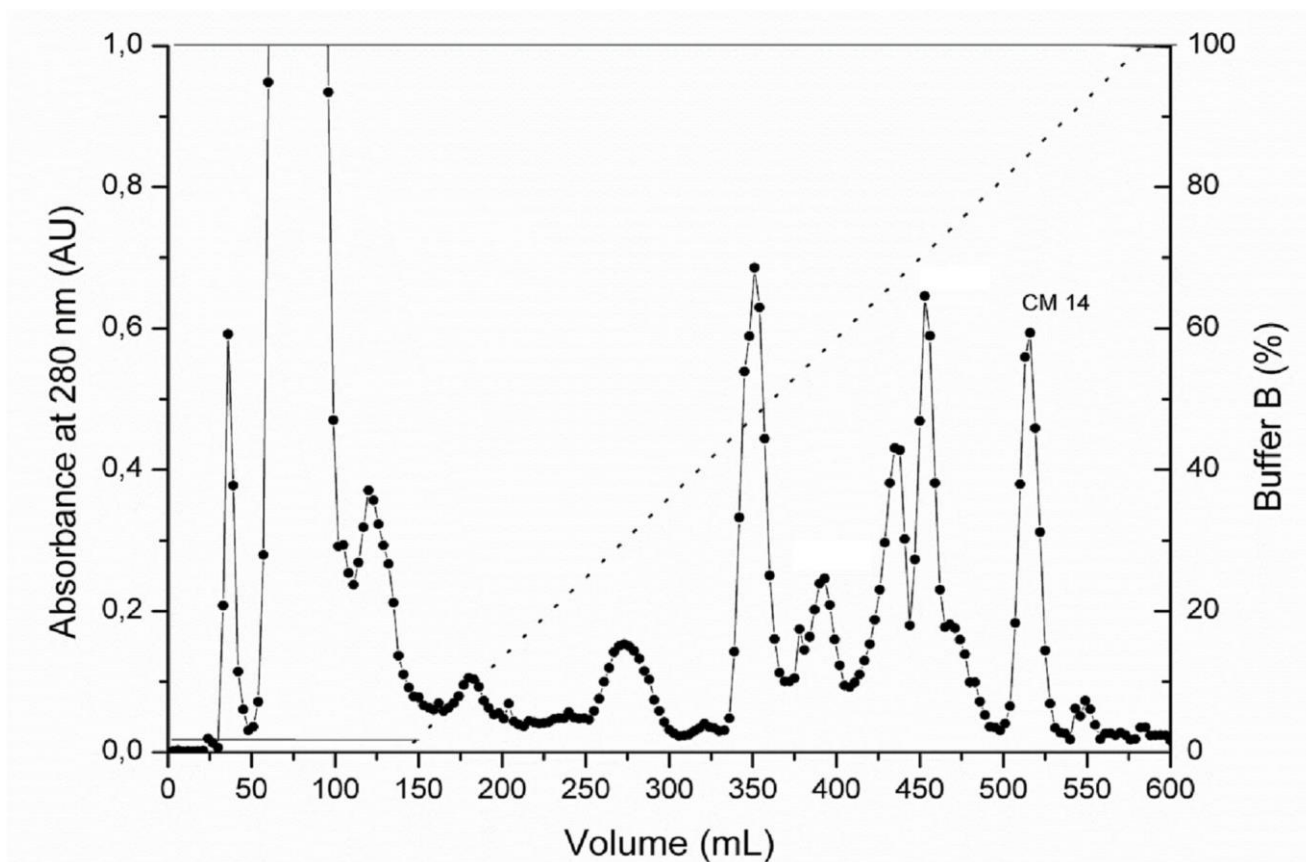


Fig. 1.

Isolation of CM14 from *Bothrops moojeni* snake venom. Chromatographic profile of *B. moojeni* crude venom (200 mg) on a CM Sepharose column, equilibrated and eluted with 50 mM ammonium bicarbonate buffer, pH 7.8, followed by a linear gradient of up to 500 mM (buffer B). Fractions were collected at a flow rate of 20 mL/h and at room temperature.

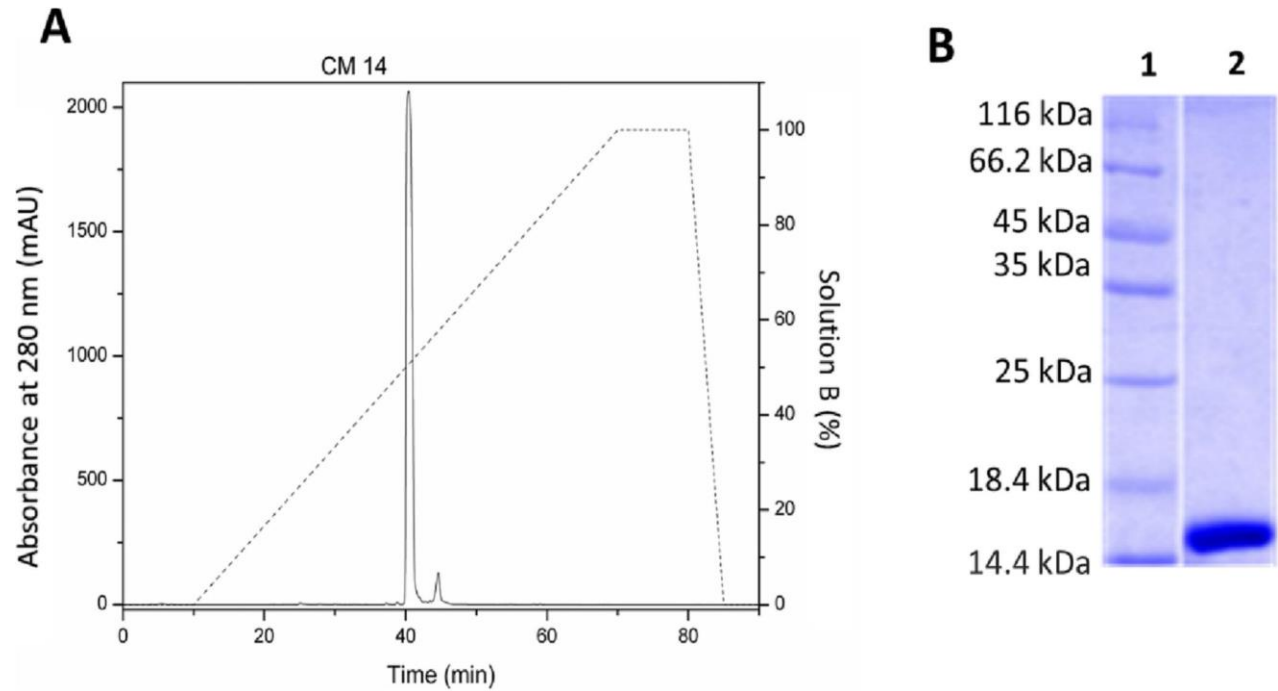


Fig. 2.

Chromatographic profile of CM14. CM14 was separated on a C18 reversed-phase column equilibrated and eluted with 0.1 % trifluoroacetic acid (TFA), and 70 % acetonitrile and 0.1 % TFA (solution B). Protein elution was achieved at flow rate of 1 mL/min with a linear concentration gradient of solution B (A). SDS-PAGE of the purified CM14 (2) under denaturing and reducing conditions. (1) Molecular weight standard: beta-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98I (25.0 kDa), beta-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa) (B).

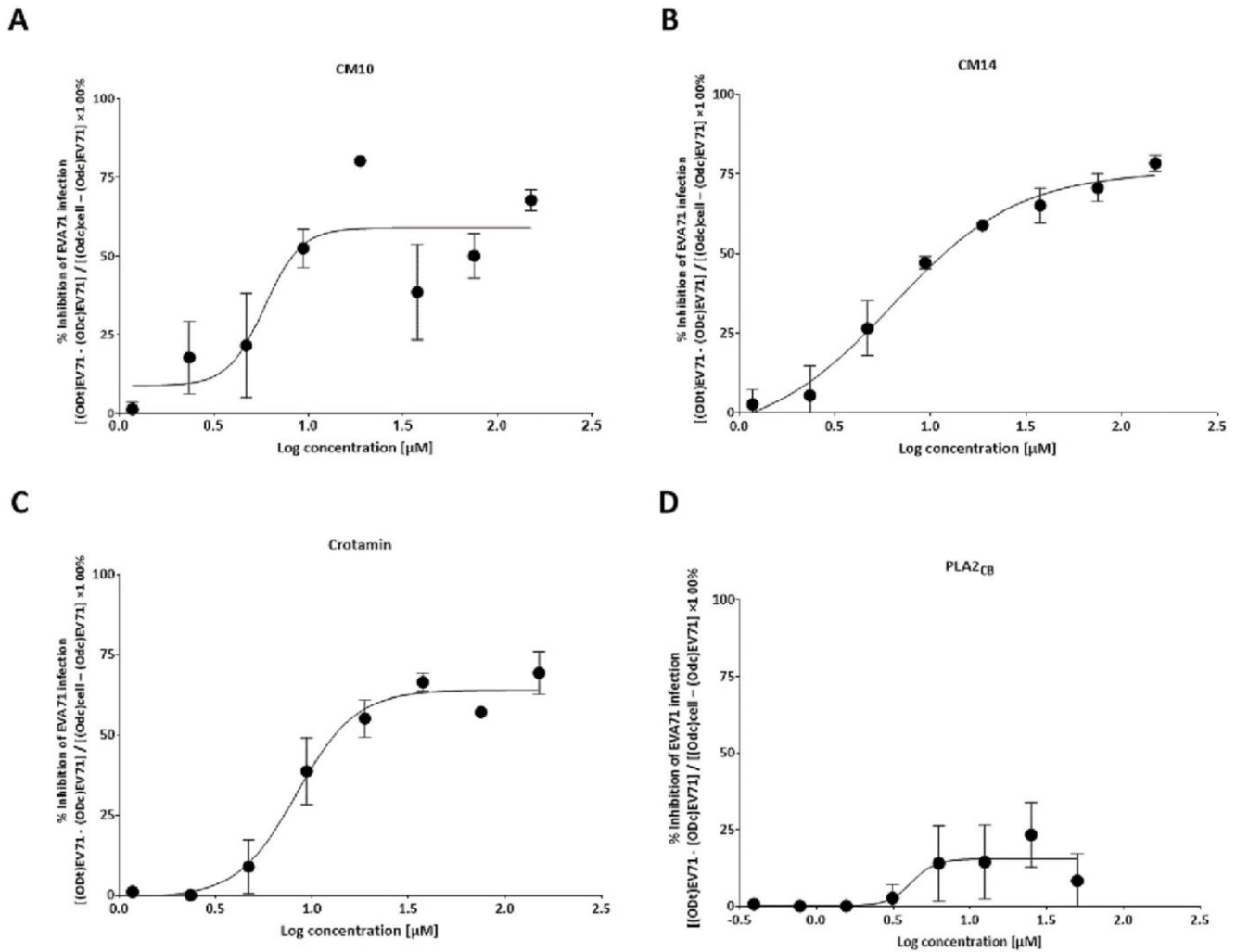


Fig. 3.

EC₅₀ of each snake venom protein. CM10 (A), CM14 (B), Crotamin (C), and PLA2_{CB} (D). Vero cells were treated with CM10, CM14, and Crotamin, with concentrations ranging from 1.2 to 150 μg/mL, and PLA2_{CB} from 0.4 to 50 μg/mL, in the presence of EV-A71 (100 TCID₅₀) and added to the cells for 72 h. PBS was used as untreated control. After 72 h, supernatant was removed and MTT solution was added to cells. The MTT solution was removed, replaced by the same volume of DMSO, and read at a wavelength of 490 nm on the GloMax plate reader (PROMEGA). The results were then transformed into percentages, and the inhibition of EV-A71 replication in cells by each protein was calculated using the following formula $\frac{[(OD_t)EVA71 - (OD_c)EVA71]}{[(OD_c)cell - (OD_c)EVA71]} \times 100\%$, where (OD_t)EVA71 is the measured optical density of treatment on EV-A71 infected cells; (OD_c)EVA71 is the optical density measured for untreated control EV-A71 infected cells; and (OD_c)cell is the measured optical density of uninfected control cells [46]. Antiviral activity was presented as % inhibition of EV-A71 replication. Results are shown as a non-linear regression analysis, indicating the percentages of the inhibition of viral replication by each protein. Mean values ± SD of two independent experiments each measured in triplicate are shown. The comparison of each curve was performed employing the F test (confidence level of 95 %), resulting in a $p < 0.0001$.

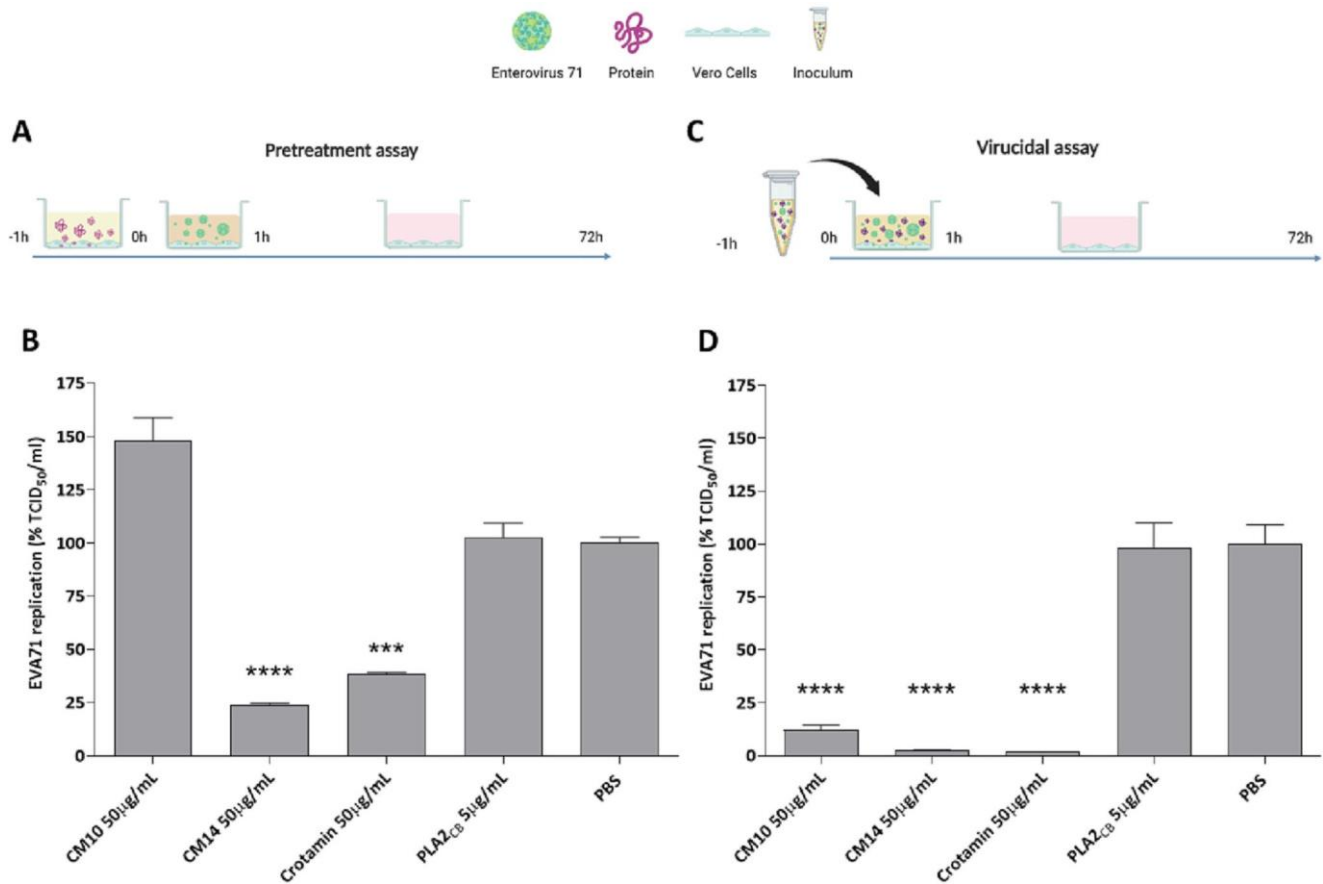


Fig. 4. Protective and virucidal effects of proteins against EVA71 infection.

EVA71 at MOI of 0.1 and the proteins CM10, CM14, and Crotamin at 50 µg/mL, and PLA2_{CB} at 5 µg/mL were added in different times to the cells and virus replication was titrated by TCID₅₀ method. For the pre-treatment assay, Vero cells were treated with each protein for 1 h, washed with PBS, and infected with EVA71 for 1 h. Then, cells were washed and replaced with fresh media and incubated for 72 h (A), and supernatant was collected, titrated, and normalized with PBS control (B). For virucidal assay, EVA71 were incubated with proteins for 1 h prior to the infection on Vero cells. Cells were added of the mixture for 1 h, washed and fresh media was added, cells were incubated for 72 h (C), supernatant was collected, titrated, and normalized with PBS control (D). PBS was used in the untreated infected control. Statistically significant differences between each compound and PBS control are shown. Significant differences from the control are denoted by *** ($p \leq 0.001$) and **** ($p \leq 0.0001$).

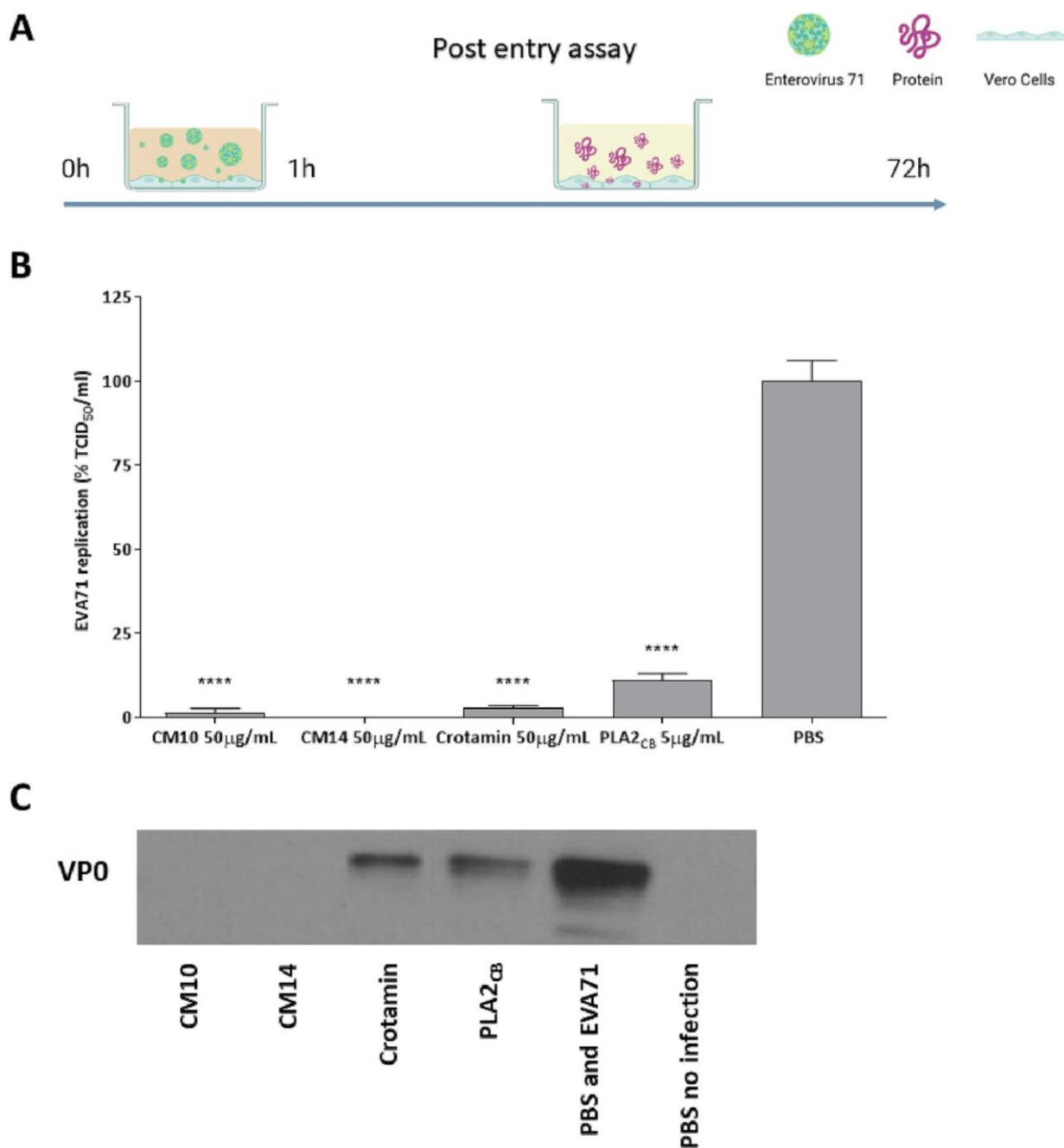


Fig. 5. Effect of CM10, CM14, Crotamin, and PLA2_{CB} on EVA71 replication.

Vero cells were infected with EVA71 at an MOI of 0.1 for 1 h, washed to the removal of non-endocytosed virus and each protein was added to the cells were incubated for 72h (A). Viral replication was measured by using TCID₅₀ method assay (B) and western blotting assays (C). PBS was used as untreated infected control. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. Statistically significant differences between each compound and PBS control are shown. Significant differences from control denoted by **** ($p \leq 0.0001$).

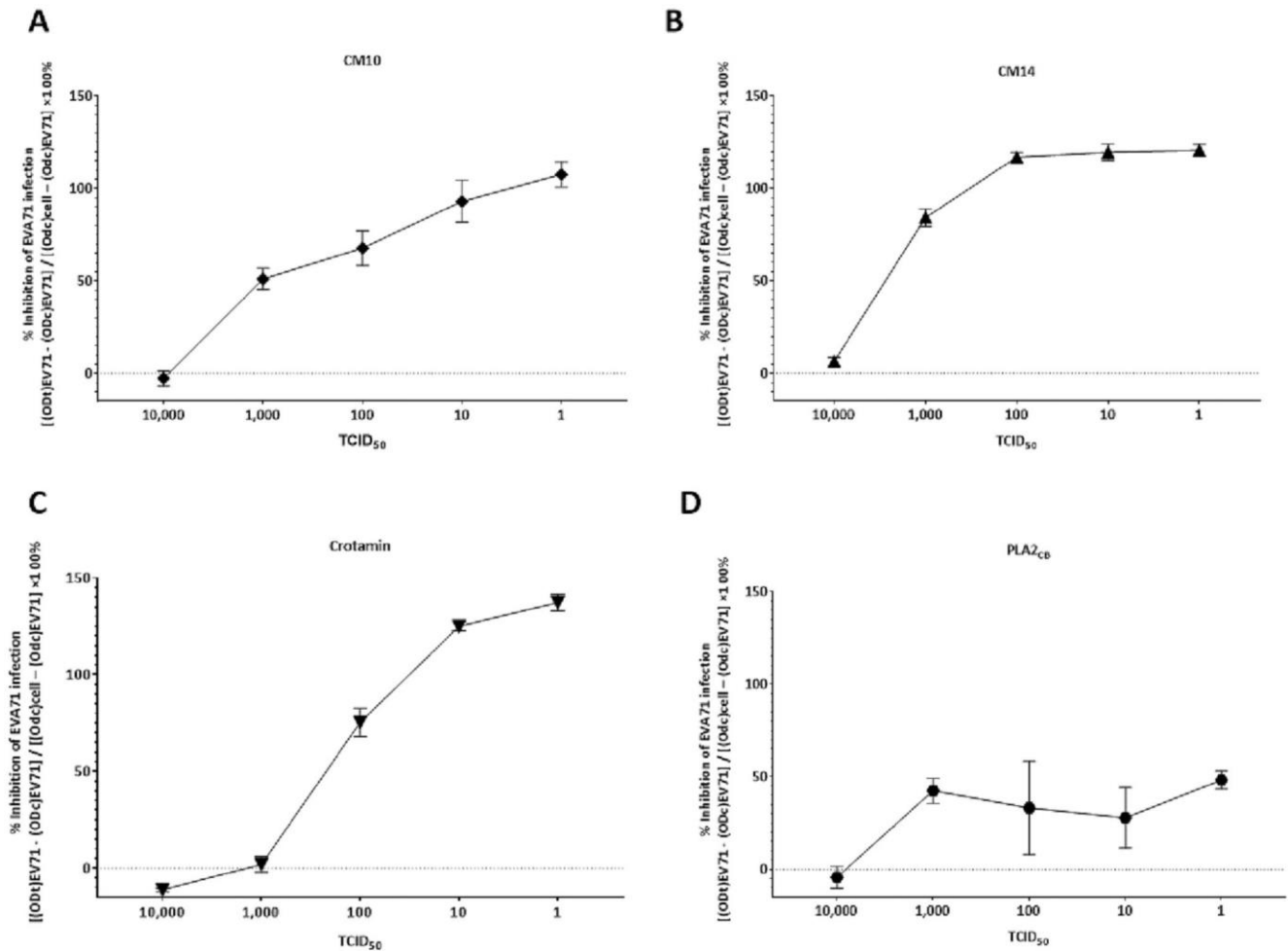


Fig. 6.

Effect of CM10, CM14, Crotamin, and PLA2_{CB} at increasing EVA71 TCID₅₀ challenge doses. Vero cells were infected with 1, 10, 100, 1000, and 10,000 TCID₅₀ of EVA71 in the presence of CM10, CM14, and Crotamin at 50 µg/mL or PLA2_{CB} at 5 µg/mL. PBS was used as untreated control. After 48 h, supernatant was removed and MTT solution was added to cells. The MTT solution was removed, replaced by the same volume of DMSO, and read at a wavelength of 490 nm on the GloMax plate reader (PROMEGA). The results were then transformed into percentages, and the inhibition of EVA71 replication in cells by each protein was calculated using the following formula $[(\text{ODtEVA71} - (\text{ODcEVA71})] / [(\text{ODc} \text{ cell} - (\text{ODcEVA71})] \times 100$ (%), where (ODt)EVA71 is the measured optical density of treatment on EVA71 infected cells; (ODc)EVA71 is the optical density measured for untreated control EVA71 infected cells; and (ODc)cell is the measured optical density of uninfected control cells [46]. Antiviral activity was presented as % inhibition of EVA71 replication. Mean values $\pm$ SD of two independent experiments each measured in triplicate are shown.

Table 1

CC50, EC50, and Selective index of each protein.

Protein	Venom Origin	CC ₅₀ (µg/mL)	EC ₅₀ (µg/mL)	SI
CM10	<i>B. moojeni</i>	427.6	6.3	67.4
CM14	<i>B. moojeni</i>	>1000	5.9	>170.8
Crotamin	<i>C. durissus terrificus</i>	>1000	8.3	>120.4
PLA2 _{CB}	<i>C. durissus terrificus</i>	52.3	4.2	12.5

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Table 2

Activity of proteins isolated from snake venoms against EVA71.

Protein	Non-toxic concentration	Cell viability (%)	Protective activity (%)	Virucidal activity (%)	Inhibition of replication (%)
CM10	50 µg/mL	104	76	98	100
CM14	50 µg/mL	107	–	88	99
Crotamin	50 µg/mL	93	62	99	97
PLA2 _{CB}	5 µg/mL	97	–	–	89

ORIGINAL ARTICLE



Dereplication of *Lantana trifolia* L. leaves and fruits by UFLC-DAD-(+)-ESI-MS/MS and its antifungal and cytotoxic activities

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Abstract

Introduction *Lantana trifolia* L. (Verbenaceae) is a shrubby plant. In folk medicine, its leaves are used in the form of infusions and syrups to treat angina, coughs, and colds; they are also applied as tranquilizer. Previous studies have reported the antimicrobial potential of the compounds present in *L. trifolia* leaves.

Objectives To report the anti-*Candida* activities of the fractions obtained from the fruits and leaves of two *L. trifolia* specimens.

Methods The *L. trifolia* fractions were submitted to UFLC-DAD-(+)-ESI-MS/MS, and the data were analyzed by using multivariate statistical tools (PCA, PLS-DA) and spectral similarity analyses based on molecular networking, which aided dereplication of the bioactive compounds. Additionally, NMR analyses were performed to confirm the chemical structure of some of the major compounds in the fractions.

Results The ethyl acetate fractions presented MIC values lower than 100 µg mL⁻¹ against the three *Candida* strains evaluated herein (*C. albicans*, *C. tropicalis*, and *C. glabrata*). Fractions FrPo AcOEt, FrPe AcOEt, and FrPe *n*But had MIC values of 1.46, 2.93, and 2.93 µg mL⁻¹ against *C. glabrata*, respectively. These values resembled the MIC value of amphotericin B, the positive control (0.5–1.0 µg mL⁻¹), against this same strain. Cytotoxicity was measured and used to calculate the selectivity index.

Conclusion On the basis of our data, the most active fractions in the antifungal assay were more selective against *C. glabrata* than against non-infected cells. The analytical approach adopted here allowed us to annotate 29 compounds, nine of which were bioactive (PLS-DA results) and belong to the class of phenolic compounds.

Keywords *Lantana trifolia* L. · Anti-*Candida* · Natural products · Molecular networking

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Introduction

Candida species are fungal pathogens that belong to the normal microbiota of 50–70% of healthy humans (Pappas et al., 2018). They underlie several infectious diseases, being the leading cause of morbidity and mortality in immunocompromised patients (Soliman et al., 2017). Treatment of diseases caused by *Candida* species includes different classes of antifungal drugs, such as amphotericin, azoles, echinocandins, allylamines, and antimetabolite antifungal drugs. Amphotericin B and fluconazole are the most used antifungal agents. *Candida* species can form biofilms, which provide them with resistance to treatment with multiple medications. Resistance to antifungals is an issue that makes selecting a drug for effective treatment a considerable challenge (Pappas et al., 2018; Rodrigues et al., 2013). In this context, searching for new compounds with antifungal properties is essential. Given that compounds from natural sources constitute the main leads for novel and potent antimicrobial agents, one strategy to overcome fungal resistance to antifungals is to employ plant extracts to guide drug discovery. About 67% of the new medicines discovered between 1981 and 2019 were based on secondary metabolites or direct derivatives of natural products (Newman & Cragg, 2020).

Traditionally, discovering bioactive compounds in plants requires complex isolation and structural elucidation steps, which are usually expensive and laborious and do not guarantee that a bioactive compound is identified fast (Cordell & Shin, 1999). In recent decades, integrative approaches that analyze large datasets have emerged as an alternative to change this challenging methodological landscape. In this scenario, metabolomics has emerged as a comprehensive approach for evaluating large datasets (metabolites) through experiments that couple separation and detection techniques, including HPLC-DAD, HPLC-MS, and HPLC-NMR (Funari et al., 2013; Pilon et al., 2020). Bioguided assays combined with libraries of compounds to evaluate extracts and fractions is another strategy that has accelerated the process of identifying biomarkers (Cordell & Shin, 1999). Bioinformatics and processing tools have evolved and taken correlation analyses to another level, allowing bioactive compounds to be proposed by correlating chemical data and biological assay results. Multivariate statistical analysis is an example of analysis that makes this possible (Li et al., 2020). In traditional medicine, *Lantana trifolia* L. (Verbenaceae), a herbaceous shrub with small spherical purple fruits commonly found in tropical and subtropical regions of America, Asia, and Africa (Silva et al., 2005), is used in the form of infusions and syrups to treat cough, cold, asthma, sinusitis, earache, fever, and stomach pain (Julião et al.,

2009; Nalubega et al., 2013). Phytochemical studies have shown that *L. trifolia* presents anti-inflammatory, analgesic (Silva et al., 2005; Uzcátegui et al., 2004), antimicrobial (Rwangabo et al., 1988), antipyretic (Silva et al., 2005), antibacterial (Julião et al., 2009), and sedative properties (Julião et al., 2010). Terpenes, steroids, flavonoids, and phenylethanoid glycosides have been identified in this species (Julião et al., 2010; Rwangabo et al., 1988). Furthermore, umuhen-gerin, a flavonoid isolated from *L. trifolia* leaves, is active against *Candida tropicalis* (Rwangabo et al., 1988).

In this study, we have evaluated the antifungal activity of the hexane, ethyl acetate, *n*-butanol, and hydroalcoholic fractions obtained from the fruits and leaves of two *L. trifolia* specimens, collected at different sites. We have analyzed the secondary metabolites of the fractions by UFLC-DAD- (+)-ESI-MS/MS (microTOFQII), and we have interpreted the data by employing molecular networking (Wang et al., 2016), in-house compound libraries, and chemometric analysis (PLS-DA) to suggest the associated biomarkers of the antifungal activity rapidly. We used a ¹H NMR-based dereplication strategy to confirm the chemical structure of the major compound in some fractions.

Materials and methods

Standard chemicals

Solvents, reagents, and standards were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

General experimental procedures

Collection and identification of botanical samples

The fruits and leaves of *Lantana trifolia* L. were collected in the cities of Peirópolis (rural district of Uberaba, Minas Gerais State) and Pontal (São Paulo state), Brazil. The plants were identified by Fátima Regina Gonçalves Salimena, PhD, from the Botany Department of the Federal University of Juiz de Fora (UFJF). Voucher specimens were deposited at the Uberlandense Herbarium of the Federal University of Uberlândia (Table S1).

Extract preparation

Fruits and leaves of *Lantana trifolia* species were dried at room temperature for seven days. The dry materials were ground in a knife mill and subjected to extraction by maceration for 48 h with ethanol (95%) at room temperature in the proportion of plant material and solvent of 1:4 (m v⁻¹). Then, the ethanol extracts were filtered (qualitative

filter paper-Qualy, 80 g m⁻²) and the solvents were removed under reduced pressure in a rotary evaporator (Buchi, model R-100) at a low temperature (≤ 40 °C). This procedure was repeated five times, producing the extracts summarized in Table S1.

The ethanol extracts of leaves and fruits (10 g) were dissolved in 60 mL of methanol/water (9:1 v v⁻¹), filtered, and subjected to a sequential liquid-liquid extraction with hexane (5 × 30 mL), ethyl acetate (5 × 200 mL), and *n*-butanol (5 × 200 mL) successively. Complete phase separation with ethyl acetate and *n*-butanol was achieved by adding 500 mL of water to the mixture before ethyl acetate separation. The solvents were evaporated under reduced pressure obtaining two fractions of leaves (Fo) and two fractions of fruits (Fr) for the two specimens (*L. trifolia*), collected in Peirópolis (Pe) and Pontal (Po), as shown in the Table S2. All the fractions were submitted to the antifungal activity test.

Antifungal activity against *Candida* spp.

The antifungal activities of the fractions of *Lantana trifolia* leaves and fruits were evaluated against microorganisms from the American Type Culture Collection (ATCC, Rockville MD, USA). Three yeast strains were tested: *Candida albicans* (ATCC 28366), *Candida tropicalis* (ATCC 13803), and *Candida glabrata* (ATCC 15126). The broth microdilution method was used to determine the minimum inhibitory concentration (MIC), which represents the lowest concentration capable of inhibiting microbial growth. The antifungal test to determine the MIC was performed according to the Clinical and Laboratory Standard Institute (CLSI M27-A3 broth dilution assay) (Clinical Laboratory Standards Institute (CLSI) 2008). Roswell Park Memorial Institute 1640 medium (RPMI) containing L-glutamine without sodium bicarbonate (pH 7.0) and supplemented with 165.0 mM morpholinepropanesulfonic acid (both from Sigma) was used. The spectrophotometric method was used to prepare the inocula at 530 nm, and the measurements were compared with a 0.5 McFarland scale to obtain a concentration of 5×10^6 CFU mL⁻¹ (colony forming units). Dilutions were performed with RPMI until reaching 5×10^2 CFU mL⁻¹ in work solution. Fractions solutions (192,000 µg mL⁻¹) were prepared in dimethyl sulphoxide (DMSO) and diluted to 12,000 µg mL⁻¹ in RPMI medium.

Serial dilutions were performed in a 96-well microtiter plates, obtaining final concentrations ranging from 1.46 to 3000.0 µg mL⁻¹. To each well, 100 µL of the inoculum suspension was added. Sterility controls of the culture medium, fractions, inocula, and DMSO were prepared. The solvent control (DMSO) was tested with concentrations ranging from 1 to 5% (v v⁻¹). Amphotericin B at concentrations ranging from 31.0 ng mL⁻¹ to 16.0 µg mL⁻¹ was used as

a positive control, and *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) strains were used as quality control to monitor assay performance. The microtiter plates were then incubated at 35 °C for 24 h. After incubation, 30 µL of 0.02% resazurin in an aqueous solution was added to each well to indicate the viability of microorganisms for MIC determination.

Cytotoxicity assay

The cytotoxicity of the fractions FoPe Hex, FoPe AcOEt, FoPe *n*But, FrPe AcOEt, FrPe *n*But, FoPo Hex, FoPo AcOEt, FrPo AcOEt, FrPo *n*But was assessed by performing an MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) (Sigma-Aldrich) assay, using naïve Vero cells, as previously described (Oliveira et al., 2018; Silva et al., 2019) with some modifications. Cells were seeded at a density of 1×10^4 cells in 96-well plates the day before the assay. Then, cells were treated with each fraction at concentrations ranging from 375 to 0.73 µg mL⁻¹. After 72 h, an MTT assay was performed, and absorbance was measured at 560 nm in the Glomax microplate reader (Promega). The 50% cytotoxic concentration (CC₅₀) was calculated using Prism (Graph Pad). The selectivity index (SI) was calculated according to the equation $SI = \log [CC_{50}]/[MIC]$ to establish the relationship between cytotoxicity and antifungal activity. Positive values indicate greater selectivity against microorganisms than toxicity to Vero cells (Case et al., 2006).

LC-MS/MS untargeted metabolomics

UFLC-DAD-(+)-ESI-MS/MS (micrOTOF-QII) Analysis

LC-MS/MS analysis was carried out on a UFLC-DAD-(+)-ESI-MS/MS (micrOTOF-QII), using an Ascentis® Express C₁₈ (15 cm × 4.6 mm × 2.7 µm) column protected by a guard cartridge of similar material, with an acetonitrile (CH₃CN) and water (H₂O) (v v⁻¹) solvent system containing 0.1% formic acid (both solvents). The flow rate was 0.5 mL min⁻¹, with an injection volume of 12 µL. Gradient elution was performed as follows: 5–100% CH₃CN for 60 min and 100% for 5 min, for a total runtime of 65 min. The UV detector (DAD) was programmed to detect between 190 and 700 nm. The oven temperature was set to 40 °C.

The ionization source parameters in the positive ionization mode were: End Plate Offset voltage of 500 V; capillary voltage of 3500 V; nebulizer at 5.5 bar; drying gas flow rate of 10.0 L min⁻¹; drying gas temperature of 220 °C. The spectrum rate of 2.0 Hz and the mass range of 50 to 1300 *m/z* were maintained throughout the analysis. The MS/MS parameters were Automated MS/MS cycle of 2 Hz

for MS1 and 4 Hz for MS2, with the selection of precursor ions in 3.0 s cycles, excluding selection after the detection of 2 spectra of the same ion or after 0.60 min, reconsidering their selection if ion intensity is twice as high as before. A collision energy ramp from 20 to 50 eV was used for the fragmentation of precursor ions. Data were analyzed using the Data Analysis® Software of the Bruker Daltonics instrument.

Chemical profiles (BPC) of ethyl acetate, *n*-butanol, and hydroalcoholic fractions of leaves and fruits in the Supporting Information (Figures S1–S3).

Data processing

Raw data were converted to .mzXML file format using ProteoWizard-MSconvert (Chambers et al., 2012) and loaded to MzMine™ (Pluskal et al., 2010), isotopic clustering and alignment (Table S3). Subsequently, the data matrix (.csv) containing quantitative MS1 data was used in the multivariate statistical analysis. A .mgf file with fragmentation profile data and a data matrix (.csv) containing quantitative data from precursor ions selected for MS2 scan was used in molecular networking.

Multivariate statistical analysis

The data matrix was centered and scaled for Pareto Variance and submitted to supervised statistical analysis, performed in SIMCA P 13.0.3.0© (Umetrics AB Malmö, Sweden). Partial Least Square–Discriminant Analysis (PLS-DA) was performed using bioactivity information obtained from the in vitro assays as Classes. The cross-validation tool validated the models using the leave-1/7-out, and raw permutation approaches. RMSECV (Root Mean Square Error of Cross-validation) was performed to confirm the predictive ability of the models. RMSECV was calculated by SIMCA software. The score plot represents the correlation between the samples and the loadings plot between the variables (Precursor ions). For each PLS-DA model, biomarkers were determined using the VIP (Variable Importance in Projection) plot and coefficient plot, selecting variables with VIP > 1 and positive coefficient values. The VIP plot indicates the importance of variables for group projection in a statistical model, and variables with VIP values greater than 1 are important for separating groups. The coefficient plot contains the regression coefficients representing the prediction vectors for the selected Y (biological activities), and the coefficients with positive values, with VIP > 1, correspond to active biomarkers (Chagas-Paula et al., 2015).

Molecular networking

Once processed in Mzmine, the MS data (.csv and .mgf files) and a metadata file (.txt) were uploaded to the Global Natural Products Social - GNPS server (<http://gnps.ucsd.edu>) (Wang et al., 2016), and a Feature-Based Molecular Networking (FBMN) (Katajamaa et al., 2006; Nothias et al., 2020; Wang et al., 2020) was created. Data were clustered with precursor ion mass tolerance of 0.02 Da and an MS/MS fragment ion tolerance of 0.02 Da. The minimum cosine score threshold for the molecular network was set to

0.7. A minimum of three peaks should be shared between the spectra and edges between two nodes at least in the top 10 cosine scores (K parameter). The maximum size of a cluster was defined as 130. The spectra were compared against GNPS spectral libraries, considering a similarity score threshold above 0.7 and at least three matched peaks. Cytoscape 3.2 (Shannon et al., 2003), was used for graphical visualization. LC-MS/MS data (raw and mzXML) were deposited in the MassIVE Public GNPS data set (<http://massive.ucsd.edu>, MassIVE number: MSV000091744). All networks can be accessed online (links in the Supporting Information).

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectra were performed on the Bruker Model Ascend™ 400 Avance III HD (9.2 Tesla) spectrometer. The fractions FrPo AcOEt, FoPo AcOEt, and FoPe AcOEt were solubilized in deuterated dimethyl sulfoxide (DMSO-*d*₆), and fractions FrPe AcOEt, FrPe *n*But, and FrP *n*But were solubilized in deuterated methanol (MeOH-*d*₄).

Results and discussion

Antifungal activity and cytotoxicity of fractions obtained from *L. trifolia* leaves and fruits

First, we carried out liquid-liquid extraction of the ethanol extracts of the leaves and fruits of two *L. trifolia* specimens, to concentrate different metabolic classes in the ethyl acetate and *n*-butanol fractions. Then, we evaluated the antifungal activity of these fractions against three *Candida* strains (*C. albicans* (ATCC 28366), *C. tropicalis* (ATCC 13803), and *C. glabrata* (ATCC 15126) by determining the minimum inhibitory concentration (MIC) (Table 1). Fractions with MIC values lower than 100.0 µg mL⁻¹, from 100.0 to 500.0 µg mL⁻¹, from 500.0 to 1000.0 µg mL⁻¹, and above 1000.0 µg mL⁻¹ are considered active, moderately active, little active, and inactive (Holetz et al., 2002).

selectivity against the *C. glabrata* than against Vero cells (SI values ranged between 0.28 and 1.49) (Table S4). Three fractions (FoPo AcOEt, FrPo *n*But, and FrPe *n*But) were more selective against *C. albicans* than against Vero cells, and two fractions (FoPo AcOEt and FrPo *n*But) showed higher activity against *C. tropicalis* than toxicity to Vero cells. Fraction FoPo AcOEt presented high activity and positive selectivity index against all the tested *Candida* strains. Due to this promising activity, we investigated the chemical profile of *L. trifolia* by UFLC-DAD-(+)-ESI-MS/MS, chemometric analysis, and molecular networking.

Multivariate statistical analysis

Initially, the PCA analysis (Figure S4) also indicated that the AcOEt extracts from both leaves and fruits exhibited a distinct chemical profile when compared to the other fractions. To evaluate which compounds (precursor ion or ion) affected the antifungal activity the most, we performed a supervised statistical analysis (PLS-DA) for each of the three fungal strains: *C. albicans* (ATCC 28366) (Fig. 1A), *C. tropicalis* (ATCC 13803) (Fig. 1B), and *C. glabrata* (ATCC 15126) (Fig. 1C). The PLS-DA models obtained high values of R²Y(cum) and Q²(cum) (Fig. 1), and the values of RMSECV are within the expected range (Supplementary material, Figures S14–S16 and Tables S5–S7). We selected the first 30 variables with the highest VIP scores and positive coefficients for the dereplication study and spectral comparison with the aid of molecular networking (Supplementary Material, Figures S5–S13 and Tables S8–S10).

Table 1 Antifungal, and cytotoxic activity for fractions of two *Lantana trifolia* specimens

Fractions	Minimum inhibitory concentration MIC (μg mL ⁻¹)		CC ₅₀ (μg mL ⁻¹)	
	<i>C. albicans</i> (ATCC 28366)	<i>C. tropicalis</i> (ATCC 13803)	<i>C. glabrata</i> (ATCC 15126)	
FoPe Hex	375	750	46.87	169.9
FoPe AcOEt	46.87	93.75	11.72	37.83
FoPe <i>n</i> But	> 3000	> 3000	46.87	88.49
FoPe HM	> 3000	> 3000	> 3000	-
FrPe Hex	> 3000	> 3000	375	-
FrPe AcOEt	93.75	93.75	2.93	84.41
FrPe <i>n</i> But	46.87	93.75	2.93	89.59
FrPe HM	> 3000	> 3000	> 3000	-
FoPo Hex	750	750	46.87	181.1
FoPo AcOEt	23.43	93.75	5.86	163.7
FoPo <i>n</i> But	3000	3000	> 3000	-
FoPo HM	750	750	750	-
FrPo Hex	> 3000	> 3000	375	-
FrPo AcOEt	93.75	93.75	1.46	43.88
FrPo <i>n</i> But	93.75	93.75	> 3000	302.3
FrPo HM	> 3000	> 3000	> 3000	-
Amphotericin B	0.25	0.25	0.12	-

Samples were tested under concentrations of 1.46–3000 μg mL⁻¹. The MICs found for the species *C. parapsilosis*, and *C. Krusei* (used as method control) were 0.5 and 1.0 μg mL⁻¹, respectively. These results validate the method according to CLSI (2008). Hexane fractions (FoPe Hex and FoPo Hex) and fruits (FrPe and FrPo Hex), ethyl acetate fractions of leaves (FoPe AcOEt and FoPo AcOEt) and fruits (FrPe AcOEt and FrPo AcOEt), *n*-butanol fractions of leaves (FoPe *n*But and FoPo *n*But) and fruits (FrPe *n*But and FrPo *n*But) and hydroalcoholic fractions of leaves (FoPe HM and FoPo HM) and fruits (FrPe HM and FrPo HM).

The ethyl acetate fractions showed higher activity against the three *Candida* strains evaluated, the MIC values of the former fractions ranged from 1.46 to 93.75 μg mL⁻¹. Against *C.*

glabrata, fractions FrPo AcOEt (1.46 μg mL⁻¹) and FrPe AcOEt (2.93 μg mL⁻¹) presented MIC values in the same order of magnitude as the MIC value of the positive control, amphotericin B (0.12 μg mL⁻¹). These fractions presented high activity even when compared to the activity of isolated compounds (MIC < 10 μg mL⁻¹) (Ríos e Recio 2005). Among the *n*-butanol fractions, fraction FrPe *n*But showed higher activity against all the tested *Candida* strains, with MIC values ranging from 2.93 to 93.75 μg mL⁻¹. As for fraction FrPo *n*But, it exhibited activity against *C. albicans* and *C. tropicalis*, with MIC value of 93.75 μg mL⁻¹, but it was inactive against *C. glabrata*. The other fractions presented moderate to weak activities against at least two of the tested *Candida* strains. We tested the fractions with MIC values lower than 100 μg mL⁻¹ in naïve Vero cells to assess their CC₅₀ cytotoxic activity (Table 1). The tested fractions showed higher selectivity against the *C. glabrata* than against Vero cells (SI values ranged between 0.28 and 1.49) (Table S4). Three fractions (FoPo AcOEt, FrPo *n*But, and FrPe *n*But) were more selective against *C. albicans* than against Vero cells, and two fractions (FoPo AcOEt and FrPo *n*But) showed higher activity against *C. tropicalis* than toxicity to Vero cells. Fraction FoPo AcOEt presented high activity and positive selectivity index against all the tested *Candida* strains. Due to this promising activity, we investigated the chemical profile of *L. trifolia* by UFLC-DAD-(+)-ESI-MS/MS, chemometric analysis, and molecular networking.

Compound annotation using molecular networking

The resulting molecular network formed by the fractions showed 2243 nodes connected by 3118 edges, as well as 237 clusters. We labeled these data by using colors to represent the collection sites Peirópolis (purple) and Pontal (yellow), the size of the nodes to represent the abundance of the ions in the mass experiments (the larger the node size, the higher the peak area), and the edge width to depict cosine similarity.

(the larger the similarity degree, the larger the edge width). We removed the ions that we had detected during blank injections (solvent) (Fig. 2). The molecular network revealed molecular families of classes of metabolites such as phenolic compounds.

Then, to discover the compounds underlying the antifungal activity of the active fractions, we employed an approach that projected the biological activities of each evaluated fraction against each *Candida* strain on the molecular network (Pilon et al., 2022; Shannon et al., 2003). The MIC values of each fraction were projected on their corresponding nodes (that is, peaks/substances that were detected in the fractions). We used different colors (node colors) to differentiate the active nodes (pink – MIC < 100 $\mu\text{g mL}^{-1}$) from the moderately active and inactive nodes (grey – MIC > 100 $\mu\text{g mL}^{-1}$). We also colored some nodes yellow to highlight the peaks (substances) with high inhibitory activity (MIC < 100 $\mu\text{g mL}^{-1}$) that were present in various fractions (Fig. 3 and Supplementary Material, Figures S18–S19).

Although this approach corresponds to bioactivity projections, it is used to guide decisions about regions/compounds to be explored and studied. In this case, the nodes colored in pink pointed to compounds with greater antifungal potential, and the nodes colored in yellow represented the common nodes of the most active samples.

Figure 3 illustrates a phenolic-type flavonoid cluster (39 nodes connected by 105 edges) of the molecular network filtered according to the MIC values against the *Candida* strains. There was a high degree of spectral similarity between the active compounds (yellow nodes) present in the different fractions and the tested *Candida* strains. This reinforced the importance of these compounds and guided us in an investigation of the structural characteristics of these nodes.

In addition, the molecular network revealed other clusters of phenolic compounds with potential antifungal properties, as shown in Figures S5–S13 (Supplementary Material, Tables S8–S10, and Figures S20 and S28–S208).

By using molecular networks, we annotated 29 flavonoid-like compounds (Pilon et al., 2019) (Table 2), the annotation level is 3 for these compounds (Sumner et al., 2007). The correlation between the biomarkers obtained by PLS-DA and VIP values (Tables S8–S10) and the molecular networks resulted in the annotation of nine candidates for the antifungal activity of the active fractions. Among the phenolic acids, chlorogenic acid (**1**), 4-coumaroylquinic acid (**5**), and 5-cafeoylshikimic acid (**6**) stood out; among the flavonoids, apigenin 7-glucuronide (**17**), scutelarein (**18**), cisosplenitin B (**21**), tricine (**26**), and chrysoplenol D (**27**) stood out; and among the phenylpropanoids, verbascoside (**10**) can be highlighted (Fig. 4). The chemical composition of the most active *L. trifolia* fractions may justify their anti-*Candida* activity. Plant extracts enriched in phenolic compounds have provided

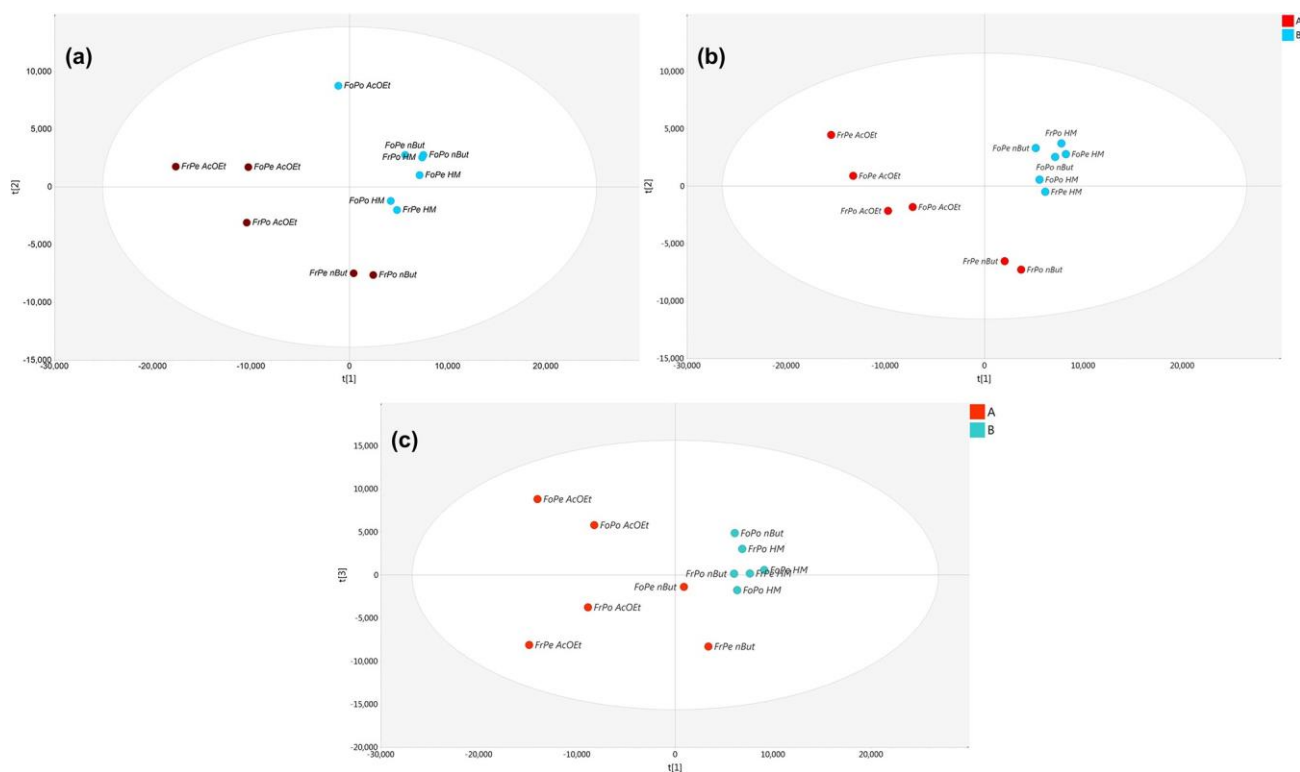


Fig. 1 Partial Least Squares Discriminant Analysis (PLS-DA) score plot of *Candida* species antifungal bioassay

Key: Active class (A-red) and inactive class (B-blue), (1 A) *C. albicans*, number of components, 5; $R^2X(\text{cum})$, 0.781; $R^2Y(\text{cum})$, 0.997 and $Q^2(\text{cum})$, 0.829. (1B) *C. tropicalis*, number of components, 5; $R^2X(\text{cum})$, 0.775; $R^2Y(\text{cum})$, 0.998 and $Q^2(\text{cum})$, 0.834. (1 C) *C.*

glabrata, number of components, 5; $R^2X(\text{cum})$, 0.778; $R^2Y(\text{cum})$, 0.996 and $Q^2(\text{cum})$, 0.879. Ethyl acetate fractions of leaves (FoPe AcOEt and FoPo AcOEt) and fruits (FrPe AcOEt and FrPo AcOEt), *n*-butanol fractions of leaves (FoPe nBut and FoPo nBut) and fruits (FrPe nBut and FrPo nBut) and hydroalcoholic fractions of leaves (FoPe HM and FoPo HM) and fruits (FrPe HM and FrPo HM)

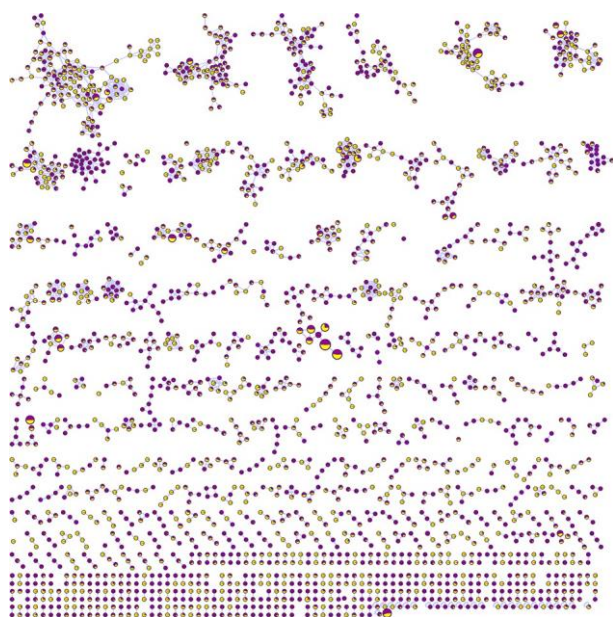


Fig. 2 Representation of molecular networking applied to UFLC- DAD- (+)-ESI-MS/MS data from *L. trifolia* using electrospray ionization in positive mode filtered by collection site. The node color chart reflects the collection site (purple for Peirópolis and yellow for Pontal), node size shows the precursor peak area and width of edge is associated with cosine similarity, (similarity degree among nodes)

relevant results against *Candida* species. In particular, phenolic acids and flavonoids at low concentrations have been recognized as inhibitors of *Candida* species growth (Martins et al., 2015).

Soliman and colleagues (Soliman et al., 2017) natural products can exert their antifungal action through several mechanisms, which include inhibiting germination, biofilm formation, and cell metabolism; impairing cell wall integrity and cell membrane plasticity; and inducing cell apoptosis.

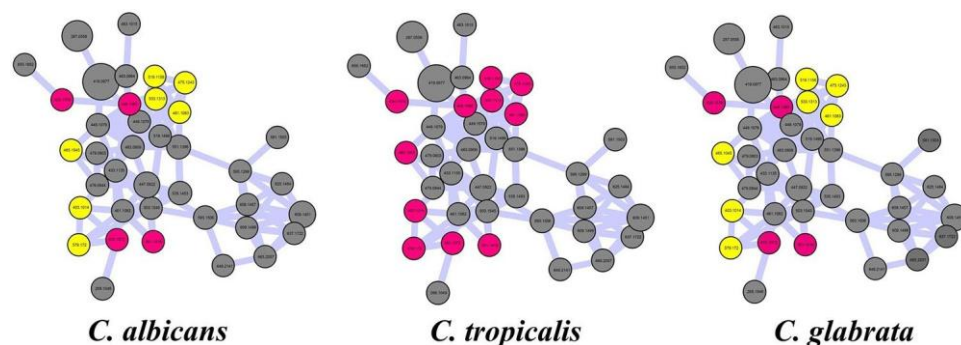


Fig. 3 Representation of molecular networking filtered by MIC values against *C. albicans*, *C. tropicalis*, and *C. glabrata*. The node size represents the intensity of the precursor ion and width of edge is associated with cosine similarity, (similarity degree among nodes), the color chart reflects MIC values against the *Candida* strains, to differentiate

Possible mechanisms of antifungal action have been reported for some classes of phenolic compounds. Regarding flavonoids, the mechanisms of action of their antifungal effects include inhibition of the efflux pump and induction of apoptosis (cell death), cell wall damage, and cytoplasmic membrane disruption. Additionally, flavonoids may inhibit the development, growth, and formation of *Candida* species colonies (Sung & Lee, 2010). The mechanism of action of phenolic acids against *Candida* species has been suggested to include interference with the fungus by rupturing the cytoplasmic membrane, thus altering membrane permeability (Silva et al., 2021; Sung & Lee, 2010).

Thus, the most active fractions obtained from *L. trifolia* leaves and fruits are rich in phenolic compounds that can act against *Candida* species, on different targets.

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) data

Molecular networking analysis pointed out the major component of fraction FrPo *n*But, and we identified this compound as verbascoside (**10**), a phenylethanoid glycoside (Supplementary Material, Figures S19–S27 and Table S11), by ^1H NMR and (+)-ESI-MS analysis of the crude fraction. The anomeric hydrogens at δ 4.38 d ($J = 7.9$ Hz; H-1'') and δ 5.19 d ($J = 1.6$ Hz, H-1''') confirmed the presence of glucose and rhamnose moieties, respectively. The signals at δ_{H} 7.59d ($J = 16.0$ Hz) and δ_{H} 6.28 d ($J = 16.0$ Hz) were typical of the caffeoyl group double bond between H-7' and H-8'. The signals at δ_{H} 6.70 d ($J = 2.0$ Hz, H-2), δ_{H} 6.56 dd ($J = 8.0$, 2.0 Hz, H-6), and δ_{H} 6.68 d ($J = 8.0$ Hz, H-5) evidenced the trisubstituted aromatic ring of the verbascoside aglycone. The (+)-ESI-MS spectrum of this compound revealed a molecular ion peak $[\text{M} + \text{H}]^+$ at m/z 625.2125 (calcd for $\text{C}_{29}\text{H}_{37}\text{O}_{15}$, 625.2127 ($\Delta -0.3$ ppm), which corresponded to the molecular formula $\text{C}_{29}\text{H}_{36}\text{O}_{15}$ of the verbascoside

the active nodes (pink – $\text{MIC} < 100 \mu\text{g mL}^{-1}$) from the moderately active and inactive nodes (grey – $\text{MIC} > 100 \mu\text{g mL}^{-1}$), and yellow for highlight the peaks (substances) with high inhibitory activity ($\text{MIC} < 100 \mu\text{g mL}^{-1}$) that were present in various fractions

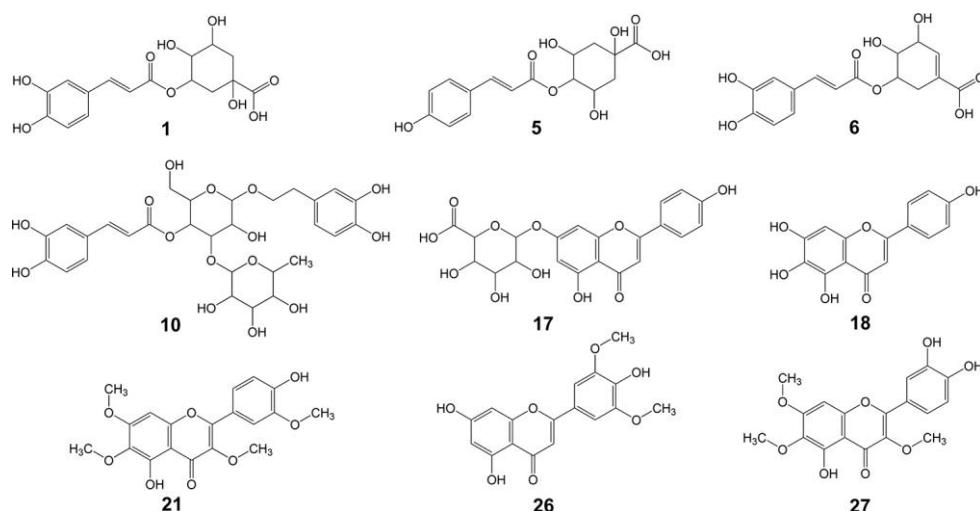
Table 2 Molecular networking annotations of compounds in <i>Lantana trifolia</i> fractions analyzed by UFLC-DAD-(+)-ESI-MS/MS (microTOFQII).							
Number	t_R (min)	$[M+H]^+$ (m/z)	Error ^c (ppm)	cosine	Molecular Formula	GNPS Annotations	References
1a	6.68	355.1039	4.2	0.92	$C_{16}H_{18}O_9$	Chlorogenic acid ^d	GNPS Library Spectrum CCMSLIB00005778073
1b	8.83	355.1034	2.8	0.97	$C_{16}H_{18}O_9$	Chlorogenic acid ^d	GNPS Library Spectrum CCMSLIB00005778073
2a	8.35	595.1662	0.8	0.86	$C_{27}H_{30}O_{15}$	Vicenin II ^d	GNPS Library Spectrum CCMSLIB00000845378
2b	9.01	595.1664	1.2	0.80	$C_{27}H_{30}O_{15}$	Vicenin II ^d	GNPS Library Spectrum CCMSLIB00000845378
3	9.45	611.1613	1.0	0.76	$C_{27}H_{30}O_{16}$	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-6-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-8-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one	GNPS Library Spectrum CCMSLIB00005745962
4a	10.39	565.1542	-1.8	0.79	$C_{26}H_{28}O_{14}$	Vicenin I ^d	GNPS Library Spectrum CCMSLIB00005721165
4b	11.37	565.1527	-4.4	0.80	$C_{26}H_{28}O_{14}$	Vicenin I ^d	GNPS Library Spectrum CCMSLIB00005721165
5	10.82	339.1081	2.1	0.87	$C_{16}H_{18}O_8$	4- <i>p</i> -coumaroylquinic acid	GNPS Library Spectrum CCMSLIB00000854975
6	11.18	337.0927	2.7	0.93	$C_{16}H_{16}O_8$	5-caffeoylshikimic acid	GNPS Library Spectrum CCMSLIB00000848306
7	11.47	581.1503	0	0.87	$C_{26}H_{28}O_{15}$	2-(3,4-dihydroxyphenyl)-5-hydroxy-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[(3,4,5-trihydroxyoxan-2-yl)oxymethyl]oxan-2-yl]oxychromen-4-one	GNPS Library Spectrum CCMSLIB00000845835
8	12.19	479.0803	-3.5	0.84	$C_{21}H_{18}O_{13}$	Quercetin 3- <i>O</i> -glucuronide	GNPS Library Spectrum CCMSLIB00000845058
9a	12.95	479.1542	-1.3	0.82	$C_{23}H_{26}O_{11}$	Calceolarioside B ^d	GNPS Library Spectrum CCMSLIB00000848884
9b	15.01	479.1550	0.4	0.85	$C_{23}H_{26}O_{11}$	Calceolarioside B ^d	GNPS Library Spectrum CCMSLIB00000848884
10a	14.11	625.2123	-0.63	0.87	$C_{29}H_{36}O_{15}$	Verbascoside ^d	GNPS Library Spectrum CCMSLIB00005716581
10b	15.01	625.2123	-0.63	0.84	$C_{29}H_{36}O_{15}$	Verbascoside ^d	GNPS Library Spectrum CCMSLIB00005716581
11	13.01	369.1197	4.6	0.90	$C_{17}H_{20}O_9$	Chlorogenic acid methyl ester	GNPS Library Spectrum CCMSLIB00000847734
12	13.24	435.1353	15.4	0.72	$C_{21}H_{22}O_{10}$	6- <i>O</i> -Caffeoylarbutin	GNPS Library Spectrum CCMSLIB00000846362
13	13.56	433.1132	0.7	0.96	$C_{21}H_{20}O_{10}$	Isovitexin	GNPS Library Spectrum CCMSLIB00000578385
14	15.60	579.1720	2.1	0.95	$C_{27}H_{30}O_{14}$	Apigenin-7- <i>O</i> -neohesperidoside	GNPS Library Spectrum CCMSLIB00000213886
15	15.92	449.1093	3.3	0.96	$C_{21}H_{20}O_{11}$	Kuromanin	GNPS Library Spectrum CCMSLIB00000408903
16	15.97	433.1135	1.38	0.97	$C_{21}H_{21}O_{10}^+$	Callistephin	GNPS Library Spectrum CCMSLIB00000409184
17	16.15	447.0922	0	0.93	$C_{21}H_{18}O_{11}$	Apigenin 7-glucuronide	GNPS Library Spectrum CCMSLIB00000848637
18	18.13	287.0558	2.8	0.91	$C_{15}H_{10}O_6$	Scutellarein	GNPS Library Spectrum CCMSLIB00000258374
19	18.25	317.0659	0.9	0.85	$C_{16}H_{12}O_7$	3- <i>O</i> -Methylquercetin	GNPS Library Spectrum CCMSLIB00000231342
20	18.61	653.2430	-1.5	0.76	$C_{31}H_{40}O_{15}$	Martinoside	GNPS Library Spectrum CCMSLIB00000848397
21	19.33	375.1092	4.8	0.85	$C_{19}H_{18}O_8$	Chysosplenitin B	GNPS Library Spectrum CCMSLIB00006525506
22	19.48	345.0974	1.4	0.81	$C_{18}H_{16}O_7$	5,3'-Dihydroxy-6,7,4'-trimethoxyflavone	GNPS Library Spectrum CCMSLIB00000311035

Table 2 (continued)

Number	t_R (min)	$[M+H]^+$ (m/z)	Error ^c (ppm)	cosine	Molecular Formula	GNPS Annotations	References
23	20.21	287.0560	3.5	0.94	C ₁₅ H ₁₀ O ₆	Luteolin	GNPS Library Spectrum CCMSLIB00000578417
24	20.61	317.0667	3.5	0.89	C ₁₆ H ₁₂ O ₇	6-methoxyluteolin	GNPS Library Spectrum CCMSLIB00000231342
25	20.98	301.0714	2.3	0.90	C ₁₆ H ₁₂ O ₆	Chrysoeriol	GNPS Library Spectrum CCMSLIB00000257836
26	21.84	331.0819	2.1	0.83	C ₁₇ H ₁₄ O ₇	Tricin	GNPS Library Spectrum CCMSLIB00005778067
27	23.15	361.0927	2.5	0.77	C ₁₈ H ₁₆ O ₈	Chrysosplenol D	GNPS Library Spectrum CCMSLIB00000848806
28	24.29	331.0825	3.9	0.87	C ₁₇ H ₁₄ O ₇	Cirsiliol	GNPS Library Spectrum CCMSLIB00000847486
29	31.65	315.0869	1.9	0.85	C ₁₇ H ₁₄ O ₆	Velutin	GNPS Library Spectrum CCMSLIB00000213961

^c Relative error, ° Represent different stereoisomeric forms (e.g., catechin, which may be catechin or *epicatechin*)

Fig. 4 Structures of the candidates potentially active determined by PLS-DA analysis and annotated using molecular networking



(Figure S37, Supplementary Material). The verbascoside chemical shifts, coupling constants, and MS data agreed with literature data (Silva et al., 2021). The ¹H NMR (Figure S27, Supplementary Material) highlights the signals corresponding to other phenylethanoid glycosides, which have almost the same pattern as the verbascoside aglycone and caffeoyl signals.

Conclusion

The fractions extracted from the *Lantana trifolia* showed significant activity against three *Candida* strains tested, with minimum inhibitory concentration (MIC) values ranging from 1.46 to 93.75 µg mL⁻¹. In particular, FrPo AcOEt (1.46 µg mL⁻¹) and FrPe AcOEt (2.93 µg mL⁻¹) exhibited MIC values similar to the positive control, amphotericin B (0.12 µg mL⁻¹), against *C. glabrata*. Furthermore, all tested *Candida* strains demonstrated higher positive Selectivity

Index values with these fractions, indicating their remarkable antifungal potential and selectivity. Additionally, UFLC-DAD-(+)-ESI-MS/MS (microTOFQII) was employed to analyze the *L. trifolia* fractions, and molecular networking and mass fragmentation patterns were used to annotate their metabolites. The correlation between secondary metabolites and biological activity was determined by PLS-DA, indicating that flavonoids may be the main responsible for the antifungal activity (Aboody & Mickymaray, 2020).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11306-023-02032-8>.

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Author contributions GBV: Design of the study and collected the plant material. GBV and TRF: data collection, performed the extraction, analysis and interpretation of the data. GBV, CCG, ACP and LGN: data collection, performed the LC-MS/MS analyses, processed and analyzed the MS/MS data, statistical analysis, networking and interpretation of the data. DOSM and ACGJ: Performed cytotoxicity assay and interpretation of the data. MBS, CHGM and LCSC: data collection, performed antifungal assay and interpretation of the data. AC: performed the NMR analysis, processed and analyzed data, networking and interpretation of the data. MP and AD: Design of the study, data collection, analysis and interpretation of the data, inspected the results. All authors wrote, reviewed, and approved the manuscript.

Declarations

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

Competing interests The authors declare no competing interests.

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Article

The Synthetic Peptide GA-Hecate and Its Analogs Inhibit Multiple Steps of the Chikungunya Virus Infection Cycle In Vitro

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Abstract: Chikungunya virus (CHIKV) belongs to the *Alphavirus* genus and is responsible for significant outbreaks worldwide. Currently, there is no approved antiviral therapy against CHIKV. Bioactive peptides have great potential for new drug development. Here, we evaluated the antiviral activity of the synthetic peptide GA-Hecate and its analogs PSSct1905 and PSSct1910 against CHIKV infection. Initial screening showed that all three peptides inhibited the CHIKV replication cycle in baby hamster kidney fibroblast cells (BHK-21) and human hepatocarcinoma epithelial cells (Huh-7). GA-Hecate and its analog PSSct1905 were the most active, demonstrating suppression of viral infection by more than 91%. The analog PSSct1905 exhibited a protective effect in cells against CHIKV infection. We also observed that the analogs PSSct1905 and PSSct1910 affected CHIKV entry into both cell lines, inhibiting viral attachment and internalization. Finally, all tested compounds presented antiviral activity on the post-entry steps of CHIKV infection in all cells evaluated. In conclusion, this study highlights the potential of the peptide GA-Hecate and its analogs as novel anti-CHIKV compounds targeting different stages of the viral replication cycle, warranting the development of GA-Hecate-based compounds with broad antiviral activity.

Keywords: chikungunya; peptides; analogs; antiviral

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Introduction

Chikungunya virus (CHIKV) is an arbovirus that belongs to the genus *Alphavirus* (family *Togaviridae*). The virions have a single-stranded and positive-sense RNA genome of approximately 11.5 kb in size, which is polyadenylated at the 3' end and has a 7-methylguanosine cap at the 5' end. The genome contains two open reading frames (ORFs): (1) a 5' ORF that encodes a nonstructural polyprotein that is processed into four nonstructural proteins (nsP1, 2, 3, and 4), which are parts of a viral replication complex [1,2], and (2) a 3' ORF encoding a polyprotein that is processed into five main structural proteins (C, E1, E2, E3, and 6 K) [3,4]. The protein nsP1 demonstrates RNA capping activities and



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participates in the formation of the viral RNA negative strand; nsP2 triggers blocking of the host transcription process and exhibits RNA helicase, RNA triphosphatase, and proteinase activities; nsP3 recruits RNA-binding proteins that play a role in the formation of CHIKV replication complexes; and nsP4 has RNA-dependent RNA polymerase activity [2,5]. Alphavirus RNA synthesis occurs in replication complexes located in proximity to the plasma membrane [2]. Nonstructural proteins are part of these replication complexes. This complex is responsible for the formation of a full-length negative-strand RNA intermediate, which is used as a template for the production of the positive strand of sub-genomic and genomic RNAs.

The genetic material of CHIKV is surrounded by a capsid (240 copies of C) with icosahedral symmetry, forming the viral nucleocapsid. The CHIKV particle has an envelope derived from the plasma membrane of host cells, to which 80 spikes formed by trimers of glycoproteins E1 and E2 are anchored [6,7]. The viral envelope originates from host cell membranes and contains lipid rafts, sphingolipids, phosphatidylserine, and cholesterol, which provide an amphipathic character and negative charge. CHIKV entry into cells is mediated by the viral glycoproteins E2 and E1 through several attachment factors—namely, heparan sulfate and phosphatidylserine receptors (TIM1 [8], TIM4, and AXL [9])—and receptors such as Mxra8 [10]. The viral glycoprotein E2 is responsible for interacting with cell receptors, including Mxra8, the main receptor for several arthritogenic alphaviruses, including CHIKV [10,11]. The CHIKV cell receptor is ubiquitously expressed in several species and cell types [12,13]. Importantly, the binding of CHIKV virions is facilitated by several other cellular components. Glycosaminoglycans (GAGs), prohibitin (PHB), and phosphatidylserine (PtdSer)-mediated virus entry-enhancing receptors (PVEERs) are cited in the literature as factors of CHIKV attachment in mammalian cells [14–16]. CHIKV enters cells by clathrin-mediated endocytosis. Subsequently, the fusion of the viral and endosomal membranes occurs in a low-pH-dependent manner [17].

CHIKV was first reported during an outbreak that occurred in Tanzania in 1953. Currently, this arbovirus is fully adapted to the urban transmission cycle, raising concern in many tropical and temperate regions [18]. Although CHIKV continues to cause significant outbreaks around the world to date, there are no specific antiviral treatments or licensed vaccines against this viral infection [19]. Several antiviral strategies against CHIKV are being evaluated, mainly in vitro, that can affect critical steps of the viral replication cycle, such as binding and entry, viral genome replication, functionality of viral proteins, virion formation, and infectivity [20–24].

Antiviral peptides that interact with virus particles or target other critical steps of viral infection could potentially be used as treatment or prophylaxis for virus infection [25,26]. Most antiviral peptides are short (10 to 50 amino acid residues), have a positive charge, and have both hydrophilic and hydrophobic characteristics [27,28]. These peptides usually act at different steps of the virus replication cycle [26]. For example, the peptide (p-BthTX-I)₂K derived from *Bothrops jararacussu* snake impaired CHIKV entry into baby hamster kidney fibroblast cells by inhibiting the attachment and internalization steps. Additionally, the same peptide also inhibited Zika virus (ZIKV) infection by interfering with the post-entry stages of the viral replication cycle, reducing the viral protein NS3, which is essential for ZIKV replication [29]. LL-37, a peptide of the Cathelicidin family, impacts the early processes of Venezuelan equine encephalitis virus (VEEV) infection [30]. Two synthetic peptides, A2 and A3, inhibited replication of VEEV and eastern equine encephalitis virus (EEEV) in vitro, and both peptides, inhibited VEEV attachment to cells [31]. Surfactin, a cyclic lipopeptide, significantly reduced the infectivity of CHIKV, Mayaro virus, and Una virus in vitro [32]. Antiviral peptides have other mechanisms of action, such as disruption of the virus envelope, blocking release in host cells, and total or partial inhibition of viral particle assembly [21,25,28].

Different studies have demonstrated that peptide conjugation is a relevant strategy for the development of new substances and increasing their biological activity [25,33,34]. The peptide Hecate (FALALKALKKKLKKALKKKAL-CONH₂) is a synthetic peptide that has a net positive charge and amphipathic characteristics, exhibiting many biological activities, with emphasis on antiviral and antitumor activity [35,36]. Gallic acid (GA) (C₇H₆O₅) is a phenolic compound found in abundance in plants that exhibits antiviral, antibacterial, and antitumor activities [37,38]. Our group has evaluated the anti-hepatitis C virus (HCV) activity of five analogs of Hecate in human hepatocellular carcinoma-derived Huh-7.5 cells. The peptide conjugated to GA (GA-Hecate) represented the most active compound, inhibiting the main stages of the HCV replication cycle. Thus, the antiviral properties of GA-Hecate encourage its use as a prototype for the development of new antivirals [35].

This study aimed to evaluate the potential of GA-Hecate and its analogs PSSct1905 (GA-FALALKALKKKL-COOH) and PSSct1910 (4-(dimethylamino)-benzoic acid-FALALKALKKKLKKALKKKAL-CONH₂) as antivirals against CHIKV. The study was performed by testing the effects of these peptides on different steps of the CHIKV infectious cycle.

Results and Discussion

Cytotoxicity of the Peptides

The structures of the peptide GA-Hecate and its analogs PSSct1905 and PSSct1910 are presented in Figure 1. The physicochemical properties and biological activities of these peptides are summarized in Table 1. The cytotoxicity of the peptides GA-Hecate, PSSct1905, and PSSct1910 was evaluated in baby hamster kidney fibroblast cells (BHK-21) and human hepatocarcinoma epithelial cells (Huh-7). We defined the maximum nontoxic concentration (MNTC), expressed in μ M, as the highest peptide concentration that kept at least 80% of BHK-21 and Huh-7 cells viable within 24 h.

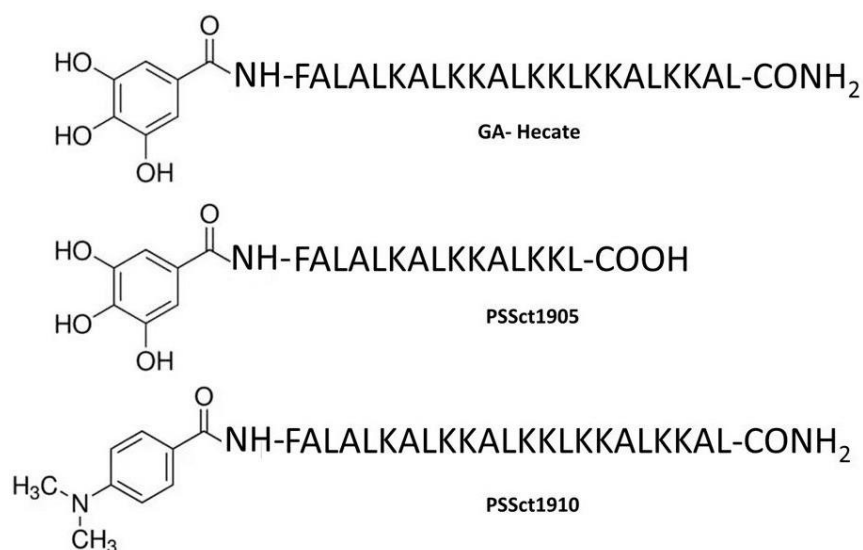


Figure 1. Structures of the peptide GA-Hecate and its analogs PSSct1905 and PSSct1910.

All peptides had higher 50% cytotoxic concentration (CC_{50}) values for Huh-7 cells than for BHK-21 cells. The MNTC of GA-Hecate and its analog PSSct1905 was 12.5 μ M or higher in these cell types (Figure S1A,B,D,E). Compared to GA-Hecate, the peptide PSSct1905 lacks the last eight amino acids in the C-terminus, a modification made on the basis of a previous observation that these amino acids were susceptible to degradation by blood proteases, decreasing the stability of the compound. Based on our analysis, this modification resulted in a modest (approximately 2-fold) increase in the CC_{50} value of the compound; the difference was more prominent in BHK-21 cells. The analog PSSct1910 was considerably more cytotoxic, with CC_{50} values of 5.4 μ M and 7.7 μ M for BHK-21 and Huh-7 cells, respectively. For both cell lines, the MNTC was 1.6 μ M (Figure S1C,F). These data indicate that the only structural change that promoted higher cytotoxicity was the replacement of GA by the (4-(dimethylamino)-benzoic acid) subunit at the N-terminal end of the peptide GA-Hecate. Additional studies of the structure-activity relationship should be performed to better understand the role of these modifications in the cytotoxic effects of these compounds.

Table 1. General characteristics and biological properties for the cationic peptides GA-Hecate, PSSct1905, and PSSct1910. MW: molecular weight. MNTC: maximum nontoxic concentration. CC_{50} : 50% cytotoxic concentration. EC_{50} : 50% effective concentration. SI: selectivity index.

BHK-21 Cells								Huh-7 Cells		
Peptide	Length	MW Charge	Net	MNTC (μ M)	CC_{50} (μ M)	EC_{50} (μ M)	SI	CC_{50} (μ M)	EC_{50} (μ M)	SI
GA-Hecate	23	2688.43	+9	12.5	23.3	5.7	4.1	221	4.5	49.1
PSSct1905	15	1808.25	+4	12.5	56.5	8.4	6.7	205	7.1	28.9
PSSct1910	23	2684.17	+9	1.6	5.4	1.1	4.9	7.7	0.2	38.5

The Peptides Strongly Impaired the Replication Cycle of CHIKV

The peptides GA-Hecate, PSSct1905, and PSSct1910 were tested at the MNTC in BHK-21 and Huh-7 cells infected with CHIKV-expressing *nanoluciferase* reporter (CHIKV-NLuc) [multiplicity of infection (MOI) 0.1] for 16 h. All three peptides exhibited significant antiviral effects against the replication cycle of CHIKV. GA-Hecate was the most active, causing inhibition of viral replication of 97.7% ($p = 0.0001$) in BHK-21 cells and 93.6% ($p = 0.0001$) in Huh-7 cells when compared to the control (Figure 2A,D). Among the analogs, PSSct1905 also showed a high level of inhibition of the CHIKV replicative cycle; the effect was slightly more prominent in Huh-7 cells (97.3% inhibition; $p = 0.0001$) than in BHK-21 cells (91.7% inhibition; $p = 0.0001$) (Figure 2B,E). Similar activities imply that the removal of KKALKKKAL amino acids from the C-terminal end of GA-Hecate has minimal effect on the antiviral activity of the compound. On the other hand, the replacement of the GA by the group with (4-(dimethylamino)-benzoic acid at the N-terminal end of the peptide GA-Hecate negatively impacted its inhibitory potential; peptide PSSct1910, harboring such a modification, resulted in 67.2% inhibition ($p = 0.0001$) of CHIKV replication in BHK-21 cells and 87.8% inhibition ($p = 0.0001$) in Huh-7 cells (Figure 2C,F). However, it should be noted that, due to its increased cytotoxicity, this compound was also used at a much lower concentration than the other two inhibitors. Indeed, PSSct1910 treatment resulted in the lowest 50% effective concentration (EC_{50}) values for CHIKV inhibition, with a higher inhibitory effect (approximately 5-fold) in Huh-7 cells ($EC_{50} = 0.2 \mu\text{M}$) than in BHK-21 cells ($EC_{50} = 1.1 \mu\text{M}$). Thus, it may still be an efficient inhibitor. All peptides had a higher selectivity index (SI) in Huh-7 cells than in BHK-21 cells. GA-Hecate was the most selective in Huh-7 cells, resulting in an SI value of 49.1.

The peptide Hecate can bind with lipids, disrupting lipid bilayers [39]. Hecate's ability to bind with lipids can trigger changes in the fluidity of the virus envelope, cause disturbances in viral envelope formation, and interfere with viral fusion and/or egress in host cells [40]. Indeed, Hecate showed antiviral activity against an unrelated enveloped virus, herpes simplex virus (HSV-1), infection in African green monkey kidney cells (Vero) by inhibiting cell fusion [41]. GA also inhibited the replication cycle of HSV in Vero cells [42]. It has also been demonstrated that this compound significantly reduced the infection of o'nyong-nyong virus (ONNV), an alphavirus closely related to CHIKV, in human skin fibroblast (HSF) cells [43]. GA exhibits antiviral activity through different mechanisms of action, such as virucidal and protective effects and interference with virus entry and replication in host cells [37,44,45]. GA-Hecate inhibited the efficiency of multiple steps in the HCV replication cycle in Huh-7.5 cells [35]. These data indicate that GA-Hecate and its analogs may impair the CHIKV replication cycle due to interference with more than one step of the CHIKV infection cycle. For this reason, we decided to evaluate which phase of the CHIKV replication cycle these peptides could inhibit.

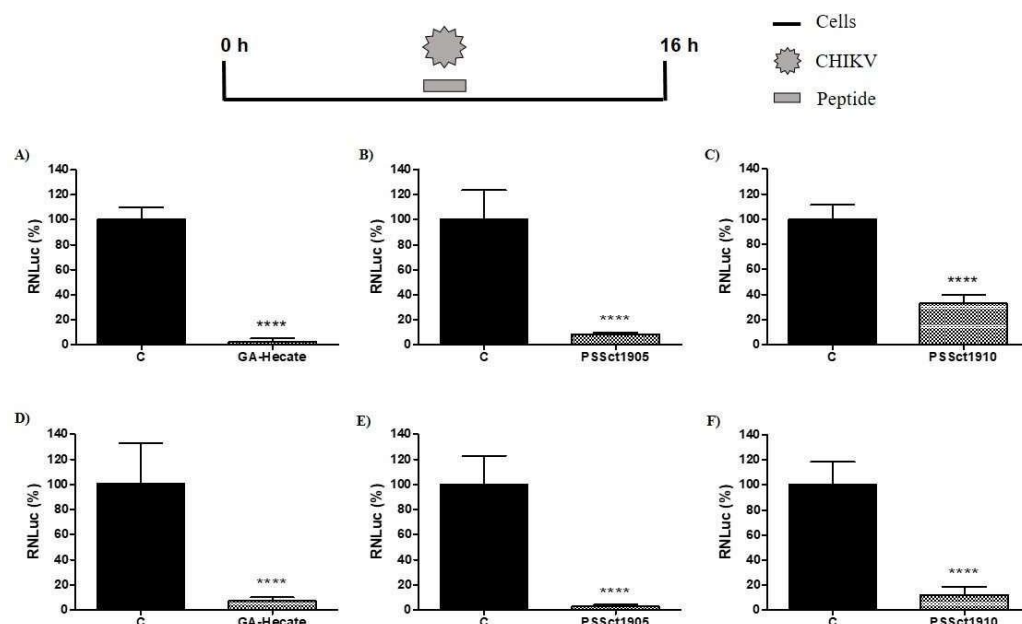


Figure 2. Inhibitory effect of the peptides on the CHIKV replication cycle in BHK-21 and Huh-7 cells. The percentages were expressed in relation to protein NanoLuciferase (NLuc) activity in cells treated with the control (C, sterile water). Luminescence signals were measured in three independent experiments, each performed in quadruplicate, and data are presented as the mean $\pm$ standard deviation (SD). (A) GA-Hecate at the MNTC (12.5 μM) in BHK-21 cells. (B) PSSct1905 at the MNTC (12.5 μM) in BHK-21 cells. (C) PSSct1910 at the MNTC (1.6 μM) in BHK-21 cells. (D) GA-Hecate at the MNTC (12.5 μM) in Huh-7 cells. (E) PSSct1905 at the MNTC (12.5 μM) in Huh-7 cells. (F) PSSct1910 at the MNTC (1.6 μM) in Huh-7 cells. ****: $p \leq 0.0001$.

Antiviral Effect of Pretreatment with the Peptides against CHIKV

To evaluate the protective effect of pretreatment of cells against CHIKV infection, BHK-21 and Huh-7 cells were treated with the peptides at the MNTC for 1 h, washed with PBS (phosphate-buffered saline), and infected with CHIKV-NLuc (MOI 0.1).

Pretreatment of cells with GA-Hecate did not result in detectable protection against CHIKV infection (Figure 3A,D), while its analog PSSct1905 was able to significantly protect BHK-21 and Huh-7 cells, reducing CHIKV infection by 65.4% (p 0.0001) and 50.6% (p 0.001), respectively (Figure 3B,E). Pretreatment with PSSct1910 had a protective effect against CHIKV in Huh-7 cells (inhibition by 46.1%; p 0.01) but not in BHK-21 cells (Figure 3C,F). Taken together, pretreatment of cells with peptides revealed that protection of cells by prophylactic treatment is not the main mechanism of action of these antiviral peptides. Nevertheless, some modest antiviral activity was observed. CHIKV infection starts with the binding of virus particles to the cellular surface, triggering attachment, and internalization [46,47]. All tested peptides also have positive charges, and, probably, in the pretreatment, the positive charges facilitate their binding to the cell. This binding may inhibit the interaction between the E2 glycoprotein and molecules on the cell surface and, consequently, the CHIKV attachment and internalization in the host cell. However, the positive charge of peptides alone could not explain all observed effects, as GA-Hecate is also positively charged yet offers no protection. It is plausible that the size difference between this peptide and the analog PSSct1905 may be responsible for different interactions with CHIKV attachment factors/receptors and these differences alter the ability of the compound to protect cells against infection.

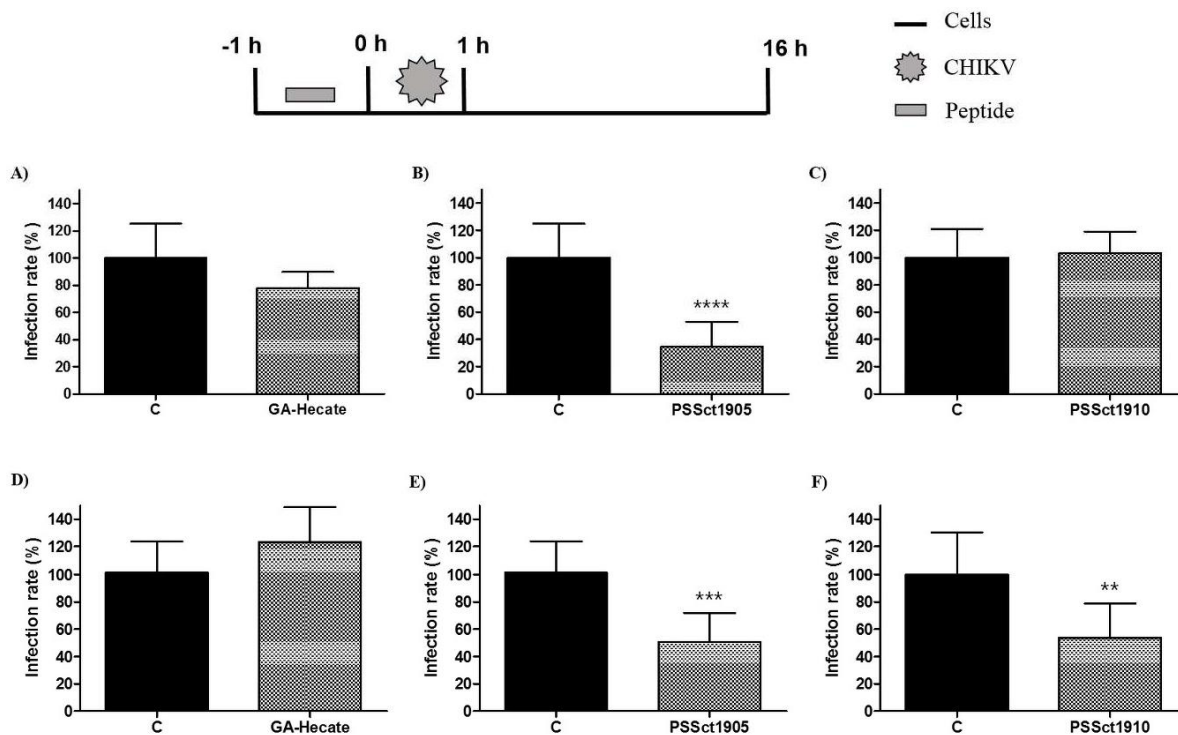


Figure 3. Protective effect of the peptides against CHIKV infection. Data were analyzed as described in Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean $\pm$ SD. (A) GA-Hecate in BHK-21 cells. (B) PSSct1905 in BHK-21 cells. (C) PSSct1910 in BHK-21 cells. (D) GA-Hecate in Huh-7 cells. (E) PSSct1905 in Huh-7 cells. (F) PSSct1910 in Huh-7 cells. C: sterile water. **: $p \leq 0.01$, ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

Virucidal Effect of the Peptides on CHIKV Virions

To investigate the ability of peptides GA-Hecate, PSSct1905, and PSSct1910 to inactivate extracellular CHIKV virions, the virus particles were treated with the peptides at the MNTC for 1 h and used for infection of BHK-21 and Huh-7 cells. In this assay, the peptides GA-Hecate and PSSct1905 reduced infection in BHK-21 cells, showing inhibitory effects of 59.4% (p 0.0001) and 21.4% (p 0.0001), respectively (Figure 4A,B). In Huh-7 cells, GA-Hecate did not inhibit viral infection (Figure 4D), while treatment with PSSct1905 resulted in 68% (p 0.0001) inhibition (Figure 4E). The peptide PSSct1910, on the other hand, only presented virucidal activity when treated virions were used to infect Huh-7 cells (80.2% inhibition; p 0.0001) (Figure 4F).

The peptides used are positively charged and amphipathic; these properties may play important roles in their activity against enveloped particles, such as the virions of alphaviruses. Amphipathic cationic peptides can interact with this virus envelope and produce a direct virucidal effect, resulting in interference with virus binding and/or fusion [48]. Similarly, a study by Ahmed and collaborators observed that a cationic peptide can interfere with virus binding by causing the aggregation of extracellular virions [30]. Therefore, it is plausible that the peptides used caused the inactivation of CHIKV virions by disrupting the viral envelope. Indeed, it was observed by Batista and coworkers that the peptide GA-Hecate was capable of generating disruption of the HCV viral mimetic envelope [35]. Interestingly, however, in this study, the impact of virion treatment clearly depended on the cell line that was infected with peptide-treated particles; for GA-Hecate, no effect

of peptide treatment was observed upon infection of Huh-7 cells, while treatment with PSSct1905 or PSSct1910 had no or minimal impact on the ability of CHIKV virions to infect BHK-21 cells. These data argue against the possibility of general destruction (or aggregation) of virions and are more consistent with milder peptide-induced damage. If so, the difference in the expression of receptors on the BHK-21 and Huh-7 cell surfaces and their affinity toward CHIKV could explain this difference. Furthermore, the experimental setup included 1 h of incubation of cells with inoculum containing both virus particles and peptides. Thus, it is possible that in the presence of peptides, the binding of virions to cells or their entry was also impaired in a cell-type-dependent manner. Therefore, the impact of peptides on these steps of the virus infection cycle was subsequently analyzed.

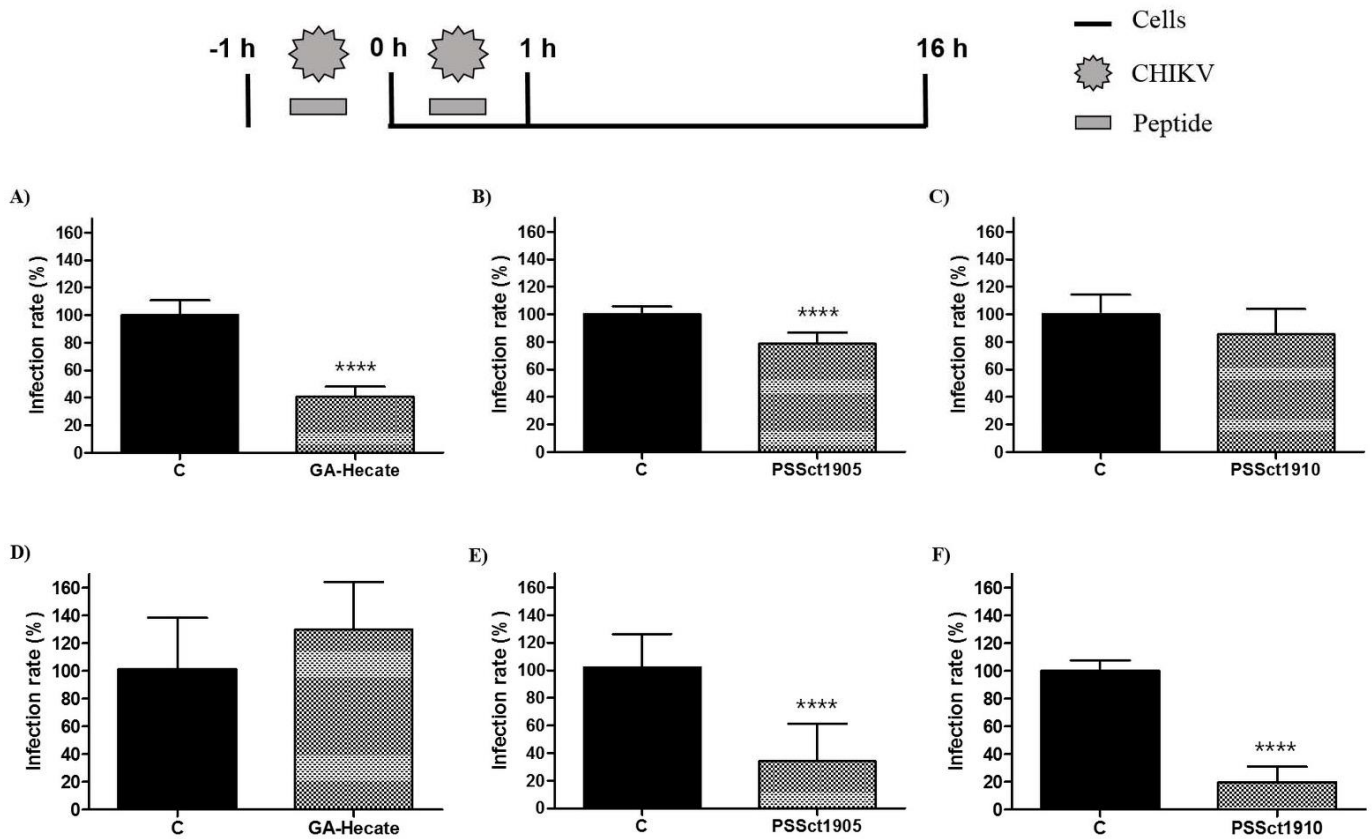


Figure 4. Effect of the peptides-treated CHIKV virions on BHK-21 and Huh-7 cells. Data were analyzed as described in Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean $\pm$ SD. (A) GA- Hecate in BHK-21 cells. (B) PSSct1905 in BHK-21 cells. (C) PSSct1910 in BHK-21 cells. (D) GA-Hecate in Huh-7 cells. (E) PSSct1905 in Huh-7 cells. (F) PSSct1910 in Huh-7 cells. C: sterile water. ****: $p \leq 0.0001$.

Antiviral Activity of the Peptides on the CHIKV Entry Steps

To evaluate the action of the peptides in the early steps of the CHIKV replication cycle, BHK-21 and Huh-7 cells were infected with CHIKV-NLuc at an MOI of 0.1 in the presence of the MNTC of GA-Hecate, PSSct1905, or PSSct1910 for 1 h, washed with PBS, and incubated with peptide-free culture medium. In this experiment, all peptides inhibited CHIKV entry into BHK-21 cells. The effect was strongest for GA-Hecate and PSSct1905, for which 77.2% ($p = 0.0001$) and 76.4% ($p = 0.0001$) inhibition were observed, respectively (Figure 5A,B). PSSct1910 was somewhat less potent, with an inhibition rate of 54.1% ($p = 0.001$) compared to the control (Figure 5C). Curiously, this trend is the same as that observed in the previous experiment (Figure 4A–C); the different inhibition rates observed in these experiments can be attributed to the 50-fold higher MOI used in the previous experiment, which makes the assay less sensitive. In Huh-7 cells, GA-Hecate failed to inhibit the early steps of CHIKV infection; instead, significant activation was observed (Figure 5D). At the same time, PSSct1905 and PSSct1910 reduced the efficiency of the entry stages of CHIKV infection and resulted in 84% ($p \leq 0.0001$) and 89.5% ($p \leq 0.0001$) inhibition, respectively (Figure 5D–F). Again, the trend is similar to that observed in the previous experiment (Figure 4D–F). Combined with data obtained for BHK-21 cells, these results strongly indicate that antiviral activities observed in the previous experiment were mostly, if not exclusively, due to inhibition of early stages of CHIKV infection. Hence, it is unclear whether the peptides have virucidal effects. In order to prove the existence or absence of virucidal properties, additional experiments would be needed.

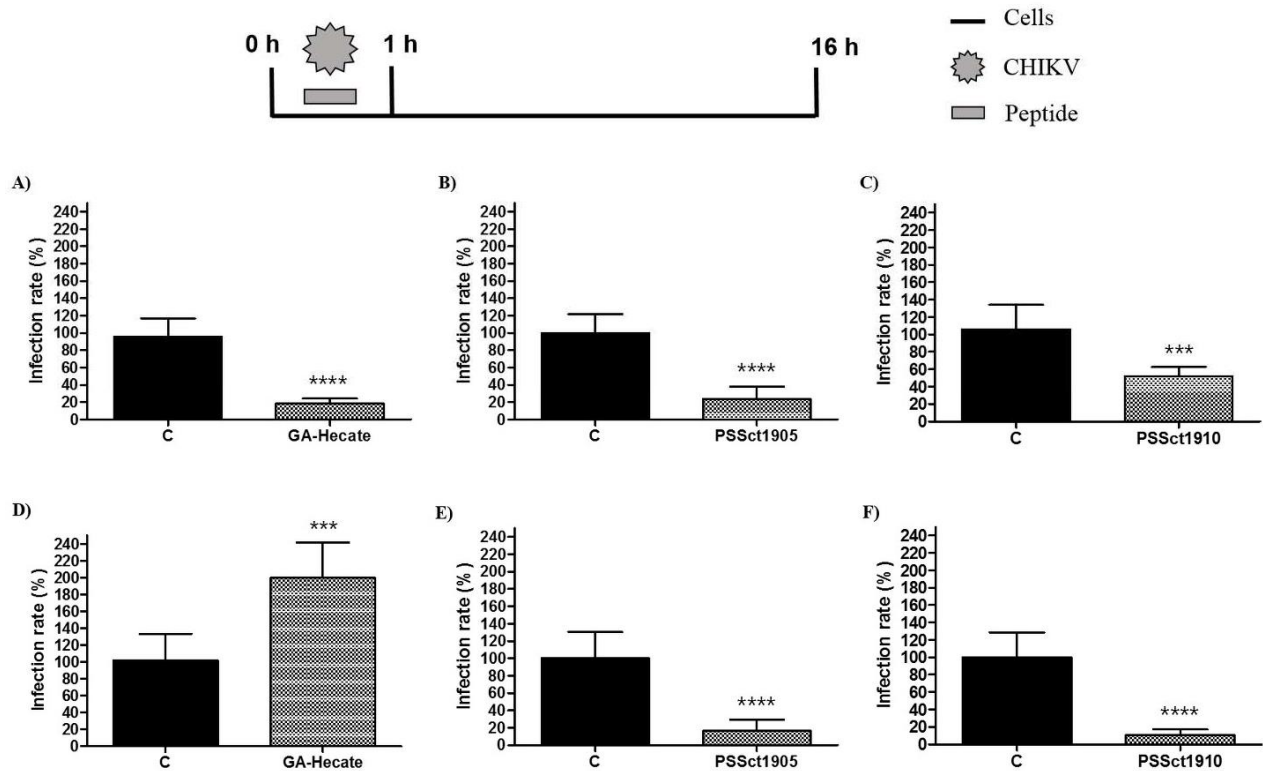


Figure 5. Effect of the peptides on CHIKV entry. Data were analyzed as described in Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean $\pm$ SD. (A) GA-Hecate in BHK-21 cells. (B) PSSct1905 in BHK-21 cells. (C) PSSct1910 in BHK-21 cells. (D) GA-Hecate in Huh-7 cells. (E) PSSct1905 in Huh-7 cells. (F) PSSct1910 in Huh-7 cells. C: sterile water. ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

Antiviral Effect of the Peptides on CHIKV Attachment

To reveal which processes involved in the entry of CHIKV infection were affected by the peptides, we first investigated their activity on CHIKV attachment. In this experiment, BHK-21 and Huh-7 cells were incubated with peptides at the MNTC and CHIKV-NLuc in amounts corresponding to infection at an MOI of 0.1 at 4 °C. At this temperature, virions interact with cell membrane receptors, but the viral particle is not able to enter the cell [49]. Subsequently, the cells were washed with PBS and incubated with a culture medium to continue the viral entry process. As GA-Hecate was unable to inhibit CHIKV entry into Huh-7 cells (Figure 5D), this combination was excluded from this analysis.

Despite demonstrating an antiviral effect on the entry of CHIKV into BHK-21 cells, the peptide GA-Hecate did not inhibit the viral attachment step (Figure 6A). These data indicate that the peptide GA-Hecate probably inhibits viral entry by interfering with the internalization of CHIKV in BHK-21 cells. In contrast, PSSct1905 and PSSct1910 inhibited the attachment of CHIKV virions to BHK-21 and Huh-7 cells. In BHK-21 cells, treatment with PSSct1910 exhibited less prominent antiviral activity (59.9% inhibition; $p = 0.01$) than PSSct1905, which resulted in 89.5% inhibition ($p = 0.001$) (Figure 6B,C). In Huh-7 cells, treatment with PSSct1905 or PSSct1910 resulted in very high levels of inhibition with minimal differences between these compounds: 93.5% ($p \leq 0.001$) and 95.3% ($p \leq 0.0001$) inhibition, respectively (Figure 6D,E). Interestingly, these effects were more prominent than those obtained for the inhibition of the complete entry stage (Figure 5B,C,E,F); the difference is probably due to different experimental conditions, such as the low temperature used in the assay that analyzed the effect of peptides on the attachment step.

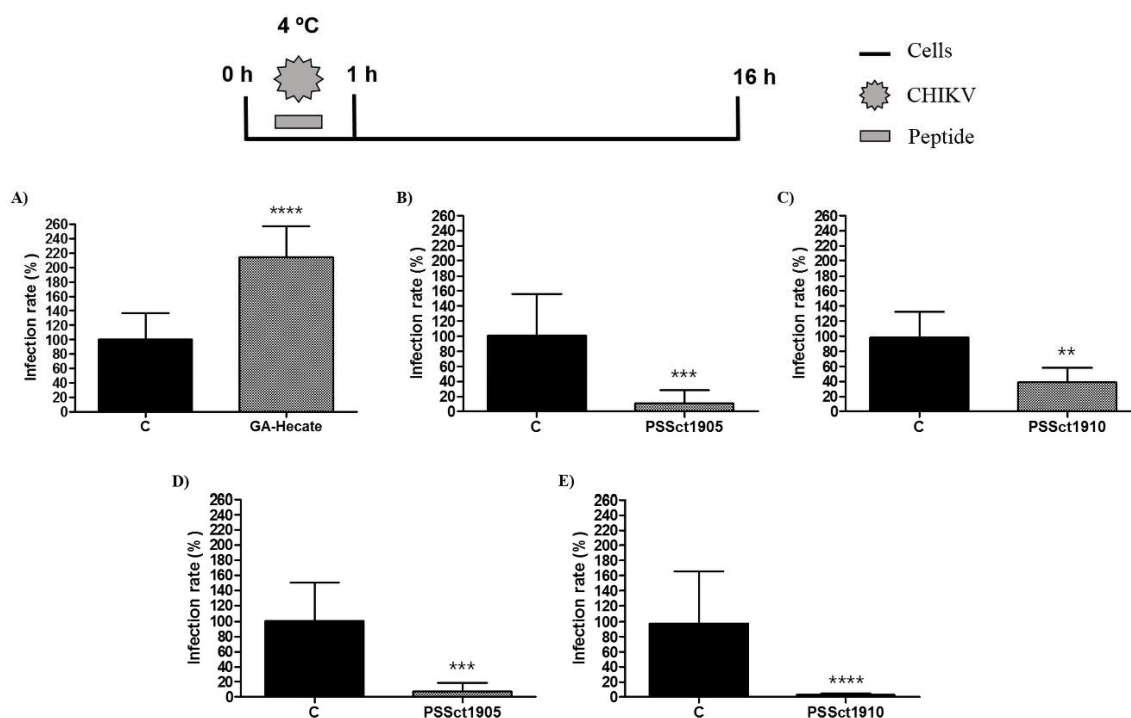


Figure 6. Effect of the peptides on CHIKV attachment. Data were analyzed as described in Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean $\pm$ SD. (A) GA-Hecate in BHK-21 cells. (B) PSSct1905 in BHK-21 cells. (C) PSSct1910 in BHK-21 cells. (D) PSSct1905 in Huh-7 cells. (E) PSSct1910 in Huh-7 cells. C: sterile water. **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

Cationic peptides can impair virus attachment to host cells by blocking cell receptors and/or by binding to virions [50]. As cited earlier, the peptides in this study have a positive charge similar to the E2 glycoprotein of the CHIKV. This may lead to competition for binding to cellular receptors, resulting in reduced attachment of virions to the cell.

Antiviral Action of the Peptides on CHIKV Internalization

In addition to attachment, the peptides GA-Hecate, PSSct1905, and PSSct1910 were tested for their ability to inhibit CHIKV internalization in BHK-21 and Huh-7 cells. For this analysis, the cells were first incubated with viral particles at 4 °C to allow viral attachment to occur; after this, the inoculum was replaced with media containing the peptides at the MNTC, and cells were placed at 37 °C to allow the internalization of the CHIKV particles. After 1 h, the cells were again washed with PBS and incubated with culture medium to continue the replicative cycle of the virus. As in the previous experiment, the combination of GA-Hecate and Huh-7 cells was excluded from this analysis.

The peptide GA-Hecate, which was unable to inhibit the attachment of CHIKV to BHK-21 cells (Figure 6A), strongly inhibited the internalization of CHIKV particles into BHK-21 cells. The observed inhibition was approximately 95.1% ($p = 0.0001$) (Figure 7A). This inhibition is apparently prominent enough to overcome the activation of CHIKV attachment, resulting in inhibition, albeit more modest, of the CHIKV entry step in general (Figure 5A). The viral internalization stage was also inhibited by PSSct1905 and PSSct1910 treatment, resulting in the inhibition of 94.5% ($p \leq 0.0001$) and 69.1% ($p \leq 0.01$), respectively, in BHK-21 cells (Figure 7B,C). High levels of inhibition were also observed in Huh-7 cells, in which treatment with PSSct1905 and PSSct1910 resulted in 85.8% ($p = 0.001$) and 87.1% ($p = 0.01$) inhibition, respectively (Figure 7D,E). Thus, regardless of the cell type, these analogs inhibited CHIKV entry by interfering with both virus attachment and internalization. Whether these two mechanisms of action can result in additive (or cumulative) effects remains unclear, as the use of different experimental conditions (most notable use of different temperatures) in assays that evaluate entry in general and attachment/internalization stages specifically does not allow direct comparison of data originating from corresponding experiments.

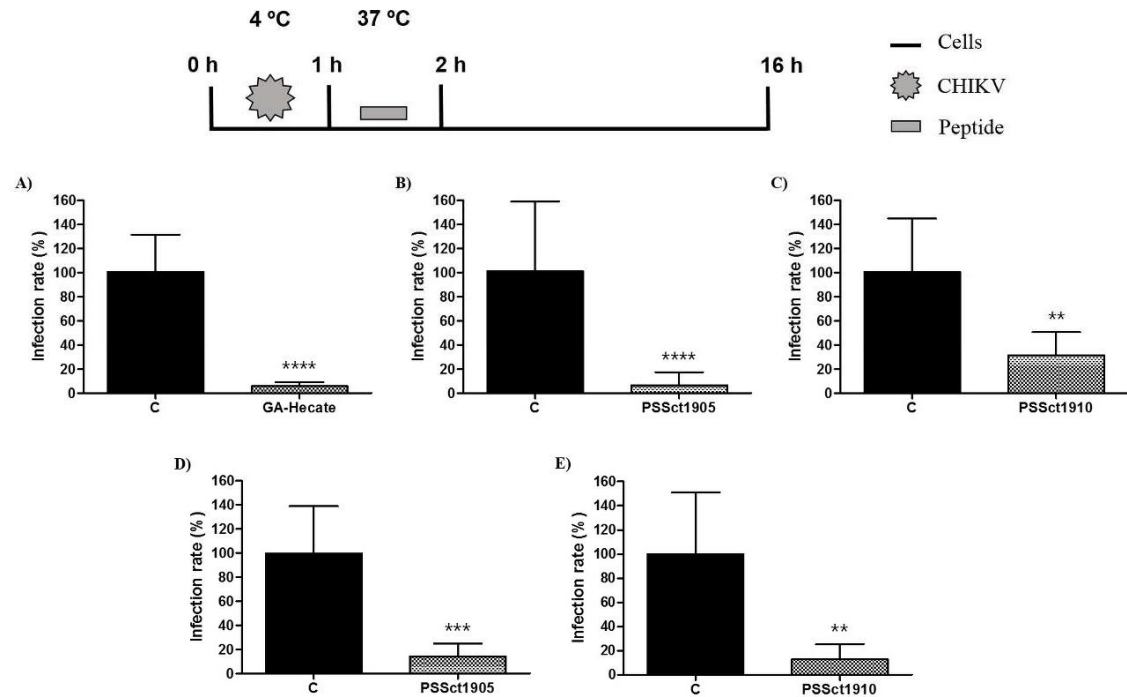


Figure 7. Effect of the peptides on CHIKV internalization. Data were analyzed as described in Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean $\pm$ SD. (A) GA-Hecate in BHK-21 cells. (B) PSSct1905 in BHK-21 cells. (C) PSSct1910 in BHK-21 cells. (D) PSSct1905 in Huh-7 cells. (E) PSSct1910 in Huh-7 cells. C: sterile water. **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

Amphipathic and positively charged peptides have been described to inhibit the intracellular trafficking of many pH-dependent enveloped viruses [51]. It is plausible that the peptides used in this study enter the cells together with the virions using endocytic vesicles by binding to cell (and/or virion) surfaces. In endocytic vesicles, they can suppress endosomal acidification, delaying or preventing the activation of pH-triggered viral fusogenic machinery, which is crucial for viral genome release into the cytoplasm. Additionally, it is possible that peptides hinder acidification via their electrostatic contributions to the endosomal lumen, resulting in resistance to the influx of hydrogen ions (driven by H^+ AT-Pase pumps). Alternatively, peptides may partially act as a buffering agent by sequestering incoming luminal protons. Finally, the peptides might regulate endosomal acidification by directly interacting with the host machinery, which drives this process [52].

Antiviral Activity of the Peptides on the Post-Entry Steps of CHIKV Infection

To evaluate the antiviral activity of the peptides in the post-entry stages of the CHIKV replicative cycle, BHK-21 and Huh-7 cells were infected with CHIKV-NLuc at an MOI of 0.1. After 1 h of incubation, the cells were washed, and media containing peptides at the MNTC was added. In this assay, GA-Hecate inhibited CHIKV infection by 80.7%

($p = 0.0001$) and 92.7% ($p = 0.0001$) in BHK-21 and Huh-7 cells, respectively (Figure 8A,D). The peptide PSSct1905 caused 92.2% ($p = 0.0001$) and 86.8% ($p = 0.0001$) reductions in viral replication in BHK-21 and Huh-7 cells, respectively (Figure 8B,E). The lowest activities were observed for the peptide PSSct1910, which reduced viral replication by approximately 35.2% ($p = 0.01$) in BHK-21 cells and 80.3% ($p = 0.0001$) in Huh-7 cells (Figure 8C,F). We suggest that the peptides used in this study may interfere with the synthesis of the CHIKV nonstructural proteins or inhibit their activity. The analysis of potential targets was outside of the scope of the current study.

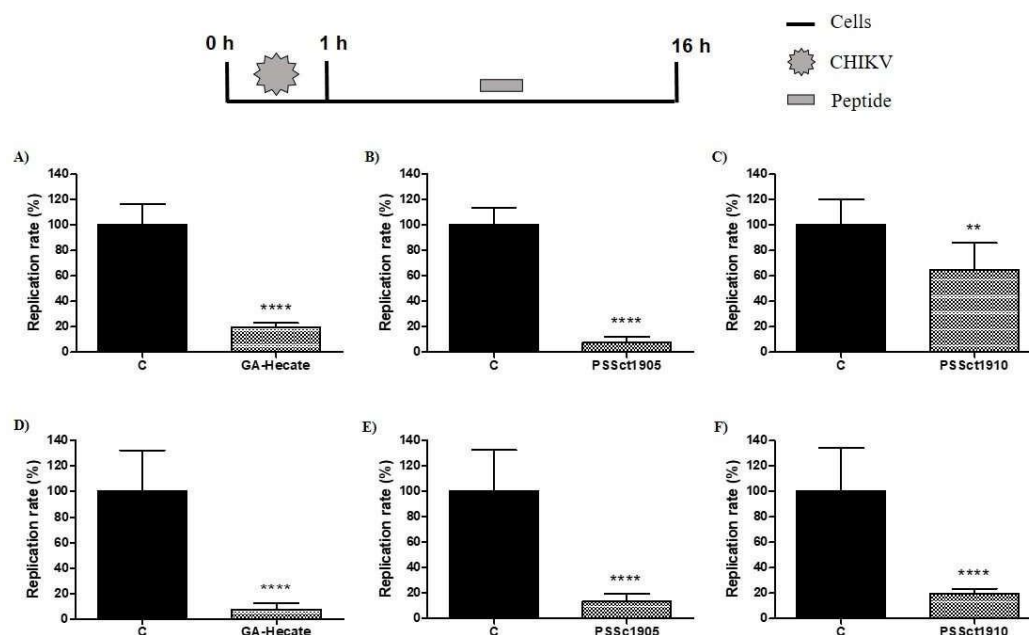


Figure 8. Effect of the peptides on the post-entry steps of CHIKV infection. Data were analyzed as described in Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean $\pm$ SD. (A) GA-Hecate in BHK-21 cells. (B) PSSct1905 in BHK-21 cells. (C) PSSct1910 in BHK-21 cells. (D) GA-Hecate in Huh-7 cells. (E) PSSct1905 in Huh-7 cells. (F) PSSct1910 in Huh-7 cells. C: sterile water. *: $p \leq 0.01$; ****: $p \leq 0.0001$.

Materials and Methods

Peptides

GA-Hecate and its analogs PSSct1905 and PSSct1910 were prepared on a TRIBUTE-UV automatic synthesizer (Protein Technologies, Tucson, AZ, USA) via solid phase peptide synthesis (SPPS) using the standard Fmoc protocol (9-fluorenylmethyloxycarbonyl) on a Rink-MBHA resin for the peptides GA-Hecate and PSSct1910 and Fmoc-Leu-Wang resin for PSSct1905 [36]. The identities of the peptides were confirmed by electrospray ionization mass spectrometry, and their purities were higher than 95%. To perform the biological experiments, the peptides were dissolved in sterile water and stored at -150°C .

Cells

BHK-21 cells (ATCC CCL-10) and Huh-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS, Gibco—Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin (10,000 IU/mL)/streptomycin (10 mg/mL) (P/S, Cultilab, Campinas, SP, Brazil). The cells were maintained in a humidified incubator at 37°C in 5% CO_2 .

Virus

The CHIKV-NLuc-expressing *nanoluciferase* reporter used for the antiviral assays is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). In the infectious plasmid, the cDNA of CHIKV-NLuc was placed under the control of the human cytomegalovirus (CMV) promoter [53]. To rescue CHIKV-NLuc, 1×10^5 BHK-21 cells seeded in 24-well culture plates (TPP, Trasadingen, Switzerland) were transfected with $1 \mu\text{g}$ of CHIKV-NLuc plasmid DNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and OPTI-MEM (reduced serum medium) (Gibco—Thermo Fisher Scientific, Waltham, MA, USA), as previously described [54]. The supernatant was collected at 72 h post-transfection (h.p.t.) and stored at -80°C .

The viral titers were determined by plaque assay, following the previously described protocol [54]. BHK-21 cells (1×10^5) were seeded in 24-well plates (TPP, Trasadingen, Switzerland). Twenty-four hours later, the cells were infected with tenfold serial dilutions of CHIKV-NLuc and incubated for 1 h at 37°C . After this, the inoculums were removed and replaced with fresh medium supplemented with 1% P/S (Cultilab, Campinas, SP, Brazil), 1% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA), and 2% carboxymethylcellulose (CMC, Sigma-Aldrich, St. Louis, MO, USA). Infected cells were incubated for 48 h in a humidified 5% CO_2 incubator at 37°C , followed by fixation with 10% formaldehyde (Merck, Darmstadt, Germany) and staining with 1% violet crystal (Merck, Darmstadt, Germany). The viral foci were counted to determine the viral titer, which was presented in plaque-forming units per milliliter (PFU/mL).

Evaluation of the Cytotoxicity Profile of the Peptides

Cytotoxicity in BHK-21 and Huh-7 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [35]. The cells (5×10^3 per well) were seeded in 96-well culture plates (TPP, Trasadingen, Switzerland). After 24 h, the cells were incubated with the peptides at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 μM for 24 h. Then, the medium containing the peptides was removed, and 100 μL of MTT (Sigma-Aldrich, St. Louis, MO, USA) diluted in DMEM (Cultilab, Campinas, SP, Brazil) (1 mg/mL) was added to each well of the plate (1 mg/mL). After 30 min of incubation at 37 °C, the medium containing MTT (Sigma-Aldrich, St. Louis, MO, USA) was removed, and 100 μL of dimethylsulfoxide (DMSO, Synth, Diadema, SP, Brazil) was added to the cells. The plate was agitated at 200 rpm. After 5 min, the absorbance was measured at a wavelength of 572 nm on a plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, Germany). The CC_{50} values were calculated using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

Analysis of the Activity of the CHIKV-NLuc-Encoded Reporter

Measurement of the activity of the virus-encoded NanoLuciferase (NLuc) reporter was used to evaluate the antiviral activity of the peptides against CHIKV-NLuc at the end of every assay with the peptides. Cells were infected with CHIKV-NLuc, and the supernatant was aspirated 16 h post-infection (h.p.i.), and each well of the plate was washed with PBS solution. Then, 30 μL of Renilla Luciferase Assay Lysis Buffer (Promega, Madison, WI, USA) was added to the cells. After 30 min, 50 μL of substrate for Renilla Luciferase (Renilla Luciferase assay reagent, Promega, Madison, WI, USA) was automatically injected in the wells into the plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, Germany), and the light intensity reading was performed. The values obtained are shown as the percentage of protein NLuc activity in the control (samples treated with sterile water).

Evaluation of the Activity of Peptides against the CHIKV Replication Cycle

The effects of the peptides on the CHIKV infectious cycle were analyzed as previously described [55]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-One, Americana, SP, Brazil) for 24 h. Then, the virus at an MOI of 0.1 and the peptides at the MNTC were simultaneously added to the cells. The plate was incubated at 37 °C for 16 h, and then antiviral activity against CHIKV was evaluated through NLuc activity. The peptides that exhibited antiviral activity at the MNTC were retested at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 μM to determine the EC_{50} values, which were calculated using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The SI was calculated using the ratio $\text{CC}_{50}/\text{EC}_{50}$.

Analysis of the Protective Effect of the Peptides against CHIKV Infection in the Pretreatment Assay

The protective effect of the peptides against CHIKV infection was evaluated as previously described [55]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-One, Americana, SP, Brazil). After 24 h, the cells were treated with peptides at the MNTC for 1 h at 37 °C. Then, the supernatant was removed, each well of the plate was washed twice with PBS, and the virus at an MOI of 0.1 was added to the cells and incubated for 1 h at 37 °C. After that, cells were washed twice with PBS again, and DMEM (Cultilab, Campinas, SP, BR) supplemented with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added to the cells. The plate was incubated at 37 °C, and the protective effect against CHIKV infection was evaluated at 16 h.p.i. by measurement of NLuc activity.

Evaluation of the Effect of the Peptides on the Extracellular CHIKV Particles

The virucidal activity of the peptides against CHIKV virions was investigated as previously described [56]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-One, Americana, SP, Brazil). A total of 5×10^4 PFU of virus was incubated with the MNTC of peptides at 37 °C for 1 h, after which the viral inoculum was added to the cells. After 1 h, the supernatant was aspirated, the wells were washed twice with PBS, and DMEM (Cultilab, Campinas, SP, BR) supplemented with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added to the cells. Luminescence levels were measured at 16 h.p.i. to analyze the virus replication rates, as explained earlier.

Analysis of the Peptides in the CHIKV Entry Steps

The peptides' action on CHIKV entry into the cells was analyzed as previously described [54,55]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-One, Americana, SP, Brazil) for 24 h. Then, the cells were infected with the virus at an MOI of 0.1 and treated with the peptides at the MNTC simultaneously for 1 h at 37 °C. Subsequently, the supernatant was removed, the cells were washed twice with PBS, and DMEM (Cultilab, Campinas, SP, BR) with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added to each well. The plate was incubated at 37 °C, and the antiviral effect against CHIKV was evaluated at 16 h.p.i. The impact of the peptides in the entry step was measured by luminescence levels.

Analysis of the Peptides on the CHIKV Attachment to the Cells

The effect of the peptides on CHIKV attachment to cells was evaluated as previously described [54,55,57]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-One, Americana, SP, Brazil). After 24 h, the plate was incubated at 4 °C for 15 min, and the cells were infected with the virus at an MOI of 0.1 and treated with the peptides at the MNTC simultaneously. The plate was incubated at 4 °C for 1 h. At 4 °C, virions interact with receptors on the cell membrane, but the viral particle is not able to enter the cell [54]. Then, the supernatant was aspirated, the wells were washed twice with PBS, and DMEM (Cultilab, Campinas, SP, Brazil) supplemented with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added. The plate was incubated at 37 °C, and the antiviral activity against CHIKV was evaluated at 16 h.p.i. The inhibitory effect of the peptides on CHIKV attachment to host cells was evaluated via the measurement of NLuc activity.

Analysis of the Peptides on CHIKV Internalization

The activity of the peptides on CHIKV internalization in cells was analyzed as previously described [57]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-One, Americana, SP, Brazil). After 24 h, the plate was incubated at 4 °C for 15 min, and the cells were infected with the virus at an MOI of 0.1 for 1 h at 4 °C. Then, the supernatant was removed, and the cells were washed twice with PBS and treated with the peptides at the MNTC for 1 h at 37 °C. Subsequently, the supernatant was aspirated, the cells were again washed twice with PBS, and DMEM (Cultilab, Campinas, SP, BR) with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added. At 16 h post-infection, cells were lysed and luciferase activity was measured.

Evaluation of the Peptides on the CHIKV Post-Entry Steps in Cells

The peptides' action on the post-entry stages of CHIKV in cells was evaluated as previously described [54,55]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-One, Americana, SP, Brazil). After 24 h, the cells were infected with the virus at an MOI of 0.1. After 1 h at 37 °C, the supernatant was removed, and the cells were washed twice with PBS and incubated with the peptides at the MNTC for 16 h. After this period, samples were harvested, and virus replication levels were quantified by luminescence using *Renilla* luciferase Assay Reagent (Promega, Madison, WI, USA).

Statistical Analysis

All the described experiments were performed in three independent events, each of which was carried out in three (cytotoxicity) and four (antiviral activity) technical replicates. The CC_{50} and EC_{50} values were determined by nonlinear regression of the dose-response curves (Log [peptide] response). The statistical significance was determined employing the paired Student's *t*-test for parametric results and the Mann-Whitney test for nonparametric data using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). A *p*-value < 0.05 was considered statistically significant.

Conclusions

Our study reports the first inhibitory effects of the peptide GA-Hecate and its analogs PSSct1905 and PSSct1910 against CHIKV infection in BHK-21 and Huh-7 cells. Antiviral activities were observed when the peptides were used as prophylactic treatment and/or were administered to CHIKV-infected cells at early and later stages of infection. These peptides exhibit potential for further development into anti-CHIKV drugs, which could help combat this mosquito-borne viral infection.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ph16101389/s1>, Figure S1. Cytotoxicity of the peptides in BHK-21 and Huh-7 cells. Peptides were incubated at different concentrations (1.6 to 100 μ M) in BHK-21 and Huh-7 cells. After 24 h, the cell viability was analyzed using the MTT method. The percentage values are expressed in relation to the results obtained in cells treated with the control (C, sterile water). (A) GA-Hecate in BHK-21 cells. (B) PSSct1905 in BHK-21 cells. (C) PSSct1910 in BHK-21 cells. (D) GA-Hecate in Huh-7 cells. (E) PSSct1905 in Huh-7 cells. (F) PSSct1910 in Huh-7 cells.

Author Contributions:

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SHORT COMMUNICATION



In vitro antiviral activity of piperidine alkaloids from *Senna spectabilis* flowers on Chikungunya virus infection

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Abstract

Background Chikungunya fever is an endemic disease caused by the Chikungunya virus (CHIKV). To date there is no antiviral treatment against this infection or licensed vaccine to prevent it. Our study aims to evaluate whether (–)-cassine (**1**) and (–)-spectaline (**2**), the main alkaloids of *Senna spectabilis*, display anti-CHIKV activity. Both compounds have been described to be biologically active against neglected tropical diseases, including malaria, leishmaniasis, and schistosomiasis, which emphasizes that these molecules could be repurposed for chikungunya fever treatment.

Methods The structures of the isolated compounds **1** and **2** were identified by NMR and HRESIMS analyses, and their antiviral activity against CHIKV was assessed by a dose–response assay employing BHK-21 cells and CHIKV-*nanoluc*, a recombinant virus carrying the *nanoluciferase* gene reporter.

Results Compound **1** presented CC₅₀ of 126.5 µM and EC₅₀ of 14.9 µM, while compound **2** presented CC₅₀ of 91.9 µM and EC₅₀ of 8.3 µM. The calculated selectivity index (SI) was 8.5 for **1** and 11.3 for **2**.

Conclusion The data presented herein show that compounds **1** and **2** have potential for being repurposed as anti-CHIKV drug. Our promising in vitro results encourage further in vitro and in vivo assays. This is the first description of the antiviral activity of compounds **1** and **2** against CHIKV infection, which can impact the development of antiviral drug candidates against chikungunya fever, which sometimes can be debilitating.

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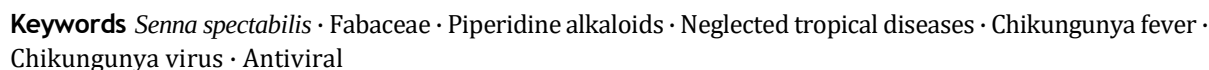
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ATR	Attenuated total reflectance
CC	Column chromatography
CC ₅₀	Cytotoxic concentration of 50%
CHIKV	Chikungunya virus
CHIKV- <i>nanoluc</i>	Chikungunya virus- <i>nanoluciferase</i>
CMC	Carboxymethyl cellulose
EC ₅₀	Effective concentration of 50%
HPLC chromatography	High performance liquid chromatography
HRESIMS	High Resolution Electrospray Ionization Mass Spectrometry
IR	Infrared
MOI	Multiplicity of infection
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
NTDs	Neglected tropical diseases
PBS	Phosphate-buffered saline
SI	Selectivity index
TLC	Thin layer chromatography

At the turn of the millennium, neglected tropical diseases (NTDs) were estimated to affect about two billion people with inadequate access to drinkable water, sanitation, and appropriate housing [1, 2]. Because several arboviruses are associated with the poorest populations in the world, diseases caused by them, such as chikungunya fever, should be included in the group of NTDs [1, 3]. The Chikungunya virus (CHIKV), transmitted to humans through the bite of female mosquitoes belonging to the genus *Aedes*, is the etiological agent of chikungunya fever [4–6]. To date, CHIKV infections have been reported in many African countries, the Indian subcontinent, and Southeast Asia. Furthermore, outbreaks have taken place not only in Africa and South-East Asia, but also in 33 countries and territories of the Americas, including Latin America where almost one million cases have been reported [3, 7]. The lack of vaccines and antiviral treatment, along with the increasing number of cases of CHIKV infections, demonstrates that searching for new therapeutic approaches is urgent [7]. One of the strategies to find new therapeutic alternatives against NTDs is to search for new molecules from natural sources [8]. *Senna spectabilis*, popularly known as “cassia-do-nordeste”, belongs to the Fabaceae family. This species is a source of rare piperidine alkaloids and has been widely

used in traditional medicine. Previous studies with the dichloromethane phase of the ethanolic extract obtained from *S. spectabilis* flowers showed that (–)-cassine (**1**) and (–)-spectaline (**2**) are the main alkaloids in this plant [9] and display activity against NTDs, including malaria [10], leishmaniasis [11], and schistosomiasis [12]. In the present study, we identified the structure of compounds **1** and **2** by NMR and MS spectra and assessed their in vitro activity against CHIKV by infecting baby hamster kidney-21 (BHK-21) cells in the presence of compounds **1** and **2**.

Materials and methods

General experimental procedures

^1H and ^{13}C NMR spectra were recorded on a Bruker Ascend™ 400 Avance III HD spectrometer (9.2 Tesla) operating at 400 MHz (^1H) and 100 MHz (^{13}C), at 25 °C. TMS ($\delta_{\text{TMS}} = 0.00$) was used as internal standard. HRESIMS spec-

tra were measured on a microTOF-Q II (Bruker Daltonics) with electrospray ionization (HRESIMS) in positive mode. Column chromatography (CC) was carried out over silica gel (63–200 μm , pore size 60 Å, Sigma Aldrich), flash silica gel (40–63 μm , pore size 60 Å, Sigma Aldrich), and RP-C₁₈ (40–63 μm , pore size 60 Å, Sorbent Technologies). Thin layer chromatography (TLC) was performed on silica gel F₂₅₄ plates (0.20 mm, Macherey–Nagel); spots were visualized under UV light (254 and 365 nm) and by spraying with the ICl₄Pt reagent. Analytical high-performance liquid chromatography (HPLC) separations were performed on a Shimadzu HPLC system equipped with LC-6AD pumps, DGU-20A5R degasser, CTO-20A oven, FRC-10A fraction collector, SIL-10AF autoinjector, SPD-M20D UV-vis, and ELSD-LT II evaporative light scattering detectors, controlled by the LC Solutions software. The column used was a Phenomenex Luna C₁₈ (250 × 4.6 mm, 5 μm), protected with the corresponding guard columns. A gradient (30 min) of increasing methanol concentration in 0.1% acetic acid aque-

ous solution (35–100%, flow rate of 1.0 mL min^{–1}) was used

as a solvent system, which was followed by final isocratic (10 min) methanol (100%). All the solvents employed in the experimental procedures were HPLC-grade or had been previously distilled. Water was purified immediately before use on a reverse osmosis system with deionizer Quimis.

Plant material

The flowers from *S. spectabilis* were collected by M. Pivatto in Araraquara (São Paulo, Brazil), in July/2010. Plant material was identified by Inês Cordeiro from Instituto de Botânica in São Paulo-SP, Brazil and a voucher specimen

(SP 384109) has been deposited in the herbarium of this institute.

Extraction and isolation

The dried flowers (7.9 kg) were ground and submitted to maceration with ethanol at room temperature. After distillation of the solvent under reduced pressure, a thick syrup (1.9 kg) was obtained. An aliquot of this extract (100.0 g) was dissolved in MeOH:H₂O (1:1, 500 mL) and then sequentially submitted to liquid–liquid extraction with *n*-hexane and CH₂Cl₂. After removal of the solvent under reduced pressure 2.6 g of *n*-hexane, 40.5 g of CH₂Cl₂, and 23.1 g of hydroalcoholic phases were obtained. Part of the CH₂Cl₂ phase (10.8 g) was subjected to CC over silica gel (63–200 μm , 20.0 × 8.0 cm i.d.) as a stationary phase and an increasing gradient of MeOH in CHCl₃ (5–100%) was employed as a solvent, and NH₄OH (0.25 mL for every 10 mL of solution) was used as a modifying agent. The fractions were analyzed by TLC and grouped accord-

ing to the *R_f* values: A1 (1.2 g), A2 (3.5 g), and A3 (4.5 g) (Fig. S1, Supporting Information). Fraction A2 (3.5 g) wa

submitted to CC over flash silica gel (40–63 μm , 20.0 × 3.5 cm i.d.) as a stationary phase and eluted using the same solvent system previously described to provide three new fractions after TLC analysis: A4 (0.5 g), A5 (2.3 g), and A6 (0.4 g) (Fig. S2, Supporting Information). Finally, the fraction A5 was subjected to CC over RP-C₁₈ (40–63 μm) and eluted with an increasing gradient of MeOH in aqueous HOAc 0.1% solution (5–100%) to yield compounds **1** (1.5 g, *t_R* 12.1 min) and **2** (100.0 mg, *t_R* 16.6 min), which were analyzed by HPLC (Figs. S3 and S4, Supporting Information).

(–)-Cassine (**1**): white solid (1.5 g); (+)-ESI-MS *m/z* 298.2750 [*M* + *H*]⁺, calculated for C₁₈H₃₆NO₂, 298.2741 (Δ –3.0 ppm); ^1H NMR (CDCl₃, 400 MHz) δ 3.67

(1H, m, H-3), 2.92 (1H, dq, *J* = 6.3; 1.5 Hz; H-2), 2.69 (1H, m, H-6), 2.42 (2H, t, *J* = 7.2, H-10'), 2.14 (3H, s, H-12'), 1.57 (6H, m, H-4a, H-4b, H-5a, H-5b, H-9'), 1.27 (19H, m, H-7, H-1'-H-8'); ^{13}C NMR (CDCl₃, 100 MHz) δ 208.91 (C, C-11'), 66.88 (CH, C-3), 57.04 (CH, C-2), 55.68 (CH, C-6), 43.27 (CH, H-10'), 35.10 (CH, C-1'), 31.0 (CH, C-4),

2

2

2

29.33 (CH₃, C-12') 29.08 (CH₂, C-8'), 28.98 (CH₂, C-6'), 28.95 (CH₂, C-5'), 28.87 (CH₂, C-3'), 28.84 (CH₂, C-4'), 28.62 (CH₂, C-7'), 25.16 (CH₂, C-5), 24.10 (CH₂, C-2'), 23.31 (CH₂, C-9'), 17.13 (CH₃, C-7).

(–)-Spectaline (**2**): white solid (200.0 mg); (+)-ESI-MS *m/z* 326.3057 [*M* + *H*]⁺, calculated for C₂₀H₃₈NO₂, 326.3054 (Δ 0.9 ppm); ^1H NMR (CDCl₃, 400 MHz) δ 3.82 (1H, br s, H-3), 3.15 (1H, q, *J* = 6.2 Hz, H-2), 2.90 (1H, m, H-6), 2.40 (2H, t, *J* = 7.4 Hz, H-12'), 2.12 (3H, s, H-14'), 2.06 (1H, m, H-4a), 1.93 (1H, m, H-1'a), 1.78 (2H, m, H-5), 1.55 (4H, m, H-4b, H-1'b, H-11'), 1.43 (3H, d, *J* = 6.2 Hz, H-7), 1.24 (18H, m, H-2'-H-10'); ^{13}C NMR (CDCl₃, 100 MHz) δ 209.48 (C, C-13'), 66.33 (CH, C-3), 57.71 (CH, C-2),

56.65 (CH, C-6), 43.82 (CH₂, H-12'), 33.36 (CH₂, C-1'), 30.79 (CH₂, C-4), 29.87 (CH₂, C-10'), 29.59 (CH₂, C-7'), 29.53 (CH₂, C-6'), 29.52 (CH₂, C-5'), 29.46 (CH₂, C-4'), 29.41 (CH₂, C-3') 29.34 (CH₂, C-8'), 29.29 (CH₂, C-9'), 29.18 (CH₃, C-14'), 25.48 (CH₂, C-2'), 23.93 (CH₂, C-5), 22.35 (CH₂, C-11'), 15.78 (CH₃, C-7).

Cell culture

Baby Hamster Kidney cells (BHK 21-ATCC® CCL-10™), which are susceptible to CHIKV infection [13], were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 100 U/mL of penicillin (Hyclone Laboratories, USA), 100 mg/mL of streptomycin (Hyclone Laboratories, USA), 1% of non-essential amino acids (Hyclone Laboratories, USA) and 10% of fetal bovine serum (FBS, Hyclone Laboratories, USA) in a humidified 5% CO₂ incubator at 37 °C.

Production of CHIKV-nanoluc reporter virus

Antiviral assays were performed using the CHIKV expressing *nanoluciferase* reporter (CHIKV-*nanoluc*), a construction based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype), placed under the control of the CMV promoter [14, 15]. Virus supernatant was produced by seeding 2.3×10^7 BHK 21 cells seeded in a cell culture flask and transfecting with 1.5 µg of CHIKV-CMV-*nanoluc* plasmid using lipofectamine 3000® and Opti-Mem medium to produce CHIKV *nanoluc*. Infectious supernatant was collected 48 h post-transfection and stored at -80 °C.

For virus titration, 5×10^5 BHK 21 cells were seeded in each of 6-well plate, 24 h before the infection, and then infected with tenfold serial dilution of supernatant containing CHIKV-*nanoluc* for 1 h at 37 °C. Cells were washed with phosphate-buffered saline (PBS) to remove the unbound virus and added of cell culture media supplemented with 1% penicillin, 1% streptomycin, 2% FBS and 1% carboxymethyl cellulose (CMC). The plates were incubated for 2 days in a humidified 5% CO₂ incubator at 37 °C, followed by cells fixation with 3% formaldehyde and staining with 0.5% violet crystal. Then, viral foci were counted to determine CHIKV-*nanoluc* titer [16].

Cell viability through MTT assay

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich), as described previously [17–19]. Briefly, BHK-21 cells were cultured in a 48-well plate and treated for 16 h at 37 °C and 5% CO₂ with each compound at concentrations ranging from 3.125 to 400 µM. Sixteen hours after treatment, the medium containing compound was removed

and MTT (1 mg/ml) was added to each well, incubated for 30 min, and then replaced with 300 µl DMSO to dissolve the formazan crystals. Absorbance was measured at 490 nm using Glomax microplate reader (Promega). Cell viability was calculated using the equation $(T/C) \times 100\%$, where T and C represented the optical density of the treated wells and control groups, respectively. DMSO was used as an untreated control.

Antiviral assays

The antiviral assays were performed as previously described [17–19], by seeding 5×10^4 BHK-21 cells into each of 48-well plates 24 h prior the infection. Compound at concentrations ranging from 3.125 to 400 µM and CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 were simultaneously added to cells for 16 h. Then, samples were harvested in Renilla luciferase lysis buffer (Promega) and *nanoluciferase* activity was measured using the Renilla luciferase Assay System (Promega) to quantify virus replication rates. The Graph Pad Prism 8.0 Software was used to calculate the effective concentration of 50% inhibition (EC₅₀) and the cytotoxic concentration of 50% (CC₅₀) value, which were used to calculate the selectivity index ($SI = CC_{50}/EC_{50}$) of compounds 1 and 2.

Statistical analysis

Individual experiments were performed in triplicate and all assays were carried out a minimum of three times in order to confirm reproducibility of data. The EC₅₀ and CC₅₀ were calculated using a non-linear regression considering log(inhibitor) versus normalized response, with variable slope (four parameters), and a comparison was also performed using the F test ($p < 0.05$). The data were also submitted to Normality and Lognormality tests using Shapiro–Wilk (W) and D'Agostino-Pearson omnibus (K2). All tests for statistical analysis were performed employing GraphPad Prism 8.

Results and discussion

Chikungunya fever is an arbovirus disease with chronic potential that encompasses social and economic aspects. To date, there is no antiviral treatment against CHIKV infection, and the development of antiviral drugs to treat chikungunya fever remains a challenge. Therefore, research aiming to identify new compounds against CHIKV is crucial. Considering the antiviral potential encompassing molecules isolated from natural sources [20], we performed chromatographic fractionation of the dichloromethane (CH₂Cl₂) phase of the ethanolic extract (EtOH) obtained from *S. spectabilis*

flowers. This procedure resulted in the isolation of the following two homologous piperidine alkaloids: (-)-cassine (**1**) and (-)-spectaline (**2**) (Fig. 1), identified by analysis of their ^1H and ^{13}C NMR spectra, which agreed with the literature data (Figs. S5–S24, Supporting Information) [10, 11, 21]. Then, we screened compounds **1** and **2** for their anti-CHIKV activity. To this end, we carried out a dose-response assay to determine the effective concentration of 50% (EC_{50}) and the cytotoxicity concentration of 50% (CC_{50}). The assay involved infecting BHK-21 cells

with CHIKV-*nanoluciferase* (CHIKV-*nanoluc*), a recombinant virus that continuously expresses the *nanoluciferase* reporter [14, 15], and treating them with compounds **1** or **2** at concentrations ranging from 3.125 to 400 μM . We evaluated the viral replication rates by measuring the *nanoluciferase* activity at 16 h post-infection (h.p.i.) (Fig. 2A). We measured cell viability in parallel with the MTT assay (Fig. 2A). In these conditions, we found that compound **1** has EC_{50} of $14.9 \pm 1.5 \mu\text{M}$ (p -value < 0.0001 , $F_{(1,22)} = 586.7$), CC_{50} of $126.5 \pm 16.2 \mu\text{M}$ (p -value < 0.0001 , $F_{(1,22)} = 1874$),

Fig. 1 Alkaloids **1** and **2** isolated from *S. spectabilis* flowers

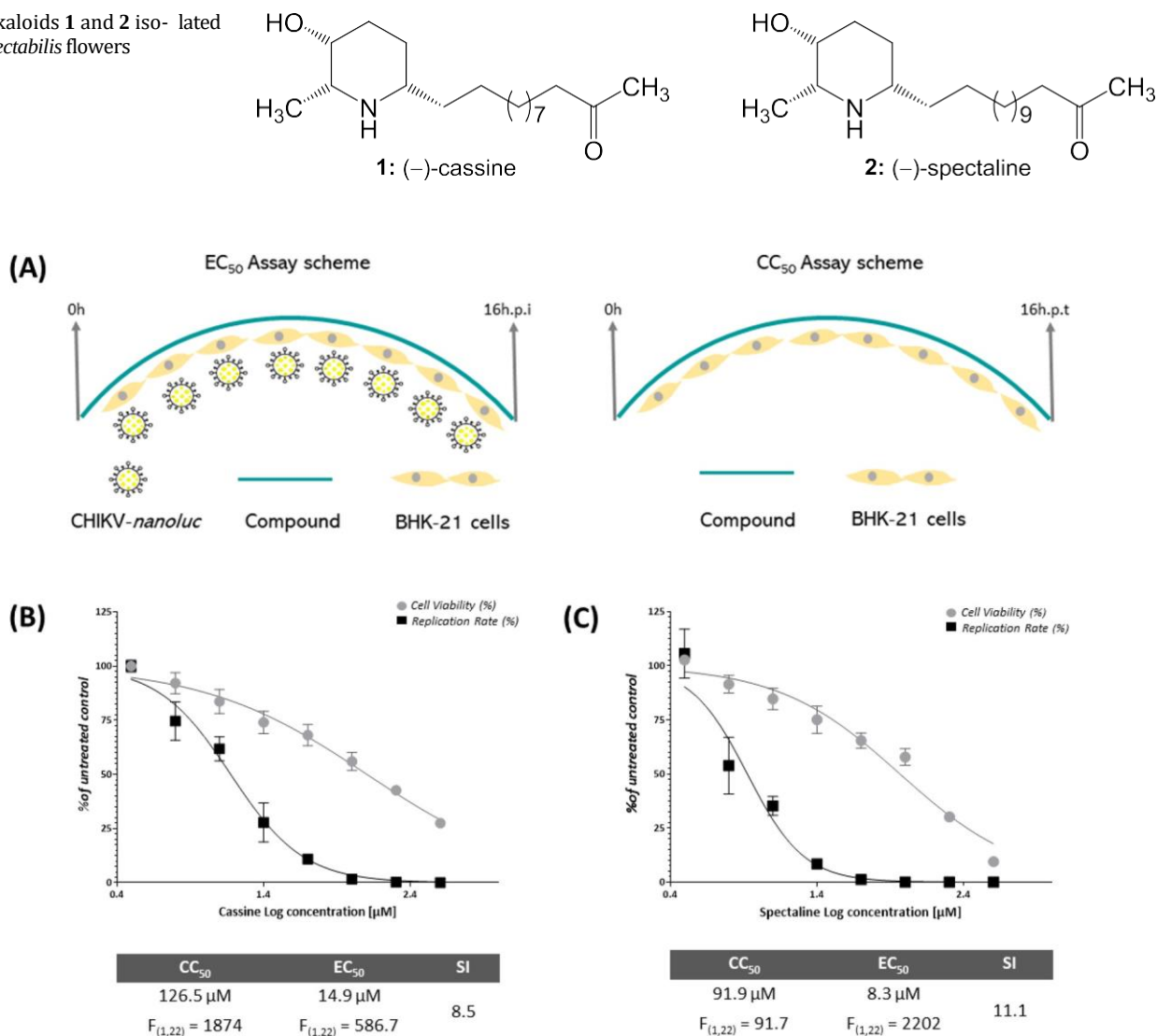


Fig. 2 The activity of **1** and **2** on the CHIKV replicative cycle. **A** Schematic representation of EC_{50} and CC_{50} assays (h.p.t. = hours post-treatment). **B** BHK-21 cells were treated with **1** at concentrations ranging from 3.125 to 400 μM in the presence or absence of CHIKV at MOI 0.1, and the EC_{50} and CC_{50} were determined. **C** BHK-21 cells were treated with **2** at concentrations ranging from 3.125 to 400 μM in the presence or absence of CHIKV at MOI 0.1, and the EC_{50} and CC_{50} were determined. For both protocols, CHIKV replication was measured by *nanoluciferase* assay 16 h.p.i. (indicated by filled

square), and cellular viability was measured using an MTT assay (indicated by filled circle). Results are shown as non-linear regression analysis, indicating the percentages of the cell viability or viral replication by each compound. Mean values $\pm$ SD of three independent experiments each measured in triplicate are shown. The comparison of each curve was performed employing the F test (confidence level of 95%), resulting in a p -value of < 0.0001 . GraphPad Prism 8 was used to create the graphics and perform statistical analysis. The figure was edited using GIMP 2.10.20 (<http://www.gimp.org>)

and selectivity index (SI) of 8.5 (Fig. 2B). As for compound **2**, it has EC₅₀ of 8.1 ± 2.4 μM (*p*-value < 0.0001, *F*_(1,23) = 91.7), CC₅₀ of 92.1 ± 13.7 μM (*p*-value < 0.0001, *F*_(1,23) = 2202), and SI of 11.3 (Fig. 2C).

Natural phytochemical alkaloids have been largely explored for their in vitro antiviral activity, and they have been shown to be promising for further analyses in vivo. Tomatidine, a natural steroidal alkaloid from tomato skin, with EC₅₀ of 1.3 μM, is a potential CHIKV inhibitor and reduces virus progeny release [22]. The antiviral activity of the alkaloid berberine against CHIKV has also been reported. Different EC₅₀ values were obtained for berberine depending on the cell line employed in the assay—HEK-293T (4.5 μM), HOS cells (12.2 μM), or CRL-2522 (35.5 μM) [23]. In this context, the EC₅₀ and CC₅₀ values obtained for **1** and **2** herein might change depending on the cell lineage that is used to determine these values. In addition, one may suggest that the absence of adverse effects in pre-clinical assays might outweigh the modest SI values obtained in vitro, related to the therapeutic significance. The effective concentration of these alkaloids for the treatment of CHIKV-infected animals and/or individuals needs to be further assessed. Additionally, SI values higher than 1.0 suggest a positive benefit and risk balance for the potential antiviral drugs [24]. Other drugs such as chloroquine, widely employed to treat *Plasmodium falciparum* infections despite the substantial adverse effects on patients, have been described as an alternative for CHIKV treatment in vitro. The SI of these drugs range from 15 to 26, depending on the type of treatment [25–27]. However, when these drugs were evaluated in vivo, the results demonstrated that a higher SI is not always correlated with beneficial clinical implications [25]. Moreover, alkaloids have been described to possess potential antiviral activity against many viruses, and their mechanisms of action varied from viral attachment and entry interference to RNA and protein synthesis inhibition [28]. Therefore, in chikungunya fever, **1** and **2** may act by interfering in one or more stages of CHIKV replication, suggesting their specificity for the viral replication process, which is important for the future development of drugs against viruses of clinical importance.

Besides that, evaluating the in vitro antiviral activity of a compound is complex and requires that potential molecules be explored, so that new treatments can be implemented. The data presented here have certain social impact because they point to a promising treatment against CHIKV, and the EC₅₀ and CC₅₀ data are important pharmacological metrics to support further in vitro assays to identify the mode of action of the compounds and their future use in pre-clinical trials.

The results presented herein demonstrate that compounds from natural sources may play an important role in the search for new medicines with antiviral activities against

CHIKV and might represent a significant contribution to the development of antiviral drugs against neglected diseases such as chikungunya fever [29].

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Author contributions Acquisition of data and results analysis: TRF, RMN, IAS, and DOSM. Drafting of the manuscript TRF, RMN, and IAS. Study design, supervision, and critical revision AD, VSB, ACGJ, and MP. All authors reviewed the manuscript.

Data availability The datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations

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

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Potential in vitro anti-periodontopathogenic, anti-*Chikungunya* activities and in vivo toxicity of Brazilian redpropolis

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Bacterial and viral infections are serious public health issue. Therefore, this study aimed to evaluate the antibacterial, antibiofilm and antiviral potential of the Brazilian Red Propolis (BRP) crude hydroalcoholic extract, fractions, and isolated compounds, as well as their in vivo toxicity. The antibacterial activity was evaluated by determining the Minimum Inhibitory Concentration and the antibiofilm activity by determining the Minimum Inhibitory Concentration of Biofilm (MICB₅₀). The viable bacteria count (Log₁₀ UFC/mL) was also obtained. The antiviral assays were performed by infecting BHK-21 cells with *Chikungunya* (CHIKV) *nanoluc*. The toxicity of the BRP was evaluated in the *Caenorhabditis elegans* animal model. The MIC values for the crude hydroalcoholic extract sample ranged from 3.12 to 100 µg/mL, while fractions and isolated compounds the MIC values ranged from 1.56 to 400 µg/mL. The BRP crude hydroalcoholic extract, oblongifolin B, and gutiferone E presented MICB₅₀ values ranging from 1.56 to 100 µg/mL against monospecies and multispecies biofilms. Neovestitol and vestitol inhibited CHIKV infection by 93.5 and 96.7%, respectively. The tests to evaluate toxicity in *C. elegans* demonstrated that the BRP was not toxic below the concentrations 750 µg/mL. The results constitute an alternative approach for treating various infectious diseases.

Despite important advances in the health area, infectious diseases have constituted a serious public health issue over time¹. One example is periodontitis, an inflammatory disease that affects tooth-supporting apparatus and which is caused by microorganisms present in dysbiosis plaque biofilms². According to Mehrotra and Singh³, about 2.6% of African Americans, 5% of Africans, 0.2% of Asians, 1% of North Americans, and 0.3% of South Americans have been diagnosed with periodontitis in its most severe form. Periodontal treatment is essential not only for dental parameters, but also to avoid other pathological conditions such as adverse reactions in pregnancy, cardiovascular and respiratory diseases, cancer, lupus, rheumatoid arthritis, diabetes mellitus, and chronic kidney disease⁴. Even if the illness can be treated with antibiotics, the infection can be aggravated in patients lacking treatment or in the presence of resistant periodontopathogenic bacteria⁵.

Viral diseases also burden the global health system due to lack of vaccines and approved antivirals to combat important human viruses, including the *Chikungunya* Fever, caused by the *Chikungunya* virus (CHIKV)⁶. CHIKV was identified in 2014 and has become hyperendemic in Brazil⁷. This virus causes dengue-like symptoms such

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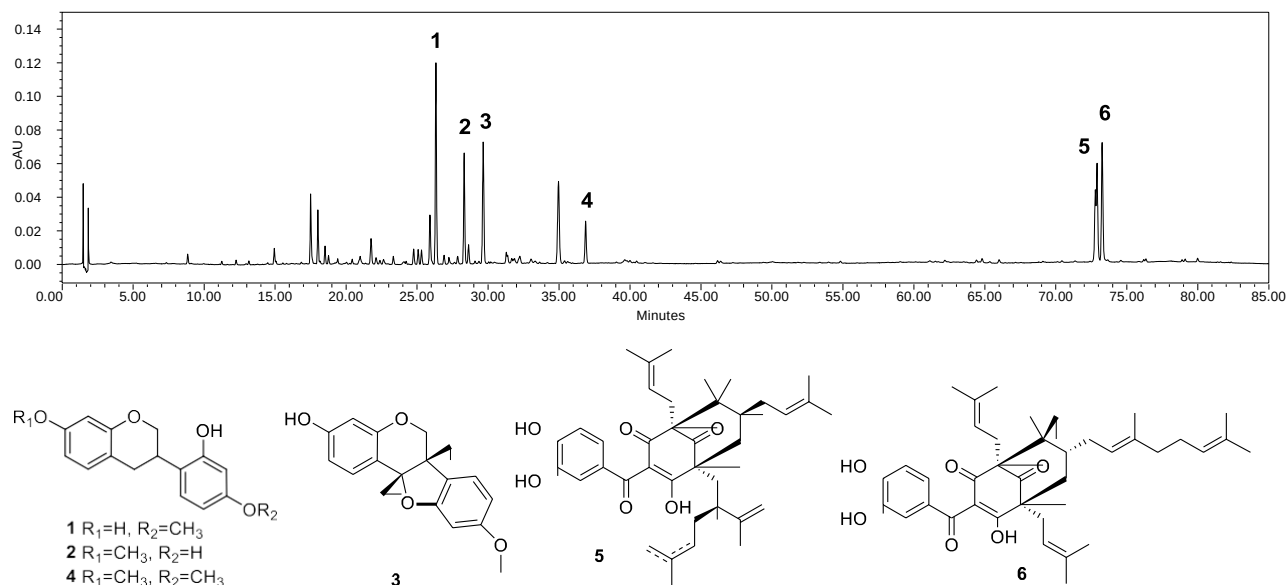


Figure 1. Chromatographic profile of Brazilian red propolis extract and chemical structures of their main compounds. Numbers correspond to: vestitol (1); neovestitol (2); medicarpin (3); 7-O-methylvestitol (4); guttiferone E/xanthochymol⁵⁹; and oblongifolin B (6).

as fever, fatigue, arthralgia, and polyarthralgia⁸. By April 2022, 28,291 suspected cases of *Chikungunya* Fever had been registered and five deaths had been confirmed in Brazil; another eight deaths are under investigation⁹. According to the World Health Organization, a considerable part of the worldwide population still depends on traditional medicine and employs natural products to treat several diseases¹⁰. Developing countries mainly use such products. In this scenario, Brazil is a valuable source of natural products given that it possesses diverse fauna and flora¹¹. Brazilian Red Propolis (BRP), a resinous material produced by *Apis mellifera* bees through the collection of the exudates of two plant species: *Dalbergia ecastaphyllum*^{12,13} and *Symphonia globulifera*¹⁴ has excellent potential for developing new medicines. BRP is currently one of the most produced and commercialized types of Brazilian propolis. It is mainly found in the Brazilian mangroves of the Northeast, especially in Alagoas and Bahia states¹⁵. BRP is composed of 50% resin, 30% wax, 10% essential oils, 5% pollen, and 5% other compounds, including secondary metabolites like flavonoids, isoflavonoids, cinnamic acid derivatives, esters, polyphenylated benzo-phenones, and some terpenes, which are considered the main biologically active constituents of this type of propolis¹⁶. The molecules isolated from BRP do not occur in any other type of propolis, which makes them rare and unique natural products¹⁷. Variations of this composition have been observed between locations. Some studies revealed that compounds such as formononetin and isoliquiritigenin are the most abundant in samples of Alagoas¹⁸. Instead, in “Canaveiras” sample, vestitol, neovestitol, medicarpin, and polyphenylated benzo-phenones have been identified as the main compounds and¹⁷. In this sense, BRP has been reported to possess antibacterial^{15,18–20} antiparasitic^{21–27}, and antiviral activities²⁸.

Considering the lack of treatment options for periodontitis and CHIKV infection, we have hypothesized that BRP and its isolated compounds are a promising candidate for treating these diseases. To the best of our knowledge, there are no data on the BRP antiviral action against CHIKV, and few studies have reported on its antibacterial action against periodontopathogenic bacteria^{13,18,28–30}. The use of BRP as a therapeutic option could reduce the use of antibiotics in periodontitis cases and become a novel antiviral strategy against CHIKV²⁸.

This study aimed to evaluate the *in vitro* antibacterial, antibiofilm, and antiviral potential of the BRP crude hydroalcoholic extract, fractions, and isolated compounds, as well as their toxicity in an *in vivo* model.

Results

BRP crude extract characterization. The chromatographic analysis revealed the presence of isoflavanes (vestitol, neovestitol, 7-O-methylvestitol), pterocarpans (medicarpin), and polyphenylated acylphloroglucinols (a mixture of guttiferone E/xanthochymol, and oblongifolin B) (Fig. 1), as main compounds of the BRP. The chromatographic profile of the fractions revealed the prominent presence of polyphenylated acylphloroglucinols in the hexane fraction, whereas the dichloromethane, ethyl acetate, and *n*-butanol fractions were composed mainly of isoflavanes (see Supplementary Figure S1).

Minimum inhibitory concentration of the BRP crude hydroalcoholic extract, fractions, and isolated compounds.

Tables 1 and 2 show the MIC results for the crude hydroalcoholic extract, fractions and isolated compounds against periodontal bacteria included in the study. The MIC values for the crude hydroalcoholic extract sample ranged from 3.12 to 100 $\mu\text{g/mL}$, for the dichloromethane fraction from 1.56 to 200 $\mu\text{g/mL}$, ethyl acetate from 12.5 to 400 $\mu\text{g/mL}$, hexane from 3.12 to 400 $\mu\text{g/mL}$, and *n*-Butanol from 100 to 400 $\mu\text{g/mL}$ (Table 1).

Minimum Inhibitory Concentration ($\mu\text{g/mL}$)					
Crude extract		Fractions			
Bacteria		Dichloromethane	Ethyl acetate	Hexane	<i>n</i> -Butanol
<i>Porphyromonas gingivalis</i> (ATCC 49417)	3.12	1.56	12.5	3.12	100
<i>Porphyromonas gingivalis</i> (clinical isolate)	12.5	25	400	100	—

<i>Fusobacterium nucleatum</i> (ATCC 10953)	100	200	400	—	—
<i>Fusobacterium nucleatum</i> (clinical isolate)	12.5	12.5	—	400	—
<i>Prevotella intermedia</i> (ATCC 15033)	6.25	6.25	200	200	200
<i>Prevotella intermedia</i> (clinical isolate)	50	100	—	400	—
<i>Actinomyces naeslundii</i> (ATCC 19039)	25	25	—	400	—
<i>Actinomyces naeslundii</i> (clinical isolate)	100	100	400	400	400

Table 1. Minimum inhibitory concentration of the Brazilian Red Propolis crude hydroalcoholic extract, and fractions against periodontopathogenic bacteria. ^aTechnique control strains: *Bacteroides fragilis* (ATCC 25285) and *Bacteroides thetaiotaomicron* (ATCC 29741)—Metronidazole: 1.47 and 2.95 µg/mL, respectively.—> 400 µg/mL was considered inactive.

Minimum Inhibitory Concentration (µg/mL)						
Isolated compounds						
	Metilvestitol	Medicarpin	Vestitol	Neovestitol	Oblongifolin B	Guttiferone E
Bacteria						
<i>Porphyromonas gingivalis</i> (ATCC 49,417)	25	50	12.5	12.5	50	1.56
<i>Porphyromonas gingivalis</i> (clinical isolate)	100	200	100	100	6.25	6.25
<i>Fusobacterium nucleatum</i> (ATCC 10,953)	50	50	25	12.5	50	200
<i>Fusobacterium nucleatum</i> (clinical isolate)	—	50	100	50	3.12	3.12
<i>Prevotella intermedia</i> (ATCC 15,033)	—	100	100	50	6.25	12.5
<i>Prevotella intermedia</i> (clinical isolate)	—	—	200	100	50	50
<i>Actinomyces naeslundii</i> (ATCC 19,039)	400	100	200	50	6.25	6.25
<i>Actinomyces naeslundii</i> (clinical isolate)	—	—	200	100	25	50

Table 2. Minimum inhibitory concentration of the BRP isolated compounds against periodontopathogenic bacteria. ^aTechnique control strains: *Bacteroides fragilis* (ATCC 25285)—Metronidazole: 1.47 µg/mL and *Bacteroides thetaiotaomicron* (ATCC 29741)—Metronidazole: 2.95 µg/mL. —> 400 µg/mL was considered inactive.

For the methylvestitol, the MIC values ranged from 25 to 400 µg/mL, medicarpin from 50 to 400 µg/mL, vestitol from 12.5 to 200 µg/mL, neovestitol from 12.5 to 100 µg/mL, oblongifolin B from 3.12 to 50 µg/mL, and guttiferone E from 1.56 to 200 µg/mL (Table 2).

Antibiofilm activity of the BRP crude hydroalcoholic extract and isolated compounds. The BRP crude hydroalcoholic extract reduced the monospecies biofilm formation of the standard strains (ATCC) and their clinical isolates (Fig. 2). Additionally, the number of viable cells in the monospecies biofilm expressed as Log₁₀ CFU/mL decreased (Fig. 2). The lowest MIC₅₀ value obtained for the BRP crude hydroalcoholic extract against the monospecies biofilms was 3.12 µg/mL against *A. naeslundii* (ATCC 19039) and *F. nucleatum* (clinical isolate) (Fig. 2e and f). Against the other evaluated monospecies biofilms, the BRP crude hydroalcoholic extract presented MIC₅₀ of 6.25 µg/mL, except for *P. intermedia* (clinical isolate), against which MIC₅₀ was 12.5 µg/mL. However, even at concentrations above MIC₅₀, we detected viable biofilm cells (Fig. 2a–f).

As for the tested isolated compounds, they also reduced monospecies biofilm formation. In the presence of oblongifolin B (Fig. 3), the lowest MIC₅₀ was 0.78 µg/mL against *A. naeslundii* (ATCC 19039) (Fig. 3c). Against the other evaluated monospecies biofilms, the MIC₅₀ values ranged from 1.56 to 6.25 µg/mL. Oblongifolin B at 6.25 µg/mL eliminated *P. gingivalis* (clinical isolate) viable cells and, at 12.5 µg/mL, it eliminated *P. intermedia* (ATCC 15033) and *F. nucleatum* (clinical isolate) viable cells (Fig. 3a,b and d).

Guttiferone E presented low MIC₅₀ (0.78 µg/mL) against *A. naeslundii* (ATCC 19,039) (Fig. 4d). Against the other evaluated monospecies biofilms, MIC₅₀ ranged from 1.56 to 25 µg/mL (Fig. 4a,b,c and e). Guttiferone E eliminated all the biofilm cells from a concentration of 3.12 µg/mL against *P. gingivalis* (clinical isolate), 6.25 µg/mL against *P. intermedia* (ATCC 15033), 25 µg/mL against *F. nucleatum* (clinical isolate), and 1.56 µg/mL against

A. naeslundii (ATCC 19039). As for *P. gingivalis* (ATCC 49417), we verified the presence of viable biofilm cells even at concentrations above MIC₅₀ (Fig. 4a).

We also assessed the activity of the BRP crude hydroalcoholic extract and isolated compounds against multi-species biofilm formed by standard strains (group 1) and clinical isolates (group 2) (Fig. 5). The BRP crude

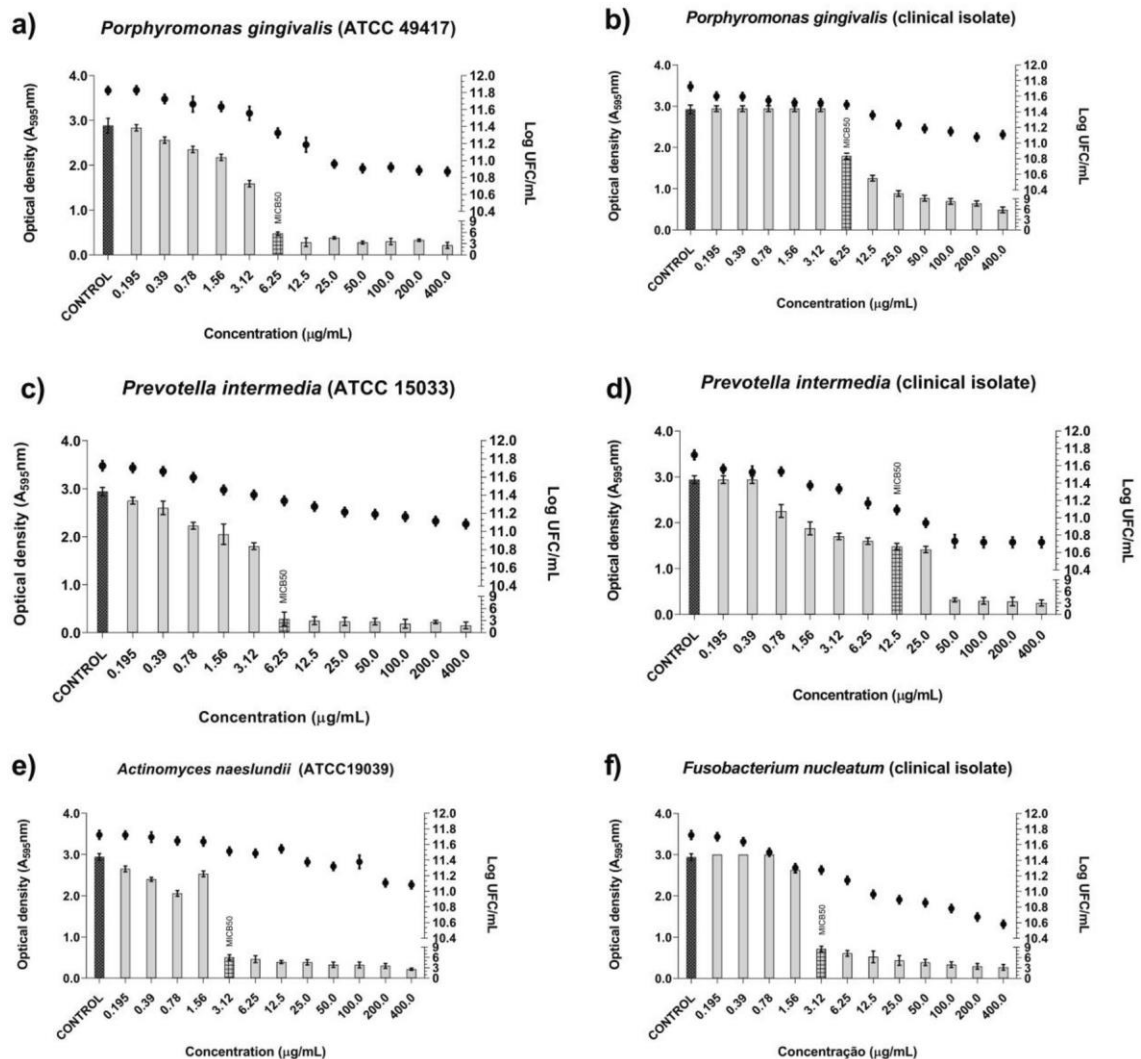


Figure 2. Antibiofilm activity of Brazilian Red Propolis crude hydroalcoholic extract samples and number of viable cells in monospecies biofilms formed by ATCC strains and clinical isolates included in the study. **(a)** *P. gingivalis* (ATCC 49417). **(b)** *P. gingivalis* (clinical isolate). **(c)** *P. intermedia* (ATCC 15033). **(d)** *P. intermedia* (clinical isolate). **(e)** *A. naeslundii* (ATCC 19039). **(f)** *F. nucleatum* (clinical isolate).

hydroalcoholic extract had MIC₅₀ of 6.25 µg/mL against the group 1 multispecies biofilm. However, even at higher concentrations, viable cells were still found in the biofilm. Similar results were found against the group 2 multispecies biofilm: MIC₅₀ was 6.25 µg/mL, and there also were viable biofilm cells above the MIC₅₀ concentration (Fig. 5A).

Concerning oblongifolin B, it had the lowest MIC₅₀ against the group 1 multispecies biofilm (1.56 µg/mL); however, at concentrations above MIC₅₀, cells remained viable in the biofilm. Against the group 2 multispecies biofilm, oblongifolin B presented MIC₅₀ of 50 µg/mL and eliminated all the biofilm cells from the biofilm at this same concentration (Fig. 5B). On the other hand, guttiferone E showed MIC₅₀ of 3.12 µg/mL against the group 1 multispecies biofilm, and 6.25 µg/mL guttiferone E eliminated all the cells from the biofilm. Against the group 2 multispecies biofilm, guttiferone E had higher MIC₅₀ (100 µg/mL), but 50 µg/mL guttiferone E also eliminated all the viable cells from the biofilm (Figs. 5C).

Regarding the control (metronidazole), the MIC₅₀ of monospecies biofilms ranged from 2.95 to 5.9 µg/mL. As for the mixed biofilms, the MIC₅₀ was 2.95 µg/mL for both the biofilm formed by group 1 and the biofilm formed by group 2 (see supplementary material Figures S2 and S3).

Effects of the BRP crude hydroalcoholic extract and isolated compounds on CHIKV replication. To further evaluate the effects of BRP extract and its isolated compounds, BHK 21 cells were treated with each extract at 50, 10 and 2 µg/mL and cell viability was measured 16 h later. The results demonstrated that cells tolerated *n*-Butanol at 50 µg/mL (98.4%), ethyl acetate at 10 µg/mL (95.9%), while the crude extract, dichloromethane, and hexane at 2 µg/mL (99.3, 99.8, and 100%, respectively), (Table 3). Through the employment of BHK-21 cells infected with CHIKV-*nanoluc*, the anti-CHIKV activities of each sample were evaluated,

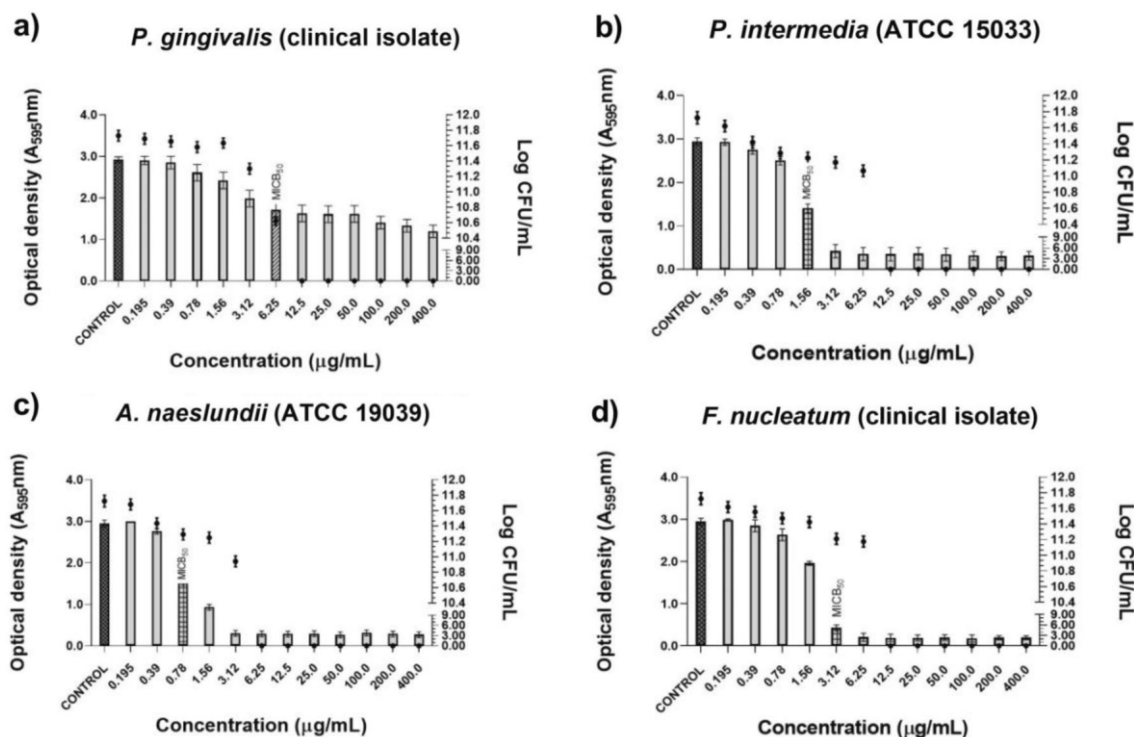


Figure 3. Antibiofilm activity of oblongifolin B and number of viable cells in monospecies biofilms formed by ATCC strains and clinical isolates included in the study. (a) *P. intermedia* (clinical isolate) (b) *P. intermedia* (ATCC 15033). (c) *A. naeslundii* (ATCC 19039). (d) *F. nucleatum* (clinical isolate).

at the maximum non-cytotoxic concentrations selected through the viability assay. The results demonstrated that *n*- Butanol significantly inhibited 69% of CHIKV replication (Fig. 6). The other samples presented no effect on CHIKV infection (Fig. 6). For the isolated substances (medicarpin, neovestitol, vestitol, oblongifolin B, methylvestitol and, guttiferone E), BHK-21 cells were treated with concentrations of each compound ranging from 32 to 0.5 µg/mL. As an outcome, the treatment with compounds in concentrations over 3 µg/mL presented cell viability rates higher than 80% (Table 4), and the highest non-cytotoxic concentration of each compound was selected for the antiviral assay. Since medicarpin, neovestitol and vestitol at 14 µg/mL presented cytotoxicity (Table 4), and at 3 µg/mL showed no antiviral activity (Supplementary Figure S4), the alternative concentration of 11 µg/mL was selected to the further assays. Therefore, the antiviral activity of medicarpin, neovestitol and vestitol was tested at 11 µg/mL, guttiferone E and oblongifolin B at 6 µg/mL, and methylvestitol at 14 µg/mL. The compounds medicarpin, neovestitol and vestitol inhibited CHIKV replication in vitro in 86%, 94%, and 97% respectively (Fig. 7).

Toxicity assessment in *Caenorhabditis elegans*. To assess the toxicity of the BRP crude hydroalcoholic extract and isolated compounds in an in vivo system, the technique for determining the lowest concentration capable of killing 50% (LC₅₀) of the larvae in relation to the incubation time was employed. Figure 8 shows the toxicity evaluation of the BRP crude hydroalcoholic extract, oblongifolin B, and guttiferone E as a function of time and concentration. The LC₅₀ of the BRP crude hydroalcoholic extract and oblongifolin B was 1500 µg/mL, determined on the second day of incubation (Fig. 8A and B). On the other hand, guttiferone E had LC₅₀ of 750 µg/mL, determined on the last day of incubation (Fig. 8C).

Discussion

For years, propolis has been used to treat infections in folk medicine, and its antimicrobial potential has been demonstrated by the scientific community¹⁵. This biological potential can be related to its differentiated chemical composition.

Sesquiterpenes, pterocarpanes, and isoflavans characterize Brazilian red propolis. Red propolis chemical composition is much different from other propolis types, such as brown propolis, which is characterized by hydrocarbons, aldehydes, and monoterpenes; and green propolis, which is characterized by polycyclic aromatic hydrocarbons, sesquiterpenes, and naphthalene derivatives³¹.

Vestitol, neovestitol, and medicarpin have been reported as major compounds in red propolis from Canavieiras, Bahia State, Brazil. On the other hand, formononetin, calycosin, biochanin A, and isoliquiritigenin were detected at lower concentrations¹⁷. Guttiferone E and oblongifolin B were described as chemical markers of red propolis¹⁴, but they appear to be at lower concentrations in the studied sample compared to the isoflavans. The triterpenes β -amyrin and glutinol have also been described in BRP from this location¹⁴.

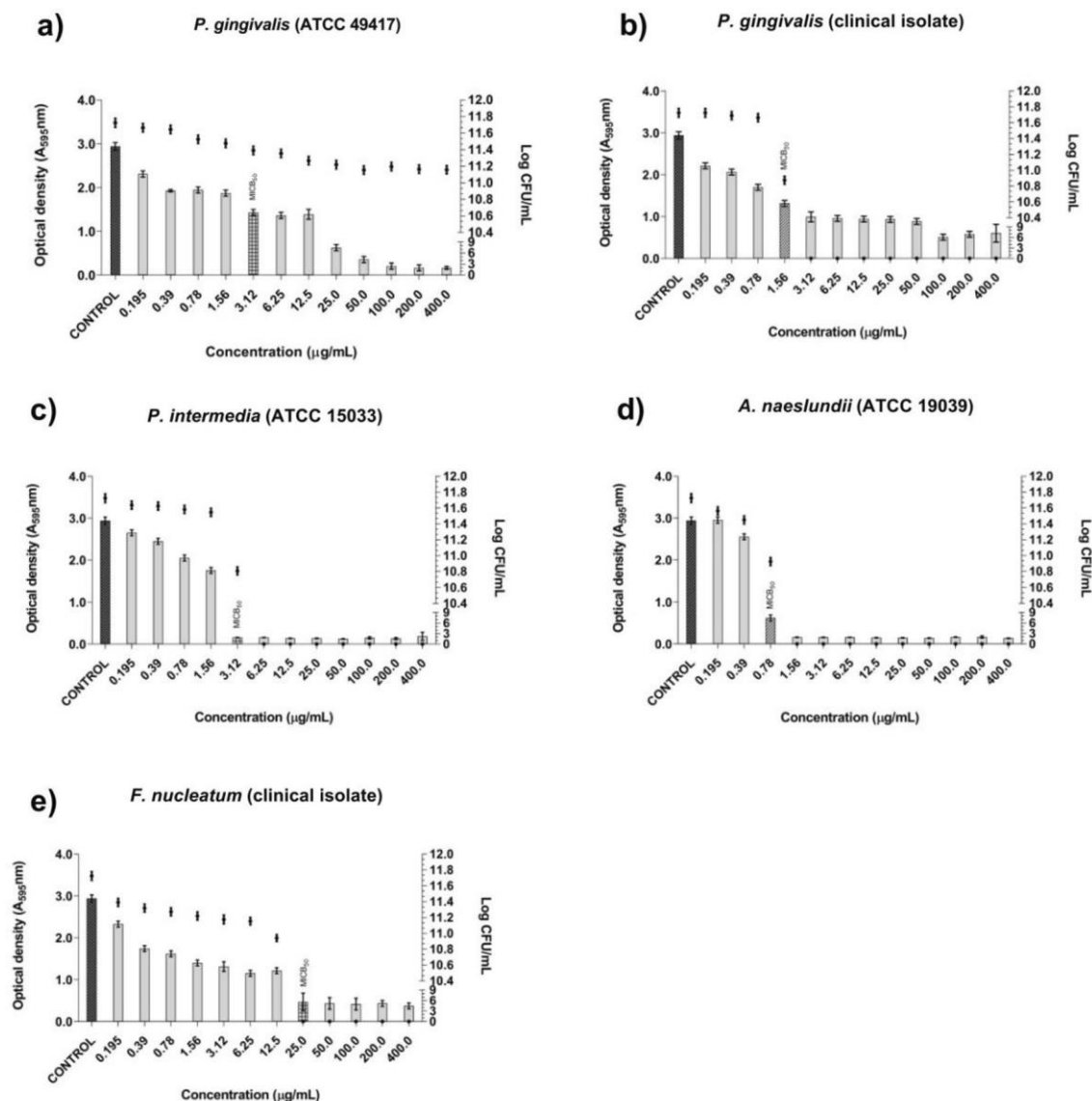


Figure 4. Antibiofilm activity of guttiferone E and number of viable cells in monospecies biofilms formed by ATCC strains and clinical isolates included in the study. (a) *P. gingivalis* (ATCC 49417). (b) *P. gingivalis* (clinical isolate). (c) *P. intermedia* (ATCC 15033). (d) *A. naeslundii* (ATCC 19039). (e) *F. nucleatum* (clinical isolate).

According to Rios and Recio³² and Gibbons³³, MIC values below 100 µg/mL for crude hydroalcoholic extract and below 10 µg/mL for isolated compounds are considered promising when evaluating the antibacterial activity of plant extracts, essential oils, and compounds isolated from natural sources. On the basis of these criteria and considering the MIC values presented here for all the evaluated BRP samples, the BRP crude hydroalcoholic extract and the isolated compounds guttiferone E and oblongifolin B displayed the best inhibition activity against most of the evaluated bacteria.

The red propolis dichloromethane fraction was not tested since the selection was based on the effect of the individual constituents of each fraction. The main compounds of hexane fraction, oblongifolin B and guttiferone E, displayed good activity at the individual testing, compared with the dichloromethane fraction individual compounds.

These samples showed antibacterial activity mainly against *P. gingivalis* (ATCC 49417), considered the most clinically important species in the development of periodontal disease³⁴ and *F. nucleatum* (clinical isolate) bacteria, also considered a relevant pathogen since it worsens gingival inflammation and tooth loss³⁵. These results demonstrated the relevance of these natural products in periodontal disease control and treatment. In this paper, the BRP crude hydroalcoholic extract, fractions (*n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol), and isolated compounds (methylvestitol, medicarpin, vestitol, neovestitol, oblongifolin B, and guttiferone E) were analyzed for their antibacterial activity against clinical isolates and the ATCC strains. The ATCC strains are more stable from a genetic viewpoint and would thus represent the bacterium species, thereby enabling comparison with other investigations. The *in vitro* assay furnishes a reliable indication of how the microorganism responds to the target agent, and extrapolation of the results for that species or even genus should be accepted. Clinical

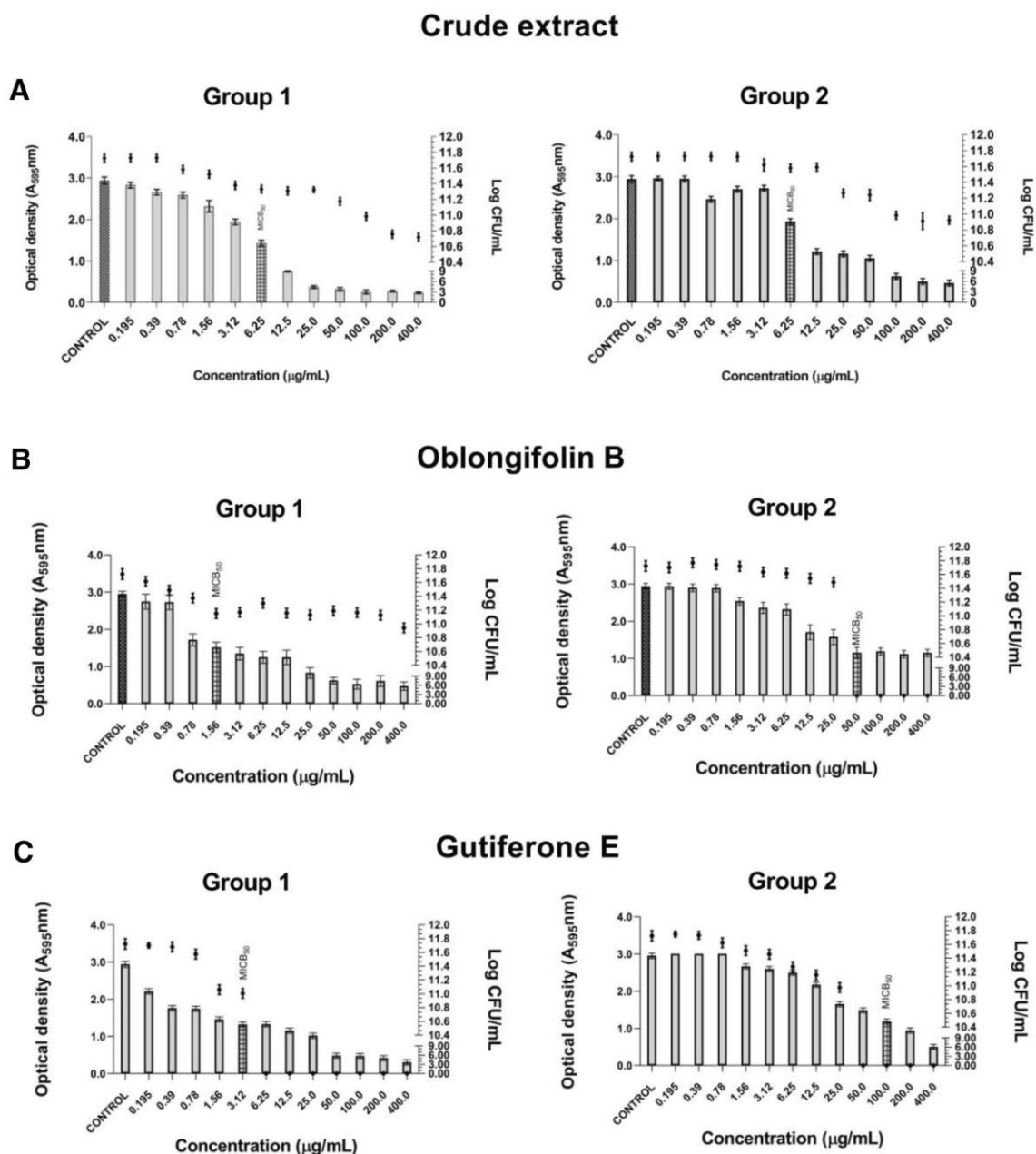


Figure 5. Antibiofilm activity of samples of Brazilian Red Propolis crude hydroalcoholic extract, oblongifolinB and guttiferone E and number of viable cells in multispecies biofilms formed by bacteria from groups 1 (standard strains) and 2 (clinical isolates). (A) Crude extract. (B) Oblongifolin B. (C) Guttiferone E.

Sample/concentration	50 µg/mL	10 µg/mL	2 µg/mL
Crude extract	57.3	83.2	99.3
n- Butanol	98.4	100.3	100.6
Dichloromethane	48.3	85.1	99.8
Hexane	45.7	83.3	100.0
Ethyl acetate	66.4	95.9	96.4

Table 3. Cell viability percentage in the presence of the BRP crude hydroalcoholic extract or fractions at 50, 10, and 2 µg/mL.

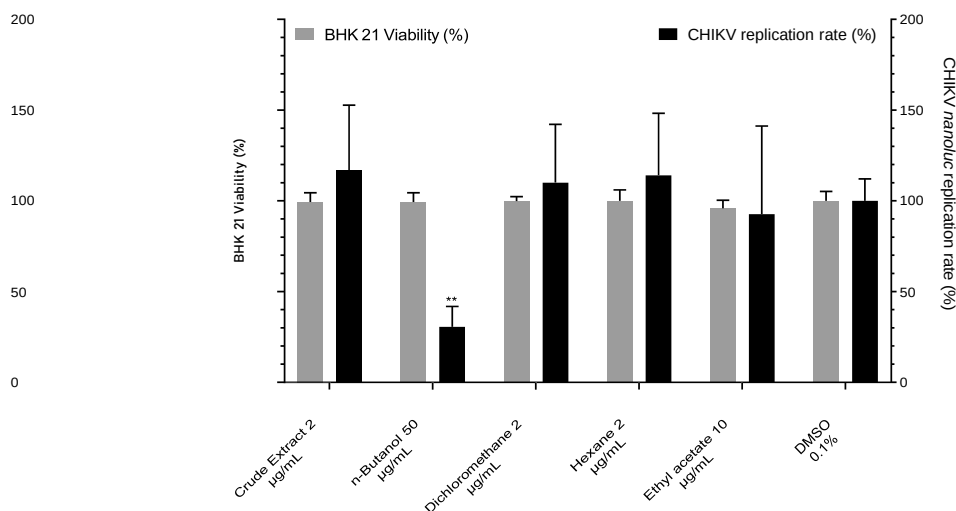


Figure 6. Cell viability and CHIKV replication rates in the presence of Brazilian Red Propolis crude hydroalcoholic extract and fractions.

Sample	Concentration (µg/mL)	Cell viability (%)
Medicarpin	14	84
	3	122
	0.5	113
Neovestitol	14	66
	3	110
	0.5	120
Vestitol	14	74
	3	117
	0.5	125
Oblongifolin B	32	72
	6	98
	1	99
Methylvestitol	14	108
	3	103
	0.5	103
Guttiferone E	32	15
	6	88
	1	90

Table 4. BHK-21 cell viability in the presence of the BRP isolated substances at concentrations ranging from 32 to 0.5 µg/mL.

isolates (also known as wild strains) are bacteria that can have their metabolism altered by environmental conditions and their genetics modified by circulation in the population, which would justify the relevance of evaluating these two types of strains. The MIC values (1.56–400 µg/mL) for the other evaluated bacteria were significantly lower as compared to literature data. Bueno-Silva et al.²⁹ evaluated the antibacterial activity of the crude extract and isolated compounds neovestitol and vestitol obtained from BRP from the same botanical origin against *A. naeslundii* (ATCC 12104), and they reported MIC values of 25, 25, and 50 µg/mL, respectively. Here, neovestitol and vestitol were not promising against several of the evaluated periodontal bacteria. Another point to emphasize is that the MIC value for the crude extract reported by Bueno-Silva et al.²⁹ against *A. naeslundii* (ATCC 12104) resembled the value we obtained against *A. naeslundii* (ATCC 19039), suggesting a species susceptibility relation for the crude extract. Santos et al.³⁶ evaluated the antibacterial activity of the aqueous hydroalcoholic extract and fractions (hexane, dichloromethane, and ethyl acetate) obtained from a different type of propolis, from the region of “Cachoeira da Prata”, Minas Gerais—Brazil, which is also collected from *Apis mellifera* bees. The tested bacteria were periodontal *F. nucleatum* (ATCC 10953), *P. gingivalis* (ATCC 33277), and *P. intermedia* (ATCC 25611). The extract gave MIC values of 1024, 256, and 256 µg/mL against *F. nucleatum* (ATCC 10953), *P. gingivalis* (ATCC 33277), and *P.*

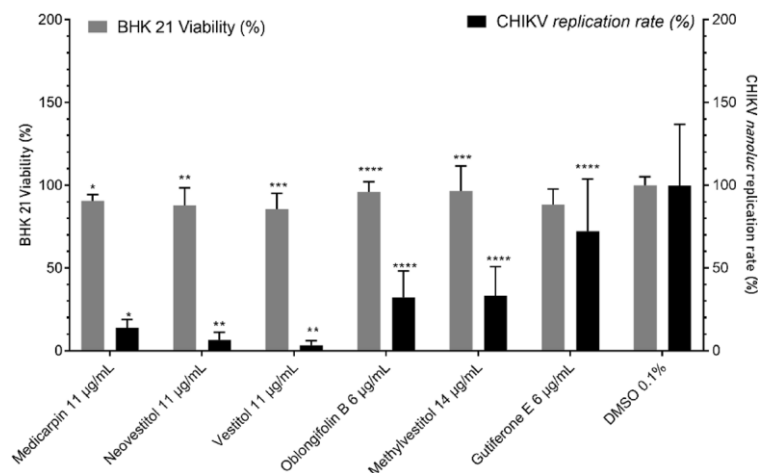


Figure 7. Effects of isolated compounds at on CHIKV infection and cell viability.

intermedia (ATCC 25611), respectively. As for the fractions, the MIC values ranged from 512 to > 1024 µg/mL. The MIC results against these bacteria were higher than those presented here for the BRP crude hydroalcoholic extract and fractions against the same bacterial species but from different strains: we found MIC values of 100, 3.12, and 6.25 µg/mL for the crude extract against *F. nucleatum* (ATCC 10953), *P. gingivalis* (ATCC 49417), and *P. intermedia* (ATCC 15033), respectively. As for the fractions, we found MIC values ranging from 12 to 400 µg/mL against the same bacteria. Therefore, compared to the results described by these authors, the BRP crude hydroalcoholic extract and fractions employed here were more effective against periodontal bacteria.

Additionally, these authors compared the MIC results they obtained with the crude extract and fractions by ANOVA analysis. They did not find any differences in the antibacterial activity of the fractions or extract against the evaluated bacteria. This corroborates our results: the BRP crude hydroalcoholic extract was more promising than the fractions and gave better values against all the evaluated bacteria, while the fractions presented anti-bacterial activity against only two bacteria (*P. gingivalis* ATCC 49417 and clinical isolate).

Shabbir et al.³⁷ evaluated the activity of the crude propolis extract collected in Skardu (Pakistan) originating from *Robinia pseudoacacia*, *Elegnus agustifolia* (Russian olive), and *Acacia modesta*, collected from *Apis mel-lifera* bees. The natural products afforded MIC values ranging from 64 to 512 µg/mL against *P. gingivalis* and *P. intermedia* clinical isolates. Our results suggested that BRP is more effective than the propolis used by Shabbir et al.³⁷ since the MIC values we obtained for the BRP crude hydroalcoholic extract against *P. gingivalis* and *P. intermedia* clinical isolates were lower (12.5 and 6.25 µg/mL, respectively). Therefore, BRP proved to have more promising activity than propolis from other countries given that it is composed of unique compounds that do not normally occur in other types of propolis¹⁵.

Inhibiting biofilm formation by these bacteria can contribute to reducing periodontitis. Indeed, Al-Ahmad et al.³⁸ described that *A. naeslundii* and *F. nucleatum* play the roles of initial colonizing bacterium and late colonizer, respectively. The latter bacterium was shown to be present at rates greater than 50% after 62 h of biofilm formation, contributing to increased inflammation and tooth loss³⁸. Other studies have evaluated the BRP monospecies antibiofilm activity against other types of bacteria. de Souza Silva et al.³⁹ evaluated the antibiofilm activity of the BRP crude hydroalcoholic extract (BRP collected in the same region as the BRP used in our study) coated on polymeric nanoparticles against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 33591), *Staphylococcus aureus* (ATCC 43300), and *Pseudomonas aeruginosa* (ATCC 27853). The free BRP crude hydroalcoholic extract and the extract coated on nanoparticles inhibited the biofilm formed by the Gram-positive strains more effectively as compared to the biofilm formed by the Gram-negative strains, with biofilm inhibitory concentration values ranging from 15.6 to 125 µg/mL against the *S. aureus* strains and from 100 to 1560 µg/mL against the *P. aeruginosa* strain. These results corroborated our findings given that we verified the lowest MIC₅₀ values against the Gram-positive bacterium *A. naeslundii* (ATCC 19039) 3.12 µg/mL for the crude extract and 0.78 µg/mL for the isolated compounds oblongifolin B and guttiferone E.

Miranda et al.¹³ evaluated the antibiofilm activity of the crude hydroalcoholic extract of BRP from the same botanical origin as the BRP used here. These authors divided the evaluated bacteria into complexes (*Actinomyces*, purple, yellow, green, orange, red, and others). At extract concentrations of 800 and 1,600 µg/mL, the authors showed a 40 and 45% decrease in the metabolic activity of the multispecies biofilms formed by these complexes, respectively. de Figueiredo et al.³⁰ also evaluated the antibiofilm activity of the BRP crude extract at 1600, 800, and 400 µg/mL and obtained 56, 56, and 57% reduction in the biofilm metabolic activity, respectively. Our study did not assess eradication, but it evaluated the ability of the BRP samples to inhibit biofilm formation. According to Wei et al.⁴⁰ inhibiting biofilm formation is much more important than eradicating it because biofilm formation inhibition prevents bacterial growth and, hence, bacterial maturation. The results presented here demonstrated that the BRP samples inhibited multispecies biofilm formation by periodontal bacteria by at least 50%. Oblongifolin B gave the lowest MIC₅₀ value (1.56 µg/mL) against the multispecies biofilm formed by the ATCC strains.

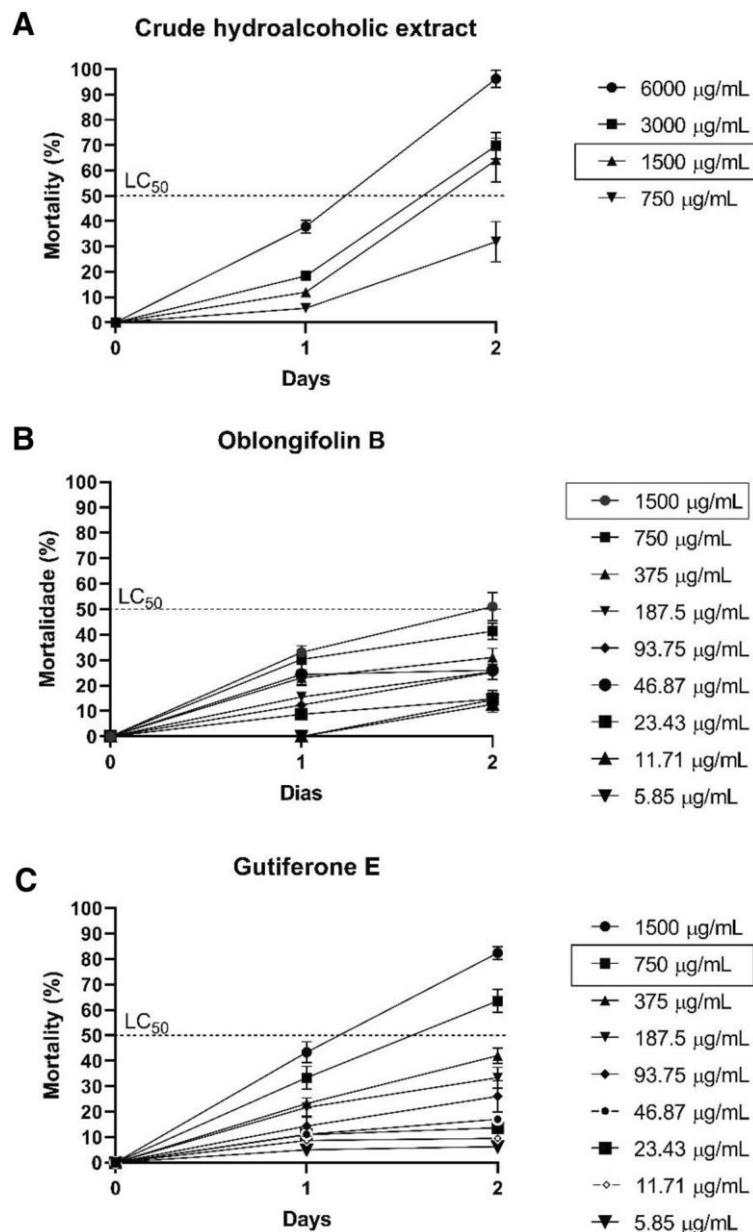


Figure 8. Evaluation of the toxicity of the Brazilian Red Propolis crude hydroalcoholic extract and isolated compounds guttiferone E and oblongifolin B in the *C. elegans* in vivo model. (A) Crude hydroalcoholic extract. (B) Oblongifolin B. (C) Guttiferone E.

As for the multispecies biofilm formed by the clinical isolates, the lowest MICB₅₀ value was 6.25 µg/mL. These results suggested that the isolated compounds oblongifolin B and guttiferone E inhibited all the viable cells of most monospecies and multispecies biofilms formed by the bacteria included in this study. This pointed out that the BRP samples can inhibit biofilm formation and reach the cells within this bacterial community, eliminating them and leaving only the glycoprotein conjugate without live cells¹³.

It is worth mentioning that the MICB₅₀ values found in this paper are relatively low, especially for clinical isolates that are generally more resistant and demand higher concentrations. However, at the lowest concentration capable of inhibiting biofilm formation by at least 50%, the so-called MICB₅₀, we demonstrated an inhibition of at least 50% of the biofilm. In other words, this did not correspond to total inhibition of the biofilm, which would probably require a higher concentration.

Propolis bioactive components such as flavonoids, esters, alcohols, essential oils, and other organic compounds have already been demonstrated to display antiviral activity against viruses such as herpes viruses (HSV-1 and HSV-2), sindbis virus, parainfluenza virus, cytomegalovirus, HIV, and *Varicella zoster* (HSV-1 and HSV-2), sindbis virus, parainfluenza virus, cytomegalovirus, HIV, and *Varicella zoster*^{41,42}. In addition to the BRP antibacterial and antibiofilm activities demonstrated in this study, we evaluated the anti-CHIKV activity of the

BRP crude hydroalcoholic extract, fractions, and pure substances. We assessed the BHK-21 cell viability in the presence of the BRP samples by the MTT assay. In drug discovery, samples are considered non-toxic when the cell viability rate is above 50%, moderately cytotoxic when the cell viability rate varies between 25 and 50%, and highly cytotoxic when the cell viability rate is less than 25%⁴³. In this study, all samples evaluated showed cell viability equal to or higher than 80% at a concentration of 50 µg/mL for the crude extract and fractions and 3 µg/mL for isolated compounds (Tables 3 and 4). All the BRP samples evaluated in this study provided BHK-21 cell viability equal to or higher than 80% (Tables 3 and 4), which was higher than the cell viability found by Rufatto et al.²⁰, (between 14.5 and 46%).

Regarding the infection assays, the isolated compounds neovestitol and vestitol furnished the most promising results, with virus infection inhibition rates of 94 and 97%, respectively (Fig. 7). Even though several natural compounds with antiviral activity, including anti-CHIKV activity, have been described, neovestitol and vestitol have not had their antiviral potential screened. Our results showed higher inhibition rates than those reported in other studies with natural molecules, such as the study of Pohjala et al.⁴⁴, who obtained an infection inhibition limit of at most 75% when they screened 356 compounds, being 123 of them natural compounds. To the best of our knowledge, there are no reports on the anti-CHIKV activity of BRP or isolated compounds. This shows the importance of capitalizing the BRP potential as candidate for antiviral treatment. Our study has pioneered evaluation of the BRP anti-CHIKV activity and has achieved expressive inhibition rates, paving the way for the development of antivirals against CHIKV as well as other viruses. For BRP to be safely applicable, its toxicity must be evaluated in different experimental models. The murine model is the most used in vivo model to assess the toxicity of treatments, but it has disadvantages such as high cost, difficult maintenance, and delay in obtaining results, among others⁴⁵. Therefore, here we evaluated toxicity by using another in vivo model, the nematode *C. elegans*, a complete animal with digestive, reproductive, endocrine, and neuromuscular systems. Apart from being small, having a short life cycle, and being easy to maintain, *C. elegans* possesses 60–80% genetic homology with humans⁴⁶. In this context, we evaluated the most promising BRP samples for their toxicity against *C. elegans*. The lowest concentration capable of killing at least 50% of the larvae (LC₅₀) was 1500 µg/mL for the BRP crude hydroalcoholic extract and oblongifolin B and 750 µg/mL for guttiferone E. These values were significantly higher than all the MIC and MIC₅₀ concentrations reported in this study.

Moreover, below this concentration, even after the larvae had been exposed to BRP samples for two days, LC₅₀ was not reached, demonstrating the non-toxic profile of these natural products. Interestingly, the LC₅₀ values of the BRP samples against *C. elegans* obtained in this study were higher than the LC₅₀ values of other types of Brazilian propolis evaluated against *C. elegans*. For example, Campos and collaborators (2015)⁴⁷ reported that propolis samples possessed LC₅₀ of 461.8 µg/mL. Here, the BRP concentrations determined as toxic were high, above the highest MIC value (400 µg/mL). Therefore, propolis is not toxic at the concentrations used in this study and can be safely employed at concentrations below 1500 and 750 µg/mL. The results obtained here are extremely relevant, because through different methodologies the antibacterial activity of Brazilian red propolis was demonstrated against a panel of periodontopathogenic bacteria. Another point to highlight is the anti-CHIKV activity of the BRP isolated compounds. *Chikungunya* infection has a high incidence and severity, therefore, the search for new treatment options is highly desirable. Our results constitute an initial step for further studies of BRP as an alternative approach for treating various infectious diseases.

Conclusion

The Brazilian red propolis used in this study has antibacterial activity against a panel of periodontopathogenic bacteria. Furthermore, its crude extract and isolated compounds oblongifolin B and guttiferone E at concentrations similar to or slightly above the MIC concentrations inhibits monospecies and multispecies biofilms by over 50%. Medicarpin, neovestitol, and vestitol strongly inhibit CHIKV infection in vitro. Besides, toxicity tests on *C. elegans* demonstrated that the crude extract, oblongifolin B, and guttiferone E are non-toxic, proving to be safe and promising so that in the future, these samples of propolis can be used as medicine.

Methods

Crude hydroalcoholic extract, fractions, and isolated compounds. BRP was collected in Canavieiras Bahia State, Brazil, in March 2019 at the Canavieiras Beekeepers Association (COAPER). BRP was frozen and extracted with 70% hydroalcoholic ethanol solution, as described by Santiago et al.⁴⁸. The BRP crude hydroalcoholic extract was partitioned with organic solvents (hexane, dichloromethane, ethyl acetate, and *n*-butanol). Authentic standards from BRP (7-*O*-methylvestitol, medicarpin, vestitol, neovestitol, oblongifolin B, and guttiferone E) previously isolated by our research group were used to characterize the samples¹⁷.

Chromatographic analysis of BRP extract and its fractions were performed on a Waters 2695 HPLC instrument, coupled to a 2998 photodiode array detector (PDA), with Empower 3 software as a controller. Chromatographic profiles were carried out on a Supelco Ascentis Express C-18 (150 × 4.6 mm, 2.7 µm) column. Mobile phase with water (A) (0.1% formic acid) and acetonitrile (B) was used as follows: 10 → 100% of B until 80 min; 100% of B in 89 min; 100 → 10% in 90 min, maintaining the condition until 95 min. The injections were performed on a flow rate of 1 mL/min, a 40 °C, and an injection volume of 10 µL. Chromatograms were recorded at 275 nm.

For the antibacterial, antiviral, and toxicity assays were used the crude hydroalcoholic extract of BRP, fractions in dichloromethane, hexane, ethyl acetate, *n*-butanol, as well as the isolated compounds guttiferone E, oblongifolin B, methylvestitol, medicarpin, vestitol, and neovestitol.

Bacterial strains, *Chikungunya* virus and animal model employed in the study. The periodontopathogenic bacterial strains employed in the antibacterial and antibiofilm activity assays were obtained from the American Type Culture Collection (ATCC); their respective clinical isolates were obtained from human peri-odontal infections. The strains included *Porphyromonas gingivalis* (ATCC 49417 and clinical isolate), *Fusobacterium nucleatum* (ATCC 10953 and clinical isolate), *Prevotella intermedia* (ATCC 15033 and clinical isolate), and *Actinomyces naeslundii* (ATCC 19039 and clinical isolate). These bacteria are part of the collection of the Antimicrobial Assays Laboratory (LEA, abbreviation in Portuguese) of the Federal University of Uberlândia (UFU) and were cryopreserved at -20°C . For the in vivo toxicity assays, the mutant strain *Caenorhabditis elegans* AU37, obtained from the Genetics Center (CGC, University of Minnesota), was used.

For the antiviral assays, a CHIKV expressing the *Nanoluciferase* reporter (CHIKV-*nanoluc*) based on the CHIKV LR2006PPY1 strain (East/Central/South African genotype) was rescued⁴⁹. The protocols were carried out as described previously⁵⁰.

Determination of the minimum inhibitory concentration⁵¹. The antibacterial activity of the BRP crude hydroalcoholic extract, fractions, and isolated compounds were evaluated by the broth microdilution method, in triplicate. The assays were conducted in 96-well microplates; the methodology recommended by the Clinical and Laboratory Standards Institute⁵², with modifications, was followed. The inoculum was standardized to the McFarland 0.5 scale and diluted to a bacterial concentration of 1.5×10^6 CFU/mL in the wells. To prepare the samples, the BRP crude hydroalcoholic extract, fractions, or isolated compounds were solubilized in 5% dimethyl sulfoxide (DMSO) and diluted in Brucella broth supplemented with hemin (5.0 mg/mL) and menadi-one (1.0 mg/mL); a twofold serial dilution with concentrations ranging from 0.195 to 400 $\mu\text{g/mL}$ was used. Control of 5% DMSO was performed, and the solvent did not interfere with bacterial growth at this concentration. It was also performed the following controls: inoculum (all the bacteria used in the test + the culture medium), to observe the viability of the bacteria; broth, to guarantee that the culture medium is sterile; and BRP sample, to guarantee that this solution is sterile. The microplates were incubated in an anaerobic chamber (Don Whitley Scientific, Bradford, U.K.) under anaerobic conditions (80% N_2 , 10% CO_2 , and 10% H_2) at 37°C for 72 h. Resazurin was used to reveal bacterial growth—the blue color indicated absence of bacterial growth, and the pink color indicated presence of bacteria⁵³. As a control technique, metronidazole from 0.0115 to 5.9 $\mu\text{g/mL}$ was used against the control bacteria *Bacteroides fragilis* (ATCC 25285) and *Bacteroides thetaiotaomicron* (ATCC 29741)⁵².

Evaluation of antibiofilm activity monospecies and multispecies by Minimum Inhibitory Concentration of Biofilm (MICB₅₀). To assess the antibiofilm activity, the BRP samples that presented the most promising MIC results against four or more bacteria were submitted to the Minimum Inhibitory Concentration of Biofilm (MICB₅₀) assay. MICB₅₀ is defined as the lowest concentration of the microbial agent that can inhibit biofilm formation by at least 50%⁴⁰ and is calculated using the following equation:

$$1 - \frac{\text{Absorbance (595nm) of the well containing the treated sample}}{\text{Absorbance (595nm) of the untreated control well}} \times 100$$

Here, MICB₅₀ was determined as described in the CLSI guidelines (2007)⁵², with modifications. First, the capacity of the analyzed strains to grow in the sessile mode was verified. All the strains at 1.5×10^6 CFU/mL formed monospecies and multispecies biofilms after incubation at 37°C for 72 h (data not shown).

For the monospecies biofilms, 100 μL of each bacterium inoculum at 1.5×10^6 CFU/mL was added to the well with the propolis samples to be evaluated at concentrations from 0.195 to 400 $\mu\text{g/mL}$ (crude hydroalcoholic extract, oblongifolin B and guttiferone E). The microplates were incubated in an anaerobic chamber at 37°C for 72 h. For the multispecies biofilms, the main periodontopathogenic bacteria found in the oral biofilm were selected and divided into two groups: group 1 consisted only of the standard bacteria (*P. gingivalis* ATCC 49417, *P. intermedia* ATCC 15033, and *A. naeslundii* ATCC 19039), while group 2 was composed only by the *P. gingivalis*, *P. intermedia*, and *F. nucleatum* clinical isolates. The antibiofilm activity of the most promising BRP samples was evaluated against the multispecies biofilm formed by group 1 bacteria and against the multispecies biofilm composed by group 2 bacteria. For this purpose, 33.33 μL of each evaluated bacterium, totaling 100 μL of bacterial inoculum, at 1.5×10^6 CFU/mL was added to the wells with the propolis samples to be evaluated at concentrations from 0.195 to 400 $\mu\text{g/mL}$ (crude hydroalcoholic extract, oblongifolin B and guttiferone E). The microplates were incubated under the same conditions as the monospecies biofilm microplates. The standard antibiotic metronidazole was used as a control at concentrations from 0.0115 to 5.9 $\mu\text{g/mL}$ with MIC₅₀ (see supplementary material, Figures S2 and S3). Control of 5% DMSO was performed, and the solvent did not interfere with bacterial growth at this concentration. It was also performed the following controls: inoculum (all the bacteria used in the test + the culture medium), to observe the viability of the bacteria; broth, to guarantee that the culture medium is sterile; and BRP sample, to guarantee that this solution is sterile. After incubation, the supernatant culture was withdrawn, and the planktonic cells were removed by washing the wells with ultrapure distilled water. Monospecies and multispecies biofilms were fixed with methanol and stained with 2% crystal violet⁵⁴. The reading was performed in a microplate reader (GloMax®) at 595 nm. Reading was performed in a microplate reader (GloMax®) at 595 nm. The experiments were carried out in triplicate and independent events.

Evaluation of the inhibition of biofilm formation by counting microorganism. This assay was performed for monospecies and multispecies biofilms according to de Souza Silva et al.³⁹, as described below. Two microplates were incubated, one for MICB₅₀ determination, and the other for microorganism count. After the microorganism count microplate was incubated, the supernatant was withdrawn, and the planktonic cells

were removed by washing the wells with ultrapure distilled water. Subsequently, supplemented Brucella broth was added to all the microplate wells, and the biofilm was detached from the well after an ultrasound bath. Then, tenfold serial dilutions were performed in each well of a 96-well microplate, and 50 μ L of each well, corresponding to each dilution available was placed on two plates of Brucella agar supplemented with horse blood (5%), hemin (5.0 mg/mL), and menadione (1.0 mg/mL). Each of the plates were fractionated into eight parts, as described by Harrison et al.⁵⁵ and incubated in an anaerobic chamber for 37 °C. After 72 h, the Colony Forming Units (CFU) count was performed in each plate. The results were expressed as Log₁₀ (CFU/mL), and the assays were independently performed in triplicate.

Mammalian cells for antiviral assays. The BHK-21 cells (fibroblasts derived from Syrian golden hamster kidney; ATCC CCL-10) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 100 U/mL penicillin (Hyclone Laboratories), 100 mg/mL streptomycin (Hyclone Laboratories), 1% dilution of stock of non-essential amino acids (Hyclone Laboratories), and 1% fetal bovine serum (FBS, Hyclone Laboratories) in a humidified 5% CO₂ incubator at 37 °C.

Cell viability through MTT for antiviral assays. BHK-21 cell

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viability in the presence of the tested BRP samples was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich) assay. BHK-21 cells were cultured in 48-well microplates and treated with different concentrations of the tested BRP sample at 37 °C for 16 h. Then, media containing the tested BRP sample was removed from the 48-well microplate. Next, 1 mg/mL MTT solution was added to each well, incubated for 30 min, and replaced with 300 μ L of DMSO to solubilize the formazan crystals. Absorbance was measured at 490 nm on a Glomax microplate reader (Promega). Cell viability was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the optical density of the treated well and control groups, respectively. DMSO was used as untreated control⁵⁰.

Antiviral activity against CHIKV infection in vitro. For initial screening of the anti-CHIKV activity of the BRP crude hydroalcoholic extract and isolated compounds, HK-21 cells were seeded at a density of 5×10^4 cells per well in 48-well microplates 24 h before the infection. CHIKV-nanoluc at a multiplicity of infection⁵⁶ of 0.1 and the tested isolated compound or extract were simultaneously added to the cells. The cells were harvested in Renilla luciferase lysis buffer (Promega) 16 h post-infection (h.p.i.), and virus replication was quantified by measuring nanoluciferase activity with the Renilla luciferase Assay System (Promega). The CHIKV replication rates were calculated according to the equation $(T/C) \times 100\%$, where T and C represent the optical density of the treated well and control groups, respectively. DMSO 0.1% was used as untreated control.

Toxicity assessment in *Caenorhabditis elegans*. Toxicity evaluation was performed for the most promising BRP samples in the CIM, using the in vivo model of *C. elegans*, according to Andrade et al.⁵⁷ and Singulani et al.⁵⁸. The *C. elegans* AU37 mutant strain was cultivated in Nematode Growth Medium (NGM) plates seeded with *Escherichia coli* OP50 and incubated at 16 °C for 72 h. After incubation, the NGM plates containing larvae and eggs were washed with M9 buffer, and the supernatant was placed in 15-mL conical tubes. A bleaching solution (hypochlorite + NaOH) was further added, to kill the adult larvae. The eggs were placed in NGM plates and incubated again at 15 °C for 24 h. Later, the NGM plates containing the larvae at the L1/L2 stages were washed with M9 buffer, and the supernatant was transferred to NGM plates seeded with *E. coli* OP50 and incubated at 16 °C for 24 h. After

synchronization, 20 μ L of the NGM plate contents containing from 10 to 20 L4 stage larvae was added to each well of a 96-well flat-bottomed microplate and incubated at 16 °C for 72 h. The BRP crude hydroalcoholic extract was evaluated from 750 to 6000 μ g/mL, and the isolated compounds oblongifolin B and guttiferone E were evaluated from 5.85 to 1500 μ g/mL. DMSO was used as solvent (final concentration $\leq 1\%$).

Larvae were counted every 24 h for three consecutive days under an inverted microscope. Larvae with movement were considered alive and static even after touching they were considered dead. For each sample, the lowest concentration that was able to kill 50% of the larvae, called Lethal Concentration (LC₅₀), was determined according to time.

Statistical analysis. Individual experiments were performed in triplicate, and all the assays were performed a minimum of three times to confirm the reproducibility of the results. Differences between the means of the readings were compared by analysis of variance (one-way or two-way ANOVA) or Student's t-test conducted with the software Graph Pad Prism 8.0 (Graph Pad Software). The *p* values ≤ 0.05 were considered statistically significant.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

N.B.S.S. designed the experiments, functions/Writing—original draft; writing—proofreading and editing. J.H.S. designed the experiments and carried out the experiments. M.B.S. designed the experiments and carried out the experiments. J.R.S.A. designed the experiments and analysed the results. R.A.S. designed the experiments and carried out the experiments. I.A.S. analysed the results and Writing—proofreading and editing. J.A.A. designed the experiments and carried out the experiments and Analysed the results and Writing—proofreading and editing. A.C.J. Analysed the results and Writing—proofreading and editing. R.P.S. Writing—proofreading and editing, methodology, supervision. S.R.A. Acquisition of financing, writing—proofreading and editing. R.C.S.V. Writing—proofreading and editing, acquisition of financing. J.K.B. Writing—proofreading and editing, acquisition of financing. R.H.P. Methodology. C.H.G.M. Conceptualization, formal analysis, methodology, supervision, validation; visualization, functions/ Writing—original draft; writing—proofreading and editing. All authors gave the final approval for publication.

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Competing interests

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Insights into the role of the cobalt(III)-thiosemicarbazone complex as a potential inhibitor of the Chikungunya virus nsP4

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Abstract

Chikungunya virus (CHIKV) is the causative agent of chikungunya fever, a disease that can result in disability. Until now, there is no antiviral treatment against CHIKV, demonstrating that there is a need for development of new drugs. Studies have shown that thiosemicarbazones and their metal complexes possess biological activities, and their synthesis is simple, clean, versatile, and results in high yields. Here, we evaluated the mechanism of action (MOA) of a cobalt(III) thiosemicarbazone complex named $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ based on its in vitro potent antiviral activity against CHIKV previously evaluated (80% of inhibition on replication). Furthermore, the complex has no toxicity in healthy cells, as confirmed by infecting BHK-21 cells with CHIKV-*nanoluciferase* in the presence of the compound, showing that $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ inhibited CHIKV infection with the selective index of 3.26. $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ presented a post-entry effect on viral replication, emphasized by the strong interaction of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ with CHIKV non-structural protein 4 (nsP4) in the microscale thermophoresis assay, suggesting a potential mode of action of this compound against CHIKV. Moreover, in silico analyses by molecular docking demonstrated potential interaction of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ with nsP4 through hydrogen bonds, hydrophobic and electrostatic interactions. The evaluation of ADME-Tox properties showed that $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ presents appropriate lipophilicity, good human intestinal absorption, and has no toxicological effect as irritant, mutagenic, reproductive, and tumorigenic side effects.

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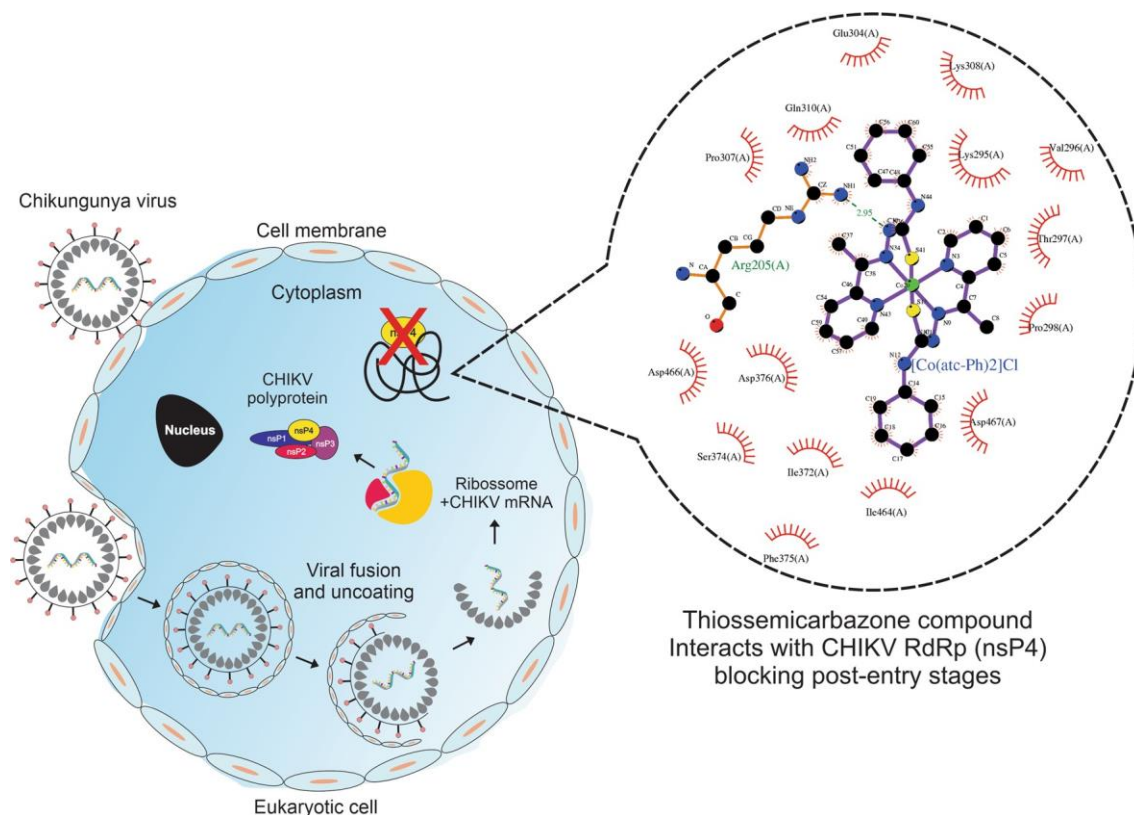
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Graphical abstract



Keywords Metal ion · Antiviral activity · Chikungunya virus · Mechanism of action · Molecular docking · ADME-Tox

Introduction

The chikungunya virus (CHIKV) is the etiological agent of chikungunya fever, a tropical disease characterized by high fever, rash, joint pain, and arthralgia [1]. The virus is transmitted through the bite of *Aedes* mosquitoes and the infection caused by CHIKV can progress to a disabling chronic disease or may cause neurological complications such as Guillain–Barre syndrome [2, 3].

CHIKV is an alphavirus that belongs to the family *Togaviridae* (ICTV, 2019). It is an enveloped virus of icosahedral capsid and a positive-sense single-strand RNA of approximately 12 kb [4]. CHIKV genome contains two open reading frames (ORFs) [4]. The 5' ORF encodes four non-structural proteins (nsP1–nsP4) and the 3' ORF is transcribed into a subgenomic RNA which is translated into five structural proteins (C, E1, E2, E3, and 6 K) [5, 6]. The non-structural proteins play substantial roles in the virus replication forming a replicase complex [7], nsP4 being defined as the CHIKV RNA-dependent RNA polymerase (RdRp). In this way, CHIKV replication is directly dependent on nsP4 activity that represents a promising target for antiviral therapeutic

[8]. Therefore, disruption or inactivity of the nsP4 activity can result in the impairment of the CHIKV replicative cycle, decreasing virus replication and also impacting in the clinical progress of Chikungunya fever.

CHIKV was first isolated in Tanzania in 1952 and, for a while, cases related to CHIKV occurred in the Southeast Asian regions, Africa, and Oceania [9]. In 2006, an outbreak was documented in some Indian Ocean islands [10] and, a year later, France and Italy reported cases of this infection [11, 12]. In 2013, an outbreak occurred in Central America, in the Caribbean Islands [13, 14]. Then, the virus spread through North and South America [15]. In Brazil, according to the Urban Arboviruses Monitoring Report, 132,205 cases of chikungunya fever were registered in 2019, with an incidence rate of 62.9 cases/per 100,000 inhabitants in 2019. Northeast and Southeast were the regions with the highest incidence rates in the Country, with 104.6 and 59.4 cases/100,000 inhabitants, respectively. In the same year, 92 deaths were confirmed by CHIKV infection, and a higher lethality rate was observed in elderly people over 60 [16]. In 2020, during the outbreak of COVID-19, the Brazilian states of Bahia, Mato Grosso, Espírito Santo, and Rio de Janeiro

showed an increase in likely cases of chikungunya, according to the bulletin released by the surveillance secretary [17]. Furthermore, according to CDC (Centers for Disease Control), over 110 countries reported CHIKV infection cases until October 2020 [18].

Currently, there is no licensed treatment against CHIKV showing the need for new molecules that possess antiviral activity [19]. The treatment of infected patients is palliative and aims to relieve the symptoms caused by the infection. According to the World Health Organization (WHO), the most appropriate drug is acetaminophen 1 g 3–4 times per day for adults, and 50–60 mg per kg per day for children [20]. Other non-steroidal analgesics are also recommended, except aspirin which can cause platelet dysfunction [21].

Thiosemicarbazones (TSCs) are a class of synthetic compounds known to have versatile chemistry, low cost of production, and few atoms waste (only molecules of water are usually released during synthesis reactions) [22]. Teitz and coworkers (1994) described the antiviral activity of *N*-methylisatin- β 4':4'-diethylthiosemicarbazone, a compound that demonstrated to disrupt the synthesis of Human Immunodeficiency Virus (HIV) structural proteins. TSCs derivatives have also demonstrated antiviral activity described against herpes simplex virus (HSV), affecting the expression of structural proteins of HSV, suppressing capsid assembly, and inhibiting cell-to-cell spread [23].

Besides that, TSCs are also recognized as excellent metal binders, therefore, being widely explored as ligands in coordination chemistry. It also provides very stable and high-quality chelating organometallics [24–26]. Interestingly, TSCs metal complexes have demonstrated antimicrobial [27], antiprotozoal [28], and anticancer [29], and antiviral activities [30]. Usually, thiosemicarbazones are coordinated with metals such as Copper(II) and cobalt(III). These metals are present in the human organism, therefore, representing pre-existing biological pathways that will regulate their therapeutic levels. For example, cobalt(III) is an essential trace element in the human body and also is a necessary component of vitamin B₁₂ [31]. Physiological speciation involving these endogenous metal ions can increase the bio-availability of these compounds, decrease the cytotoxicity and even improve their biological activity [32].

Considering our previous findings on the antibacterial action and preliminary anti-CHIKV activities of the complexes of the type $[\text{Co}^{\text{III}}(\text{atc-R})_2]\text{Cl}$ (R = methyl, Me or phenyl, Ph), as well as the free ligands [33], here we further characterized the anti-CHIKV activity of the cobalt(III) coordination compound with the thiosemicarbazone ligand $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ (Fig. 1) and exploited its potential mode of action. Our data show that cobalt(III)-conjugated thiosemicarbazones may provide an interesting source of compounds for the development of future antivirals to treat chikungunya fever.

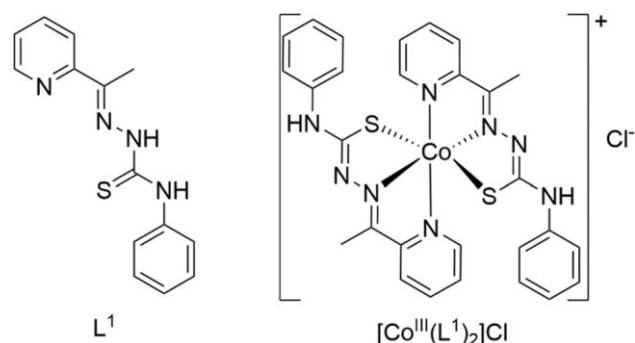


Fig. 1 Chemical structure of the TSC free ligand L^1 and its cobalt(III) complex $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$

Materials and methods

Preparation of the compounds

The compounds studied here were previously synthesized and characterized as described by Fernandes and coworkers [34]. L^1 was prepared by refluxing 4-phenyl-3-thiosemicarbazide and 2-acetylpyridine (1:1) in ethanolic solutions [35]. Briefly, the reaction of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and L^1 (1:2) in ethanol (15 mL) provided $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ (Fig. 1). The products were characterized and purities evaluated [33, 34]. The characterization of the compounds was previously certified by techniques such as ^1H and ^{13}C NMR, high-resolution mass spectrometry, LC–MS/MS and fragmentation study, and previously reported [34]. The compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C for the biological assays. Dilutions of the compounds in a complete medium were made immediately prior to the experiments to reach a maximum final concentration of 0.1% DMSO. For all the assays performed, cells were treated with DMSO 0.1% as untreated control.

Cell culture

BHK-21 cells (fibroblasts derived from Syrian golden hamster kidney; ATCC® CCL-10™), which are susceptible to CHIKV infection [36], were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 100 U/mL of penicillin (Hyclone Laboratories, USA), 100 mg/mL of streptomycin (Hyclone Laboratories, USA), 1% of non-essential amino acids (Hyclone Laboratories, USA) and 1% of fetal bovine serum (FBS, Hyclone Laboratories, USA) in a humidified 5% CO_2 incubator at 37°C .

Rescue of CHIKV-nanoluc reporter virus

The CHIKV expressing *nanoluciferase* reporter (CHIKV-*nanoluc*) used for the antiviral assays were designed from a CHIKV sequence based on CHIKV isolate LR2006OPY1 (East/Central/South African genotype). The cDNA plasmid of CMV-CHIKV-*nanoluc* contains a CMV promoter, SV40 terminator, and a sequence encoding *nanoluciferase* protein inserted into the region encoding the C-terminal domain of viral nsP3 protein [37, 38]. The production of CHIKV-*nanoluc* virions was carried out as previously described [39–41]. Briefly, 2.3×10^7 BHK-21 cells seeded in a cell culture flask were transfected with 1.5 μ g of CMV-CHIKV-*nanoluc* plasmid, using lipofectamine 3000® and Opti-Mem medium to rescue CHIKV *nanoluc* reporter virus. Forty-eight hours post-transfection, the supernatant was collected and stored at -80°C .

To determine the viral titer, 5×10^5 BHK-21 cells were seeded in each of 6 wells plate 24 h prior to the infection. Then, the cells were infected with tenfold serially diluted supernatant of CHIKV *nanoluc* for 1 h at 37°C . The inoculum was removed, and the cells were washed with phosphate-buffered saline (PBS) to remove the unbound virus and added of cell culture media supplemented with 1% penicillin, 1% streptomycin, 2% FBS and 1% carboxymethyl cellulose (CMC). Infected cells were incubated for 2 days in a humidified 5% CO_2 incubator at 37°C , followed by fixation with 3% formaldehyde and stained with 0.5% violet crystal and the viral foci were counted to determine CHIKV-*nanoluc* titers [42].

Cell viability through MTT assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich) assay. BHK-21 cells were cultured in 96 well plate and treated with concentrations of each compound for 16 h at 37°C with 5% of CO_2 . Twenty-four hours post-treatment, compound-containing media was removed and MTT solution at 1 mg/mL was added to each well, incubated for 1 h, and replaced with 100 μL of DMSO to solubilize the formazan crystals. The absorbance was measured at 560 nm on Glomax microplate reader (Promega, USA). Cell viability was calculated according to the equation $(T/C) \times 100\%$, in which T and C represented the optical density of the treated well and control groups, respectively. DMSO was used as untreated control. The cytotoxic concentration of 50% (CC_{50}) was calculated using GraphPad Prism 8 (Graph Pad Software).

Antiviral activity assays

BHK-21 cells were seeded at a density of 5×10^4 cells per well into 48-well plates 24 h prior to the infection. CHIKV-*nanoluc* [38, 41] at a multiplicity of infection (M.O.I) of 0.01 and compounds at the non-cytotoxicity concentration were simultaneously added to cells. Samples were harvested in Renilla luciferase lysis buffer (Promega, USA) at 16 h post-infection (h.p.i.) and virus replication levels were quantified by measuring *nanoluciferase* activity using the Renilla luciferase Assay System (Promega, USA). The effective concentration of 50% inhibition (EC_{50}) was calculated using GraphPad Prism 8. The values of CC_{50} and EC_{50} were used to calculate the selectivity index ($\text{SI} = \text{CC}_{50}/\text{EC}_{50}$).

To evaluate the protective activity of the compound, cells were pre-treated for 1 h with 50 μM of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$, extensively washed with PBS to remove the compound, and infected with CHIKV-*nanoluc* (M.O.I. of 0.01). The effect of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ on early stages of infection was analyzed by incubating the virus and compound simultaneously with BHK-21 cells for 1 h. Then, the compound was removed, and cells were added of fresh medium. Additionally, to investigate the activity of the compound in the post-entry stages of the viral replicative cycle, cells were infected with CHIKV for 1 h, washed extensively with PBS to remove the unabsorbed virus, and added of compound-containing media. Luminescence levels were measured 16 h.p.i. to analyze the virus replication rates.

Intercalation assay

To investigate whether the compound interacts with the double-stranded RNA (dsRNA), an experiment using the previously described protocol [43, 44] was performed. Briefly, fifteen nanograms of the dsRNA were incubated with 50 μM of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ at room temperature for 45 min and electrophoresed on a 1% agarose gel prior to analysis by densitometry. DMSO and Doxorubicin (DOX) at 100 μM were used as the untreated and positive control, respectively.

CHIKV nsP4 cloning, overexpression, and purification

The coding region of chikungunya non-structural protein 4 (nsP4) (GenBank KP164572.1; PROT-ID AJY53709.1) was cloned into pET-SUMO expression vector, generating the nsP4_pET-SUMO/LIC expression vector. Rosetta (DE3) *E. coli* (Novagen) cells were transformed with nsP4_pET-M11/LIC and grown in TB (Terrific Broth) medium, supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol at 37°C . The cell growth was monitored until reaching the optical density ($\text{OD}_{600\text{nm}}$) of 1.0. The protein expression was induced by adding 1 mM of Isopropyl

β -D-1-thiogalactopyranoside (IPTG), at 18 °C and maintained for 16 h. Cells were harvested by centrifugation and cell pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, and 10% glycerol). Cells were lysed by sonication and cell debris was separated by centrifugation. The nsP4 was purified using an AKTA Purifier System (GE Healthcare). The first purification step was affinity chromatography using a HisTrap HP 5.0 ml column (GE Healthcare) and the elution was performed using 250 mM imidazole. Concomitantly, the buffer was exchanged through dialysis and the His-tag-SUMO was cleaved by TEV protease overnight at 4 °C. Another affinity chromatography step was performed using the same system to collect the protein after cleavage. The protein was concentrated, and a final purification step was done through size-exclusion chromatography on an XK 26/1000 Superdex 75 column (GE Healthcare) pre-equilibrated in buffer 50 mM Tris pH 8.0, 200 mM NaCl and 5% glycerol. The final protein sample was analyzed by SDS-PAGE electrophoresis to confirm its purity. The concentration was determined spectrophotometrically in a Nanodrop 1000 spectrophotometer.

MicroScale thermophoresis (MST)

Experiments were performed on a Monolith[®] NT.115 instrument (Nanotemper technologies). The nsP4 was labeled on cysteine residues with NT-647-Maleimide dye (Nanotemper Technologies) using the Monolith NTTM Protein Labeling Kit RED-MALEIMIDE as per the manufacturer's instructions. The cys-labeled nsP4 was used to perform MicroScale Thermophoresis experiments, at the final concentration of 25 nM. An initial binding test was carried out with compound [Co^{III}(L¹)₂]Cl at the concentration of 500 μ M, to check the interaction between the protein and the compound. The assay control was performed using cys-labeled nsP4 with 5% DMSO. Then, a serial dilution of the [Co^{III}(L¹)₂]Cl from 500 to 0.015 μ M (15 nM) was performed to obtain the binding curve. The dissociation constant (K_d) was obtained by fitting the binding curve with the Hill function, using GraphPad Prism 8 (Graph Pad Software).

Molecular docking analysis

The 3D structure of [Co^{III}(L¹)₂]Cl was obtained by DFT at the pbe0/def2-tzvp [45] level of theory using ORCA 4.2.0 [46, 47]. Auxiliary basis sets AuxJ (def2/J) [48] and AuxC (def2-svp/C) [49] were also used. Optimized structures were rendered using Chemcraft (graphical software for visualization of quantum chemistry computations, <https://www.chemcraftprog.com>). A representative sequence of nsP4 extracted from the viral polyprotein (UniProt id: Q8JUX6) was modeled using the RoseTTAFold [50] on the Rosetta online server (<https://rosetta.bakerlab.org/>). The nsP4

tridimensional model was assessed using ERRAT [51], Ramachandran Plot [52], and Verify 3D [53] tools in the SAVES v6.0 server (<https://saves.mbi.ucla.edu/>). The nsP4 binding site was predicted using COACH [54] based on the RoseTTAFold-predicted structure. COACH is a meta-server approach that combines multiple function annotation results to generate ligand binding site predictions. COACH results indicate a binding site similar to the site where the Uridine 5'-Triphosphate (UTP) interacts with the crystal structure of HCV ns5B polymerase (PDBid: 4RY5). Thus, [Co^{III}(L¹)₂]Cl was docked with the modeled protein using GOLD [55]. GOLD performs a search for the best interacting pose of the chosen molecule in the receptor binding site using a genetic algorithm (GA) and the scoring function ChemPL. The binding site was defined by the UTP position based on the 4RY5 structure and extrapolated to the modeled nsP4. The GA parameters were set to the default values for the run, with M-L bonds treated as zero-order bonds. The poses generated are then ranked and the solution with the best score was chosen. The intramolecular interactions in the best-ranked pose were analyzed using DS Visualizer (BIOVIA, Dassault Systèmes, Discovery Studio Visualizer, v 20.1, San Diego: Dassault Systèmes, 2020).

ADME-Tox predictions

The free online platforms SwissADME (www.swissadme.ch) and OSIRIS Property Explorer[®] (http://www.cheminfo.org/Chemistry/Cheminformatics/Property_explorer/index.html) were used to evaluate the parameters of absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox).

Statistical analysis

Individual experiments were performed in triplicate and in a minimum of three times to confirm the reproducibility of the results. Differences between means of readings were compared using analysis of variance (one-way or two-way ANOVA) or Student's *t*-test using GraphPad Prism 8 (Graph Pad Software). *P* values ≤ 0.05 were considered to be statistically significant.

Results and discussion

CHIKV is an emerging arbovirus with a high impact on public health in tropical and non-tropical areas. In recent years, outbreaks have occurred around the world affecting many patients with chikungunya fever, the disease caused by CHIKV [2, 56, 57]. Currently, there is no antiviral therapy against CHIKV, demonstrating the need to identify new anti-CHIKV compounds [19].

In this context, thiosemicarbazones (TSCs) have previously been demonstrated to possess antiviral activity against HIV and HSV by disrupting the synthesis of structural proteins and also capsid assembly [23, 25]. Additionally, TSCs are described as possessing interesting motifs for metal binders and thus being coordinated with metals such as copper(II) and cobalt(III) [58, 59]. Cobalt(III) is an endogenous metal, and therefore, the bioavailability of cobalt(III)-like compounds can be increased, as well as their biological properties, in therapeutic protocols for human diseases [31, 60]. Considering the chemical versatility of the synthesis of TSC and the biological properties of cobalt(III)-like compounds, we previously analyzed complexes of the type $[\text{Co}^{\text{III}}(\text{atc-R})_2]\text{Cl}$ which demonstrated interesting antibacterial and antiviral activities [33]. Here, we characterized the anti-CHIKV activity of the $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$, a Cobalt(III) coordination compound with the thiosemicarbazone ligand with promising antiviral properties.

$[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ inhibits CHIKV infection in vitro

We previously demonstrated that the treatment of CHIKV-infected cells with the cobalt(III)-conjugated thiosemicarbazone $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ (Fig. 1) reduced CHIKV infection, and cell viability was not affected compared to the treatment with its organic ligand [33]. In this work, $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ had its antiviral activity characterized using

a recombinant CHIKV engineered to express the *nanoluciferase* reporter (CHIKV-*nanoluc*) (Fig. 2A). To assess the effect of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ on cell viability and virus infection, we performed a dose-response assay to determine the effective concentration of 50% (EC_{50}) and the cytotoxicity concentration of 50% (CC_{50}) values for this complex. BHK-21 cells were infected with CHIKV-*nanoluc* and treated with $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ at concentrations ranging from 0.14 to 300 μM , and viral replication levels were evaluated by measuring the nanoluciferase reporter activity at 16 h.p.i. (Fig. 2B). Employing this range of concentrations, it was determined that the $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ has an EC_{50} of 19 μM , CC_{50} of 62 μM , and SI of 3.26 (Fig. 2B). For further analysis, cells were treated with $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ at 50 μM , which significantly inhibited 94% of the CHIKV infection (cell viability > 80%) (Fig. 2C).

Our results showed that $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ significantly reduced virus post-entry to host cells at non-toxic concentrations, and therefore, the effect of this complex was further evaluated. As shown by other authors, and in agreement with our results, TSCs had their antiviral activity previously described against HIV and HSV, in a post-entry manner. The authors suggested that the mechanism of action for these compounds was related to the disruption of the synthesis of the structural protein and also capsid assembly [23, 25]. Additionally, Langsjoen et al. showed that the gold salt auranofin displayed antiviral activity against CHIKV with

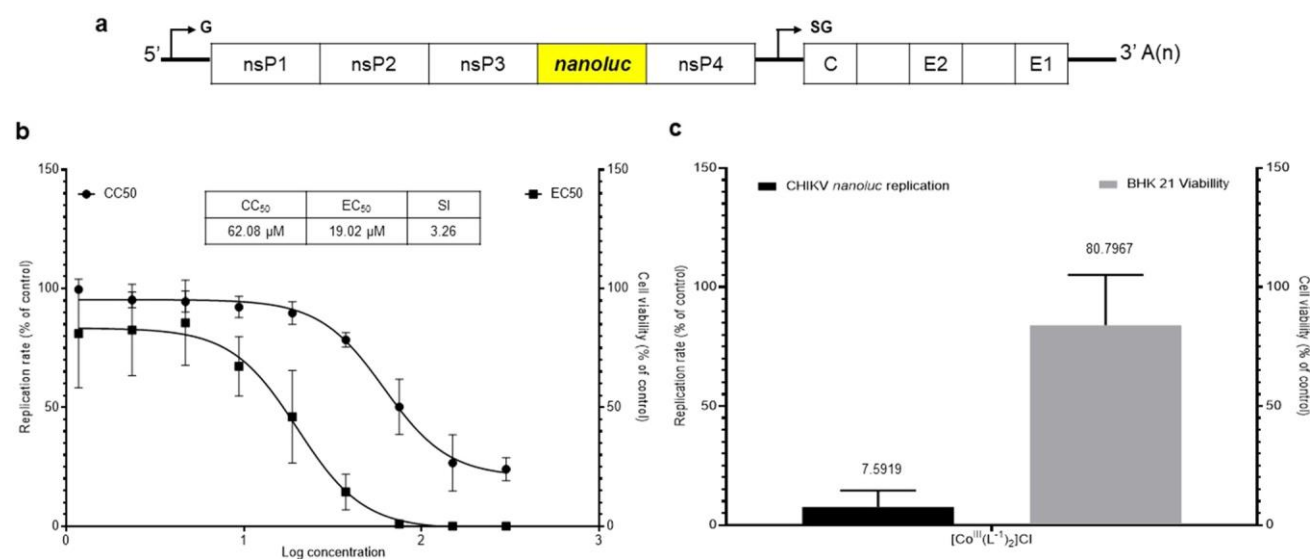


Fig. 2 A Schematic representation of the engineered Chikungunya virus *nanoluciferase* genome (CHIKV-*nanoluc*). **B** The effective concentration (EC_{50}) and cytotoxic concentration (CC_{50}) of $[Co^{III}(L^1)_2]Cl$. BHK-21 cells were treated with increasing concentrations of $[Co^{III}(L^1)_2]Cl$ ranging from 0.14 to 300 μM . CHIKV replication was measured by *nanoluciferase* activity (indicated by ■) and cellular viability was measured using an MTT assay (indicated by ●). Mean values of three independent experiments each measured in quad ruple including the standard deviation are shown. **C** BHK-21 cells were infected with CHIKV-*nanoluc* and simultaneously treated with $[Co^{III}(L^1)_2]Cl$ at 50 μM for 16 h. After treatment, cells were lysed and *nanoluciferase* levels were measured to assess the CHIKV replication rate. An MTT assay was carried out in parallel. Mean values of a minimum of three independent experiments each measured in triplicate are represented a Therapeutic Index (TI) of 104.5 12 h.p.i, aiming to target oxidative folding pathways [61].

$[Co^{III}(L^1)_2]Cl$ is a post-entry inhibitor of the CHIKV replicative cycle

To analyze the effects of $[Co^{III}(L^1)_2]Cl$ on different stages of the CHIKV replicative cycle, viruses and compounds were added to BHK-21 cells at different times. First, cells were pre-treated with $[Co^{III}(L^1)_2]Cl$ for 1 h, washed to remove the compound, and were infected with CHIKV-*nanoluc* (Fig. 3A). To assess the effect on the early stages of infection, CHIKV-*nanoluc* and $[Co^{III}(L^1)_2]Cl$ were simultaneously added to the BHK-21 cells for 1 h, followed by repeated cell washes to remove the inoculum and addition of fresh media (Fig. 3B). To investigate the interference with post-entry steps of infection, cells were first infected with CHIKV-*nanoluc*, washed to remove the unbound virus, and then a media-containing compound was added (Fig. 3C). The results obtained from all these experimental conditions demonstrated that $[Co^{III}(L^1)_2]Cl$ at 50 μM inhibited up to 94% of the CHIKV post-entry replication step (Fig. 3C) but had no effect on the early stages of the replicative cycle (Fig. 3B) or in protecting cells from infection (Fig. 3A). Despite being widely studied as antifungal [62], antibacterial [63], and largely exploited in cancer therapy [64], metal complexes are not studied nearly as much as antiviral agents in comparison to other applications such as anticancer [65], and only a few studies have reported the antiviral activity of cobalt(III) complexes [66]. In the latest 1990, a

cobalt chelate series (CTC) (General formula $[Co(acacen)(L)_2]^+$) of cobalt(III) complexes were studied as antiviral molecules against Herpes Simplex I virus (HSV-I) in vitro. The drug Doxovir™ (also named Co-1 or CTC-96 in the literature) significantly inhibited HSV-I infectivity in Vero cells at 5 $\mu g\ mL^{-1}$ [67]. In the early 2000, Schwartz and coworkers reported that Co-1 at 50 $\mu g\ mL^{-1}$ inhibited the early stages of HSV-1 infection in Vero cells, impairing virus penetration in the initial fusion stage between cell and virus. They have also found that this compound inhibited Varicella-zoster virus (VZV) plaque formation [68]. The antiviral activity of Co-1 was also described against Human Adenovirus type 5 (Ad5) in cell culture, acting as a virucidal compound, and also in rabbit model [69].

Another cobalt complex, the CTC compound Co-sb, showed to inhibit HIV-1 in vitro by Louie and Meade, by disrupting zinc finger structures, inhibiting transcription factors, and also interactions of some viral proteins with zinc fingers domains [70]. Moreover, the cobalt(III) complex sodium hydrogen butylimino bis-8,8-[5-(3-oxa-pentoxo)-3-cobalt bis(1,2-dicarbollide)]di-ate($[Co(NH_2C(NH)-NHC(NH)NH^sBu)_3]^3+$) showed antiviral activity against HIV-I, inhibiting HIV-I protease interactions in the hydrophobic sites of the protein, with EC_{50} 0.25 μM in PM-1 cells [71, 72]. This compound also showed antiviral activity against H1N1 virus, reducing H1N1 cytopathic effect in MDCK cells, with EC_{50} of 125 $\mu g/mL$ and SI of 8 [73].

What is more, there are only two research articles reporting cobalt(III) complexes as antiviral agents against arboviruses: Miranda and coworkers described the activity of

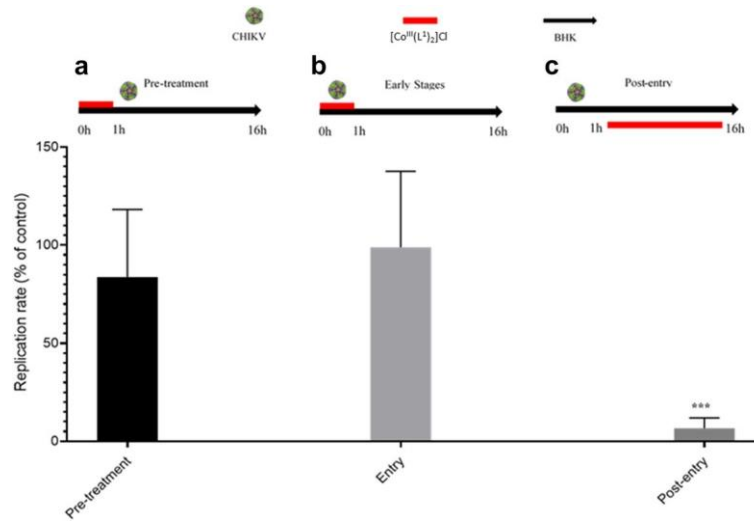


Fig. 3 $[\text{Co}^{\text{III}}(\text{L1})_2]\text{Cl}$ impairs post-entry stages of the CHIKV replicative cycle. A BHK-21 cells were pre-treated with $[\text{Co}^{\text{III}}(\text{L1})_2]\text{Cl}$ for 1 h, washed to completely remove the compounds, and were infected with CHIKV-nanoluc. B BHK-21 cells were infected with CHIKV-nanoluc and simultaneously treated with $[\text{Co}^{\text{III}}(\text{L1})_2]\text{Cl}$ 50 μM for 1 h. Then, cells were washed to remove the virus and compound and were replaced with fresh media. C BHK-21 cells were infected with CHIKV-nanoluc for 1 h, washed to remove unbound virus and added fresh media-containing compound. For all protocols, nanoluciferase activity levels were measured 16 h.p.i. Mean values of a minimum of three independent experiments each measured in triplicate are represented. *** $P < 0.01$ were considered significant

this class of metal complexes against Dengue virus (DENV) and Yellow fever virus (YFV) [73]; and our previous data published on CHIKV [33]. The compounds investigated by Miranda and coworkers were the cobalt(III) complex of protoporphyrin IX (CoPPIX) and tinprotoporphyrin IX (SnPPIX) [74]. For DENV-2, DENV-3 and YFV CoPPIX presented EC_{50} of 3.91, 0.77 and 3.74 $\mu\text{mol l}^{-1}$ in HepG2 cells, while SnPPIX presented 3.26, 0.85 and 0.37 $\mu\text{mol l}^{-1}$. For DENV, the compounds seem to disrupt or impair protein synthesis, and block virus adsorption and penetration in BHK 21 cells, with the same MOA that inhibited YFV.

Further compounds (non-metals) were reported with antiviral activity against post-entry stages of CHIKV replicative cycle. For example, the guanosine analog Ribavirin, described as a substrate of RdRp of HCV [75, 76], disrupted CHIKV replication with EC_{50} of 3.41 μM in Vero cells, 2 days post-infection [77]. Additionally, the 6-azauridine, an uridine analog used to treat psoriasis, also impaired CHIKV replication by disrupting CHIKV RNA replication. Briolant and coworkers showed that this compound possesses SI of 204 in Vero cells, 8.5 times higher than ribavirin used in the same study as a positive control [78].

Insights on mechanisms of action of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$

Based on the activity of the complex on CHIKV post-entry stages, we investigated whether this compound could interact with the viral dsRNA. During the chikungunya replicative cycle, a double-strand RNA (dsRNA) is formed as replicative intermediate molecules. Considering that some compounds possess antiviral activity by intercalating into this dsRNA [43] and that many metal complexes are DNA intercalators [79, 80] or DNA binders [81], we assessed the dsRNA interaction capabilities of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ by agarose

gel electrophoresis. $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ was incubated with 15 nM of dsRNA and analyzed in an agarose gel. Doxorubicin (DOX) at 100 μM and DMSO 0.1% were used as positive and untreated controls, respectively. Since $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ had no intercalation activity, ethidium bromide was able to intercalate to the dsRNA and the band was visualized as observed in the untreated control (Fig. 4). Densitometry analysis confirmed that the cobalt(III) conjugated compound did not intercalate with dsRNA (Fig. 4).

Aiming to access initial information about a possible target for $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ action, the CHIKV nsP4 was synthesized and purified in vitro (Fig. 5A). Thereby, the interaction between nsP4 and $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ was evaluated by Microscale Thermophoresis (MST). The binding affinity curve was obtained using a serial dilution of the compound to obtain information on the dissociation constant (kd). The results demonstrated that the binding affinity curve presented well-defined bound and unbound states, suggesting the occurrence of interaction between the CHIKV nsP4 and the $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ (Fig. 5B). Through the obtained curve, the estimated $k_d \pm \Delta k_d$ for this interaction was $8.35 \pm 0.681 \mu\text{M}$. To achieve evidence on the mechanism of action of this compound, we performed a dsRNA interaction assay. During the CHIKV replication cycle, a dsRNA is formed as a replicative intermediate molecule during the synthesis of a negative sense sRNA which is a template for the next viral replication stages. Considering that some compounds that acted on post-entry stages of virus replicative cycle demonstrated antiviral activity intercalating in dsRNA species [43], we investigated the interference of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ with a dsRNA, however, $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ seems to not act by this mechanism.

Knowing that replication is a very important process in the virus replication cycle, we investigated the interaction

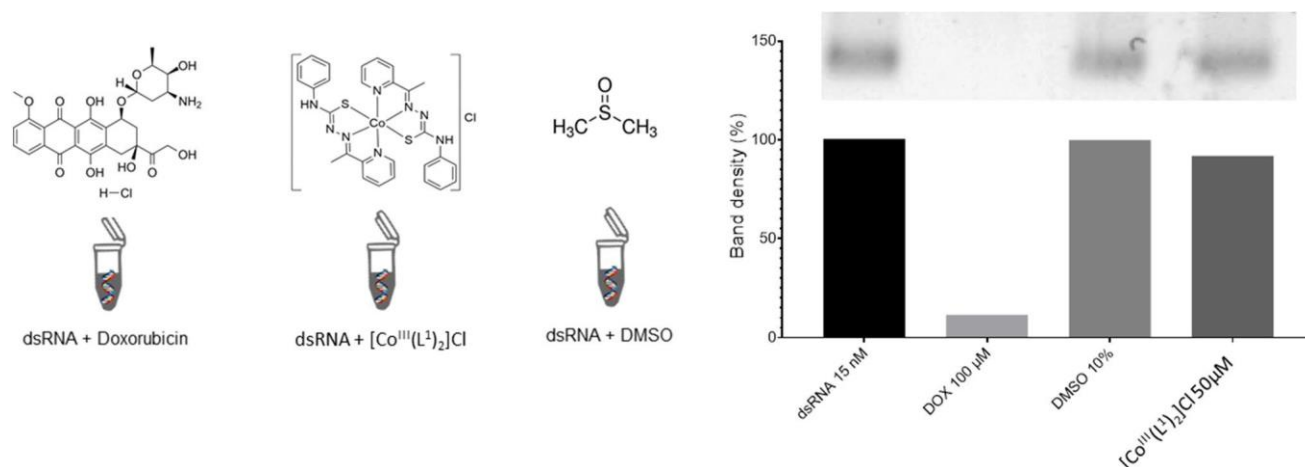


Fig. 4 $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ does not intercalate into dsRNA. $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ was incubated with 15 nM of dsRNA for 45 min and submitted to electrophoresis using a 1% agarose gel (run in $1 \times \text{TAE}$ buffer) stained with ethidium bromide, followed by densitometry analysis. DMSO 0.1% and Doxorubicin (DOX) at 100 μM were used as untreated and positive controls, respectively

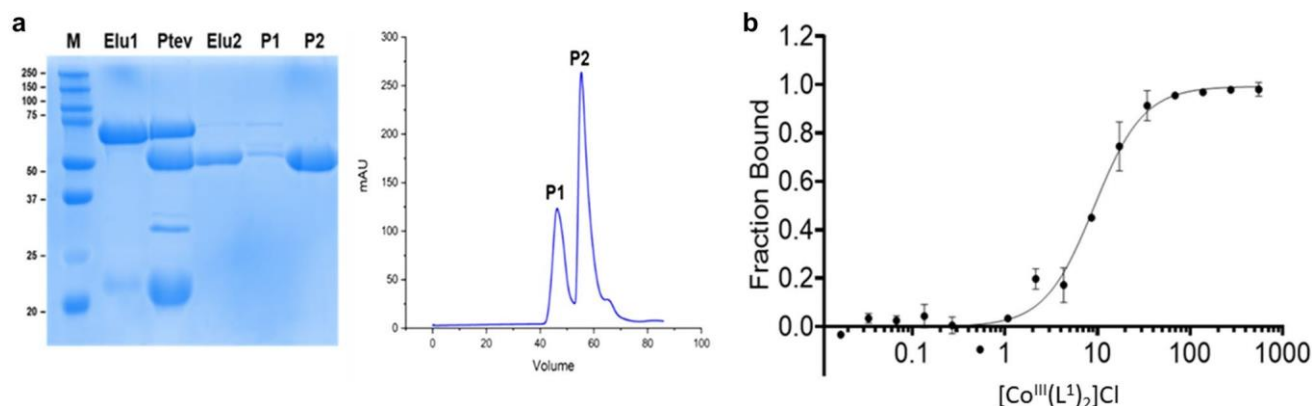


Fig. 5 $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ interacts with CHIKV nsP4. **A** SDS-PAGE Electrophoresis gel of nsP4 purification. M: Molecular weight marker; Elu1: Elution of His-tag-SUMO-nsP4 (approximately 70 kDa) by affinity chromatography step; Ptev: sample after incubation with TEV protease, indicating the cleavage of His-tag-SUMO; Elu2: Elution of nsP4 (54.54 kDa) after TEV protease cleavage; The P1 and P2 refer to the two peaks in the chromatogram (right) of the gel filtration step, corresponding to the eluted fractions. The purified nsP4 was eluted in P2 (54.54 kDa). **B** Binding affinity curve of nsP4 and $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$, obtained by Microscale Thermophoresis experiments. Estimated $k_d \pm \Delta k_d = 8.35 \pm 0.681 \mu\text{M}$.

of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ with the non-structural protein 4 (nsP4), a nsP which plays an essential role in the replicative complex activity. The nsP4 is an RNA-dependent RNA polymerase (RdRp) responsible for the synthesis of new RNA strands that will be incorporated into the viral progeny resulting from the replicative cycle. As it is crucial for viral replication, the RdRp has been an important target for antiviral therapies. Studies have shown compounds with antiviral activity against CHIKV replication, but only a few were documented suggesting nsP4 as a target. Some examples include benzimidazole [82], hesperetin-A derivatives [83], and favipiravir [84]. The availability of only a few studies for nsP4 can be explained due to the lack of structural information about this protein, such as the absence of an experimentally solved three-dimensional structure of CHIKV nsP4, which limits studies on the search for specific inhibitors based on this target.

Thus, to investigate whether this protein could be a possible target for $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$, we cloned, overexpressed, and purified CHIKV nsP4, and interaction studies were carried out by MicroScale Thermophoresis with different concentrations of the compound. Our data show that $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ interacted with CHIKV nsP4, suggesting that it might be a potential mechanism of antiviral action of this complex. Furthermore, molecular docking analysis suggested that the compound potentially interacts with CHIKV nsP4 through hydrogen bonding and electrostatic interactions in the Uridine 5'-Triphosphate (UTP) binding site, and probably impairing the initiation and elongation of new viral RNA, corroborating with their *in vitro* results.

Molecular docking analysis

In view of CHIKV nsP4 as a possible target for $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ complex, molecular docking was performed for a better understanding of the nature of the interactions between this protein and the compound. To this end, a prediction of the nsP4 protein structure was performed using the RoseTTA-Fold method and the structure was validated using Verify 3D, ERRAT, and Ramachandran Plot formalism (Supplementary Figs. 1–3). The 3D structure of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ was obtained by DFT (Fig. 6A). The compound $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ was then submitted as a ligand for molecular docking analysis using the nsP4 protein model as a receptor. The COACH software suggested the Uridine 5'-Triphosphate (UTP) binding site as the probable spot for the $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ interaction. The best scored pose (ChemPL = 66.92) was selected (Fig. 6B). Analyzing the intramolecular interactions, it can be observed a hydrogen bond involving the N-H group of Arg205 amino acid (2.95 Å). Furthermore, hydrophobic interactions with Pro298 and Lys295 amino acids (4.95 Å and 4.11 Å, respectively) were predicted, along with aromatic-sulfur interaction with Cys506 amino acid (5.30 Å). Finally, van der Waals interactions were observed with Pro307, Gln310, Glu304, Lys308, Val296, Thr297, Asp467, Ile464, Phe375, Ile372, Ser374, Asp376, and Asp466 (Fig. 7A and B).

ADME-Tox predictions

The evaluation of ADME-Tox properties of L^1 and $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ complex was carried out to verify the

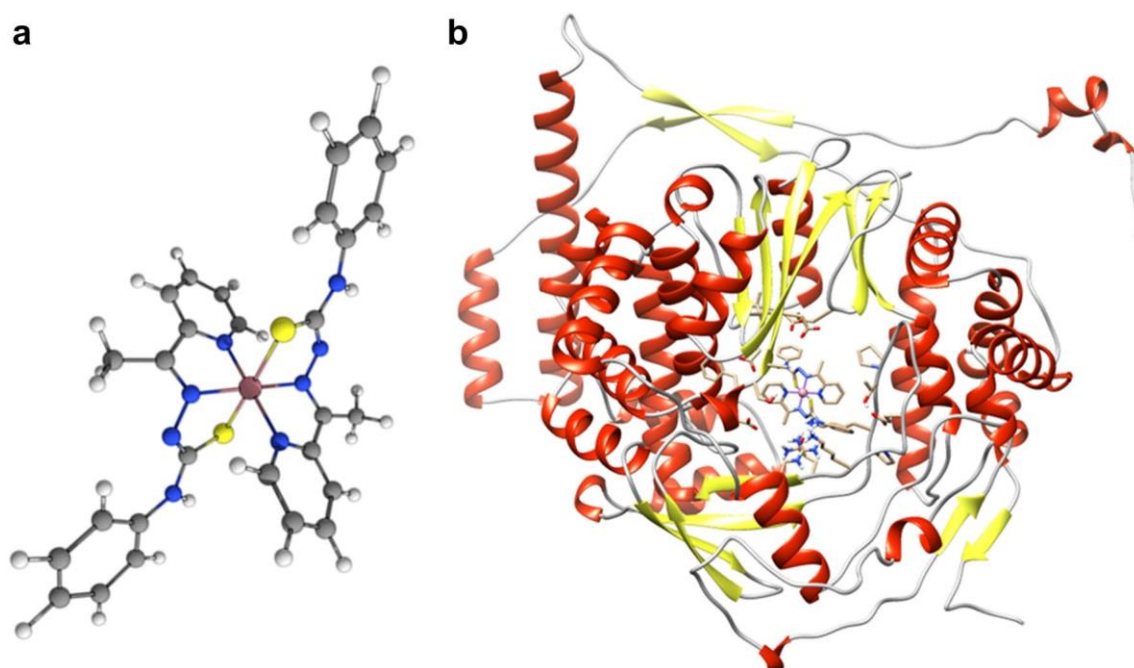


Fig. 6 Prediction of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ and CHIKV NSP4 structures. **A** DFT-optimized structure of compound $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ at the pbe0/def2-tpv level of theory as implemented in ORCA 4.2.0. Color code: white—hydrogen; gray—carbon; blue—nitrogen; yellow—sulfur; light pink—cobalt. **B** The predicted nsP4 structure interacting with $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$

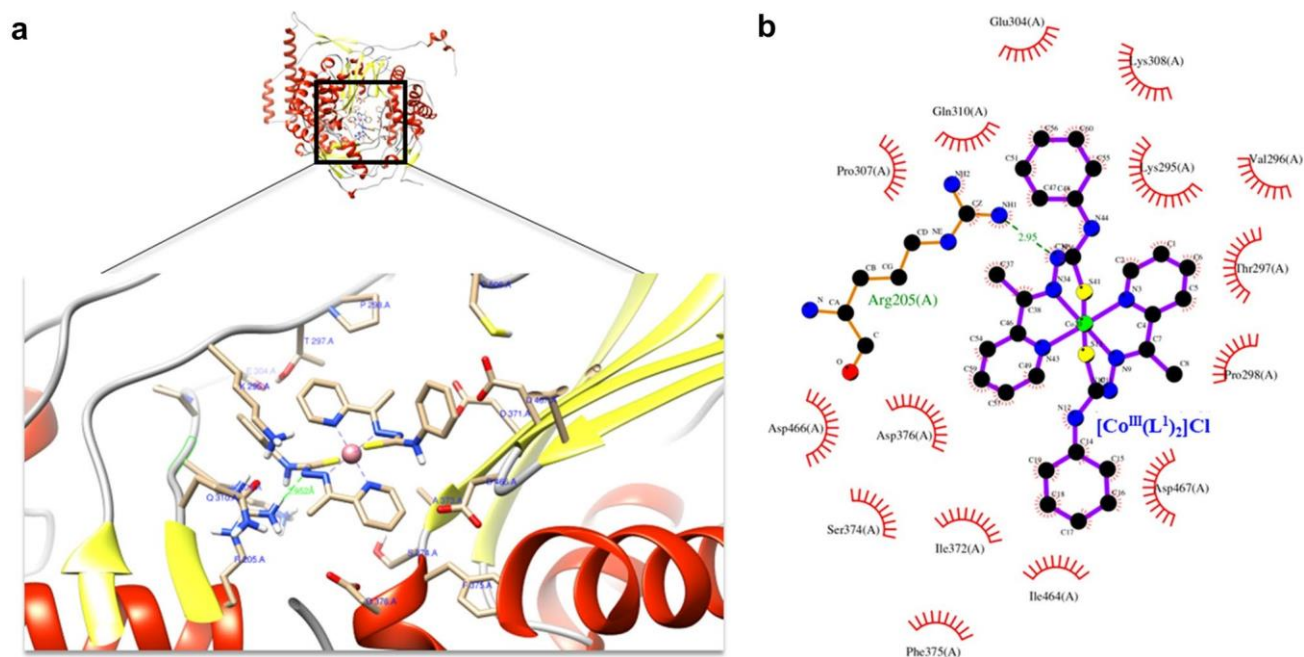


Fig. 7 In silico analysis of potential interactions between $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ and CHIKV NSP4. **A** Best ranked pose obtained by the molecular docking of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ (represented in red) into the CHIKV nsP4 model. **B** CHIKV NSP4 and $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ atomic interactions. Greendashed line indicates hydrogen bond, magenta, and light magenta dashed lines indicate hydrophobic interactions, yellow dashed lines indicate electrostatic interactions, orange dashed lines indicate pi-sulfur interactions, and numbers in black indicate the interaction distances in angstroms (Å)

physicochemical properties as lipophilicity, water-solubility, human intestinal absorption (HIA), blood–brain barrier penetration (BBB), inhibition of isoenzymes belonging to the CYP450 system and toxicological effect (irritant, mutagenic, reproductive and tumorigenic). The results are shown in Tables 1 and 2.

According to the results listed in Table 1, the L^1 is in according to Lipinski's rules while $[Co^{III}(L^1)_2]Cl$ complex breaks one of Lipinski's rules because of its higher molecular weight ($MW > 500$). Other important parameters are LogS, MR, and TPSA concerning to hydrophobicity of a given compound, and, consequently, the ability to cross plasmatic membranes and permeate cells. Those parameters provide information about the absorption and distribution of a drug in the organism [85]. According to the results shown in

Table 1 In silico evaluation of Lipinski parameters of L^1 and $[Co^{III}(L^1)_2]Cl$ complex

Parameters	L^1	$[Co^{III}(L^1)_2]Cl$
Physicochemical properties		
Formula	$C_{14}H_{14}N_4S$	$C_{30}H_{30}N_8S_2ClCo$
MW ^a	270.35 g/mol	661.13 g/mol
Num. rotatable bonds	5	6
Num. H–bonds acceptors	2	2
Num. H–bonds donors	2	2
MR ^b	81.92	191.87
TPSA ^c	81.40 Å ²	133.96 Å ²
Lipophilicity $\log P_{d,e}$	2.49	2.16
Water solubility		
$\log S$	−3.44	−9.07
Class ^f	Soluble	Poorly soluble
Druglikeness		
Lipinski ^g	Yes	No: $MW > 500$
Bioavailability score	0.55	0.55

aMW: Molecular Weight

bMR: Molar Refractivity

clog P = partition coefficient between n-octanol/water

d/w

fClass = Ali classes: insoluble < −10 < poor < −6 < moderately solu-

ble < −4 < soluble < −2 < very soluble < 0 < highly

gLipinski = $MW \leq 500$; $\log P_{o/w} \leq 5$; H–bond donors ≤ 5 ;

H–bond acceptors ≤ 10

Table 2 Data on the main pharmacokinetic and toxicology properties of L^1 and $[Co^{III}(L^1)_2]Cl$ complex

Compound	HIA	BBB	CYP3A4 inhibitor	CYP2D6 inhibitor	Reproductive	Mutagenic	Tumorigenic	Irritant
L^1	High	No	No	No	High	High	Low	Low
$[Co^{III}(L^1)_2]Cl$	No	No	No	Low	Median	Low	Low	

Table 1, the L^1 and $[Co^{III}(L^1)_2]Cl$ complex could show good lipophilicity and the ability to cross plasmatic membranes. Although this is an initial study, these results reveal that $[Co^{III}(L^1)_2]Cl$ has the potential to be used as a metal drug. Furthermore, these data may be useful for future studies in the case of evaluating the absorption and biodistribution of this complex and its analogs in vivo.

The L^1 and $[Co^{III}(L^1)_2]Cl$ complex were also evaluated for human intestinal absorption (HIA), blood–brain barrier penetration (BBB), and inhibition of isoenzymes belonging to the CYP450 system as a preliminary theoretical study (Table 2). The results showed that both candidates had high absorption in the intestine, none of the compounds were able to cross the blood–brain barrier as well as none inhibited the CYP2D6 and CYP3A4 isoenzymes belonging to the CYP450 system. Furthermore, the toxicological effects (irritant, mutagenic, carcinogenic, and tumorigenic) were also evaluated. In general, the L^1 has higher toxic effects than the $[Co^{III}(L^1)_2]Cl$ complex, which demonstrates the importance of complexation with a trace metal and shows that the introduction of the metal cobalt ion reduces the toxic effects of the free ligand [86].

Although the complex presents some unsatisfactory results as a potential to be carcinogenic and does not follow all the rules of Lipinski, it still represents a candidate to the development of antiviral drugs. Some drugs used to treat diseases also violate specific rules and continue to be used as medications, such as ivermectin, tenofovir disoproxil fumarate (TDF), ceftadizime, and isoniazid. Acarbose, Chloroquine, Lopinavir, Amodiaquine are predicted by the

cTPSA: Topological Polar Surface Area

dConsensus log Po/w = Average of all five predictions

web server AdmetSAR 2.0 as hepatotoxic and mutagenic [87].

Conclusion

In summary, we have shown that the $[Co^{III}(L^1)_2]Cl$ inhibits the post-entry stage of CHIKV infection, potentially by interacting and interfering with the nsP4 activity. Due to the lack of studies with CHIKV nsP4, there is no positive control for in vitro assays, and also, there is no well-established

structure yet. Therefore, we suggest that $[Co^{III}(L^1)_2]Cl$ might

interact with nsP4 and interferes with its activity, however, we acknowledge that interpretation of these experiments is

challenging and further studies are necessary.

It is also important to emphasize the broad antimicrobial activities of this compound since it has already shown activity against *Mycobacterium tuberculosis* [34], *E. faecalis*, *S. salivarius*, *S. sanguinis*, *S. mitis*, *S. mutans*, and *L. paracasei* [33]. In this way, this compound could be applied for the treatment of multiple diseases caused by such bacteria and the chikungunya virus, which can be interesting for the pharmaceutical industry and generate great social impact. Although metallodrugs are widely exploited in cancer treatment, some antimicrobials and skin diseases, there is no metallodrug licensed to treat any virus infection. Herein we presented data about the antiviral activity and suggest potential MOA of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ against chikungunya, thus reinforcing the relevance of these results to the current state of antiviral research and the potential of this class of metal complexes as a proposed therapeutic molecule candidate to be further explored.

It is worth mentioning that there are only a few published works using Cobalt(III) coordinated compounds as therapeutic molecules, highlighting the relevance to the published data exploring such combination. From a vast bibliography, with several papers exploring this coordination, we can then make a better scientific judgment whether the application of these complexes is worthwhile or not for in vivo and clinical trials, reinforcing the application of these data for the research landscape of therapeutic targets against chikungunya fever. The conclusions obtained from molecular docking studies demonstrated a significant binding of the complex with CHIKV NSP4 structures which is consistent with the in vitro antiviral activity. Furthermore, the antiviral activity of $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{Cl}$ against other viruses which impact human health may be the target of future investigation. Considering the pivotal role of RdRp in *Alphavirus* replication, our results may contribute to future studies on inhibitors of nsP4, representing a relevant contribution to the field of antiviral research against CHIKV. The significant antiviral profile together with ADME-Tox results provides an interesting approach for future in vivo studies.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00775-022-01974-z>.

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Data availability All data generated or analyzed during this study are included in this published article and, its supplementary information files.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chikungunya virus entry is strongly inhibited by phospholipase A2 isolated from the venom of *Crotalus durissus terrificus*

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Chikungunya virus (CHIKV) is the etiologic agent of Chikungunya fever, a globally spreading mosquito-borne disease. There is no approved antiviral or vaccine against CHIKV, highlighting an urgent need for novel therapies. In this context, snake venom proteins have demonstrated antiviral activity against several viruses, including arboviruses which are relevant to public health. In particular, the phospholipase A_{2CB} (PLA_{2CB}), a protein isolated from the venom of *Crotalus durissus terrificus* was previously shown to possess anti-inflammatory, antiparasitic, antibacterial and antiviral activities.

In this study, we investigated the multiple effects of PLA_{2CB} on the CHIKV replicative cycle in BHK-21 cells using CHIKV-*nanoluc*, a marker virus carrying *nanoluciferase* reporter. The results demonstrated that PLA_{2CB} possess a strong anti-CHIKV activity with a selectivity index of 128. We identified that PLA_{2CB} treatment protected cells against CHIKV infection, strongly impairing virus entry by reducing adsorption and postattachment stages. Moreover, PLA_{2CB} presented a modest yet significant activity towards post-entry stages of CHIKV replicative cycle. Molecular docking calculations indicated that PLA_{2CB} may interact with CHIKV glycoproteins, mainly with E1 through hydrophobic interactions.

In addition, infrared spectroscopy measurements indicated interactions of PLA_{2CB} and CHIKV glycoproteins, corroborating with data from *in silico* analyses. Collectively, this data demonstrated the multiple antiviral effects of PLA_{2CB} on the CHIKV replicative cycle, and suggest that PLA_{2CB} interacts with CHIKV glycoproteins and that this interaction blocks binding of CHIKV virions to the host cells.

Chikungunya virus (CHIKV), a member of the genus *Alphavirus*, family *Togaviridae*¹, is the causative agent of Chikungunya fever². CHIKV virions comprise an icosahedral capsid with a positive single stranded RNA genome of approximately 12 kb³ surrounded by a lipid envelope with E1, E2 and E3 glycoproteins in its surface^{4,5}. CHIKV was first identified in 1950 in Tanzania, Africa and related to Chikungunya fever in 1955^{6,7}. CHIKV is transmitted through the bite of *Aedes aegypti* and *Aedes albopictus* mosquito^{8,9}, and, therefore, have been associated to the epidemics in tropical and subtropical regions¹⁰. Since then, CHIKV outbreaks have been identified in many regions including islands of Indian Ocean, India, South-East Asia, France, Italy and the Americas¹¹. In Brazil, the first cases of Chikungunya fever were documented in 2014, and since then, the disease became an endemic¹². From January to June of 2020, 48,316 cases and 11 deaths by CHIKV were notified¹³. Chikungunya fever symptoms include fever, nausea, fatigue, arthralgia and polyarthralgia¹⁴. In some rare cases, infected individuals can develop hepatitis, myocarditis, and encephalopathy, ultimately leading to death

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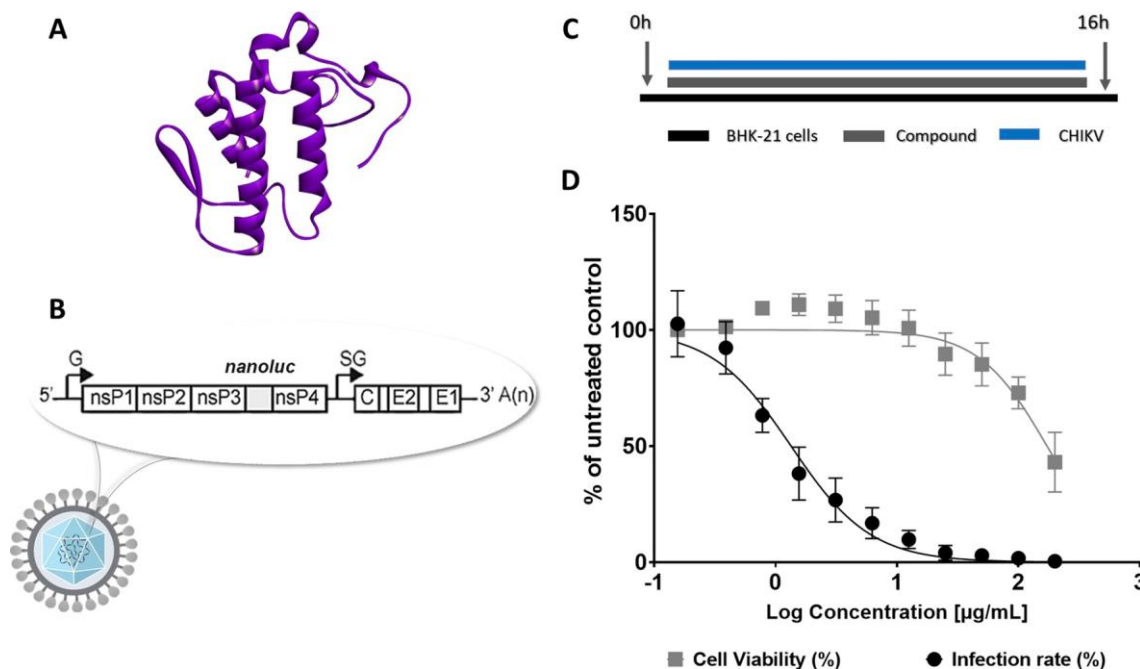


Figure 1. The activity of Phospholipase A2CB (PLA2CB) against CHIKV infection. **(A)** Phospholipase A2CB protein structure (PDB: 3R0L). **(B)** Schematic representation of CHIKV-*nanoluc* genome. **(C)** Schematic description of infectivity assay. **(D)** BHK-21 cells were treated with concentrations of PLA2CB ranging from 0.195 to 200 $\mu\text{g/mL}$. CHIKV replication was quantified by measuring of *nanoluciferase* activity (indicated by black dot) and cellular viability was measured using an MTT assay (indicated by grey square). The mean values of three independent experiments each measured in triplicate including the standard deviation are shown. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

of patients^{14,15}. Unlike other arboviruses, CHIKV infection can result in chronic symptoms lasting for months or years, resulting in a disabling disease^{16,17}. There are no approved antiviral drugs against CHIKV infection, as a consequence the treatment is often palliative and symptomatic, based on analgesics, non-steroidal anti-inflammatory, rest, and hydration¹⁸.

Given that many approved drugs employed in the treatment of infectious and chronic diseases originated or derived from natural sources^{19,20}, it is reasonable to hypothesize that natural compounds may also be exploited to generate antiviral drugs. In this context, proteins isolated from snake venoms represent promising drug leads, since they are a complex mixture of lectins, oxidases, disintegrins, metalloproteins, and phospholipases A2 (PLA2s)^{21,22}. From these, PLA2s, in its turn, are members of a secreted phospholipases, which can act in the cell membranes and play several roles in biological systems^{23–25}.

The snake venom isolated from *Crotalus durissus terrificus* has numerous constituents such as crotoxin, crotamin, neurotoxin, among others^{26,27}. Crotoxin is the major constituent of the *C. d. terrificus* venom. It is characterized as a protein complex composed by two noncovalent subunits, the basic subunit phospholipase A2 (PLA2CB), and the acid subunit crotoxin^{28,29}. Subsequently, PLA2CB is approximately 14 kDa protein which possess anti-inflammatory, antiparasitic, and antibacterial properties^{30,31}. PLA2CB has also presented activity towards viruses such as hepatitis C (HCV)³², Rocio (ROCV), Mayaro (MAYV), Dengue (DENV) and Yellow Fever (YFV)^{33,34}. Russo and coworkers expressed and purified two recombinant PLA2CB (rPLA2CB) and partially assessed its anti-CHIKV activity. It was found that rPLA2CB proteins possess lower antiviral activity and higher cytotoxicity profile than the native protein, probably due to nine additional amino acid residues present in their sequences³⁵. Considering these previous results, herein we performed thorough in vitro evaluation of the effects of the native PLA2CB on the CHIKV replication cycle.

Results

PLA2CB strongly impairs CHIKV infection in vitro. We investigated the anti-CHIKV activity of the PLA2CB (Fig. 1A) using BHK-21 cells and a recombinant CHIKV that expresses a *nanoluciferase* reporter (CHIKV-*nanoluc*) (Fig. 1B)^{36,37}. First, the PLA2CB antiviral activity was evaluated by performing a dose-response assay to determine the effective concentration of 50% (EC₅₀) and cytotoxicity of 50% (CC₅₀). BHK-21 cells were infected with CHIKV-*nanoluc* and simultaneously treated with PLA2CB at concentrations ranging from 0.195 to 200 $\mu\text{g/mL}$ in two-fold serial dilutions, and viral replication was assessed 16 h post-infection (h.p.i.) (Fig. 1C). In parallel, cell viability was assessed by an MTT assay. PLA2CB was found to be able to inhibit virus replication to greater than 99%, while the cell viability at the highest concentration tested was 43%. It was determined that PLA2CB has the EC₅₀ of 1.34 $\mu\text{g/mL}$, CC₅₀ of 172 $\mu\text{g/mL}$, and the Selectivity Index (SI) of 128 (Fig. 1D). Thus, PLA2CB acts as strongly inhibitor of CHIKV infection with high SI value.

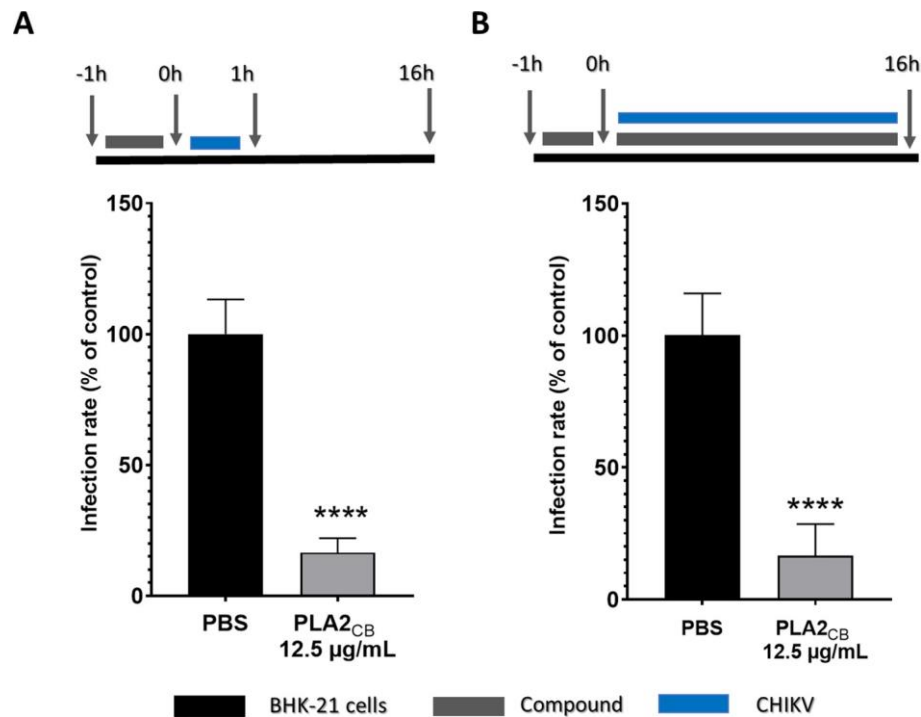


Figure 2. PLA2_{CB} protects cells against CHIKV infection. **(A)** BHK-21 cells were treated with PLA2_{CB} at 12.5 µg/mL for 1 h. Then, cells were washed and infected with CHIKV-*nanoluc* at MOI 0.1 for 1 h. The compound-containing medium was removed and replaced with a fresh medium. **(B)** BHK-21 cells were treated with the PLA2_{CB} at 12.5 µg/mL for 1 h, then were infected with CHIKV-*nanoluc* in the presence of PLA2_{CB}. For both assays, CHIKV replication was measured by *nanoluc* activity at 16 h.p.i.. Schematic representation of each time-based assay as indicated by BHK-21 cells (black bars), PLA2_{CB} (grey bars), and CHIKV-*nanoluc* (blue bars). Mean values ± SD of a minimum of three independent experiments each measured in triplicate. (****) P < 0.0001. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

PLA2_{CB} strongly inhibits early stages of CHIKV infection. Time-of-addition type of experiments were used to analyze the effect of PLA2_{CB} on different stages of CHIKV replication. For all of these assays, cells were treated with PLA2_{CB} at 12.5 µg/mL, a concentration that inhibited virus replication by ~91% without affecting cell viability (Fig. 1D).

To assess the protective effects of PLA2_{CB} against CHIKV infection, cells were pretreated with PLA2_{CB} for 1 h at 37 °C, washed extensively with PBS to remove the compound and infected with CHIKV-*nanoluc* for 1 h. Then, the supernatant was removed, cells were added of fresh medium and luciferase levels were measured 16 h.p.i. (Fig. 2A). PLA2_{CB} significantly reduced CHIKV-*nanoluc* infection by 84% (p < 0.01), demonstrating a robust protective effect (Fig. 2A). The protective effect did not increase when the compounds was present for all duration of the experiment (Fig. 2B), demonstrating that pre-treatment of cells with PLA2_{CB} inhibited CHIKV replication. This data suggests that PLA2_{CB} acts by protecting cells against infection and/or by affecting early stages of CHIKV infection.

To further evaluate the PLA2_{CB} effect on CHIKV entry to the host cells, virus and PLA2_{CB} were simultaneously added to BHK-21 cells for 1 h at 37 °C, cells were washed with PBS and replaced with fresh medium (Fig. 3A). PLA2_{CB} demonstrated to decrease 95.3% of CHIKV replication (p < 0.0001), indicating that this compound strongly inhibited the CHIKV-*nanoluc* entry (Fig. 3A). Combining this treatment with 1 h pre-incubation of the inoculum containing PLA2_{CB} and CHIKV at 37 °C further increased inhibition that reached over 99% (Fig. 3B), indicating that PLA2_{CB} also possesses virucidal activity. To analyze the effect of PLA2_{CB} on CHIKV attachment, virus and compound were first incubated with the cells at 4 °C for 1 h. At this temperature, virus particles were able to attach to the cellular receptors, but not entry into the host cells. Cells were then washed with PBS, fresh medium added, and incubated at 37 °C (Fig. 3C), to allow the continuation of the entry process. Data obtained from this assay also showed strong inhibition of CHIKV attachment by reducing virus entry by 98.2% to the cells (p < 0.0001) (Fig. 3C). Post-attachment was evaluated by including an additional incubation of 30 min at 37 °C to the previous protocol (Fig. 3D), showing that the inhibition remained strong reaching 95.2% (p < 0.0001) (Fig. 3D). Taken together this data indicates that PLA2_{CB} possesses a robust virucidal activity and the ability to block virus entry to host cells.

PLA2_{CB} moderately affect post-entry steps of CHIKV infection. Two assays were used to analyze effects of PLA2_{CB} on post-entry stages of CHIKV infection. Using CHIKV-*nanoluc* it was found that, if added after virus infection, compound cause relatively modest, 64% reduction of CHIKV replication (p < 0.0001).

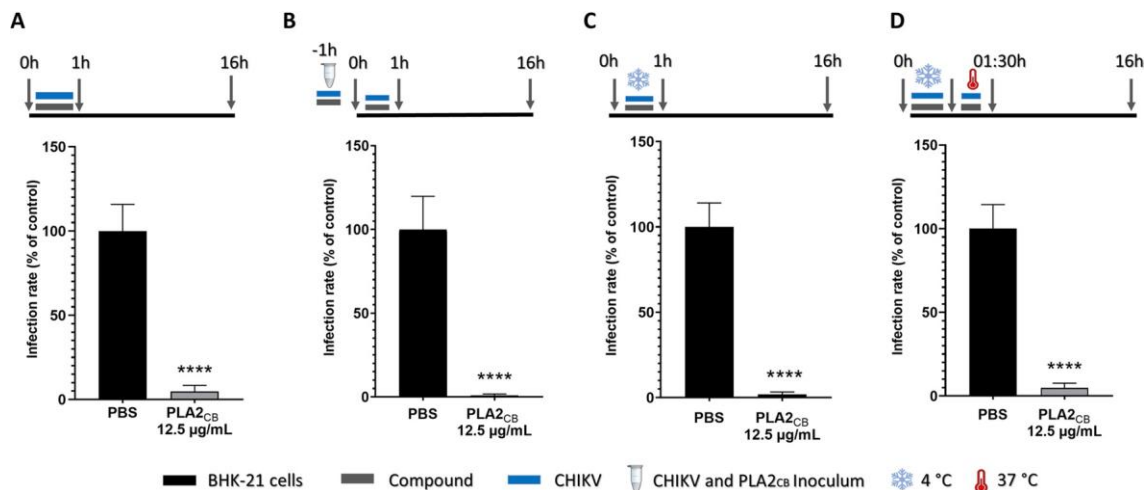


Figure 3. PLA2_{CB} has virucidal activity and blocks CHIKV entry to the host cells. **(A)** BHK-21 cells were infected with CHIKV-*nanoluc* (MOI 0.1) and simultaneously treated with PLA2_{CB} at 12.5 µg/mL for 1 h. Cells were washed and replaced by fresh medium. **(B)** CHIKV-*nanoluc* and PLA2_{CB} at 12.5 µg/mL were incubated for 1 h at 37 °C and then for one extra hour with the cells. Then, virus and compound were removed, cells were washed with PBS, and added fresh medium. **(C)** BHK-21 cells were infected with the virus and simultaneously treated with PLA2_{CB} at 12.5 µg/mL for 1 h at 4 °C. The cells were washed to remove virus and compound and replaced with fresh medium. **(D)** BHK-21 cells were infected with virus and simultaneously treated with PLA2_{CB} at 12.5 µg/mL for 1 h at 4 °C. Then, cells were incubated for additional 30 min with compound and virus at 37 °C, washed with PBS and replaced by fresh medium. For all assays, CHIKV replication was measured by *nanoluc* activity at 16 h.p.i. Schematic representation of each time-based assay as indicated by BHK-21 cells (black bars), PLA2_{CB} (grey bars), and CHIKV-*nanoluc* (blue bars), CHIKV and PLA2_{CB} inoculum (microtube), incubation at 4 °C (ice crystal) and incubation at 37 °C (thermometer). Mean values ± SD of a minimum of three independent experiments each measured in triplicate. (****) P < 0.0001. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

(Fig. 4A). To reveal the effect of PLA2_{CB} on virus RNA replication in the absence of production and spread of virions, BHK-CHIKV-NCT cells were used. This stable cell line contains CHIKV replicon which continuously expresses viral nonstructural proteins and two reporters: *Renilla* luciferase from nonstructural region and EGFP via activity of viral subgenomic promoter. Measurement of activities of these reporters allows the evaluation of the effect of PLA2_{CB} on replication complexes formed during the replication stage as well as on production and translation of subgenomic RNAs. Treatment of BHK-CHIKV-NCT cells with PLA2_{CB} at 12.5 µg/mL for 72 h after treatment revealed the reduction of *Renilla* luciferase expression by 58% without causing detectable cytotoxicity (Fig. 4B); this data confirms observation made using CHIKV-*nanoluc*. Furthermore, the levels in EGFP expression were also reduced as seen in Fig. 4C, indicating decrease of subgenomic RNA synthesis and/or translation. Taken together, these results suggest that PLA2_{CB} inhibits post-entry stages of infection possibly interfering with functioning of CHIKV nonstructural proteins.

Molecular docking reveals possible interactions between PLA2_{CB} and CHIKV glycoproteins. Results of inhibition assays clearly demonstrate that PLA2_{CB} inactivates CHIKV virions and impairs their binding to host cell suggesting interaction of compound with outer surface of virion. Therefore, a molecular docking assay was performed to investigate interactions and reveal potential binding mode between PLA2_{CB} and CHIKV glycoproteins. In a blind molecular docking, PLA2_{CB} was predicted to interact with the E1 and E2 of the glycoprotein complex, with global energy of −0.57 kJ/mol after refining (Fig. 5).

The 2D interactions between PLA2_{CB} and CHIKV glycoproteins showed that PLA2_{CB} mainly interacts with E1 glycoprotein, forming thirty hydrophobic interactions (residues Ile63, Gln33, Pro19, Phe109, Gly31, Ala55, Val18, Lys60, Arg114, Phe23, Trp30, Trp61, Leu3 in PLA2_{CB} and residues Gln353, Lys132, Leu34, Val269, Ser35, Asn389, Arg134, Asn140, Tyr390, Leu136, Gln260, Gly12, He344, Glu32, Arg340, Ser355 in E1 glycoprotein) (Fig. 6). Also, PLA2_{CB} formed 3 hydrogens bonds with E1, Ser113 and Asn270 (2.30 Å), Asn58 and Glu343 (2.95 Å) and His1 and Glu341 (2.18 Å) (Fig. 6). PLA2_{CB} may also form one hydrogen bond with E2 glycoprotein (between Arg11 and Glu 334 (2.07 Å), plus five hydrophobic interactions (Asn105, Lys104, Gly106 in PLA2_{CB} and Asn273, Lys270 in E1) (Fig. 7).

PLA2_{CB} causes molecular changes in CHIKV glycoprotein. To further investigate the interactions between PLA2_{CB} and CHIKV particles, infrared spectroscopy spectral analysis and vibrational analysis among the virus and PLA2_{CB} was performed. Representative means of the infrared spectrum of CHIKV, PLA2_{CB}, and CHIKV plus PLA2_{CB}, which is the bio fingerprint region representing proteins, lipids, nucleic acids, and glyco-proteins are shown in Fig. 8A. A representative infrared average spectrum of second derivative analysis from CHIKV virions, PLA2_{CB}, and CHIKV virions plus PLA2_{CB} is displayed in Fig. 8A. In the second derivative

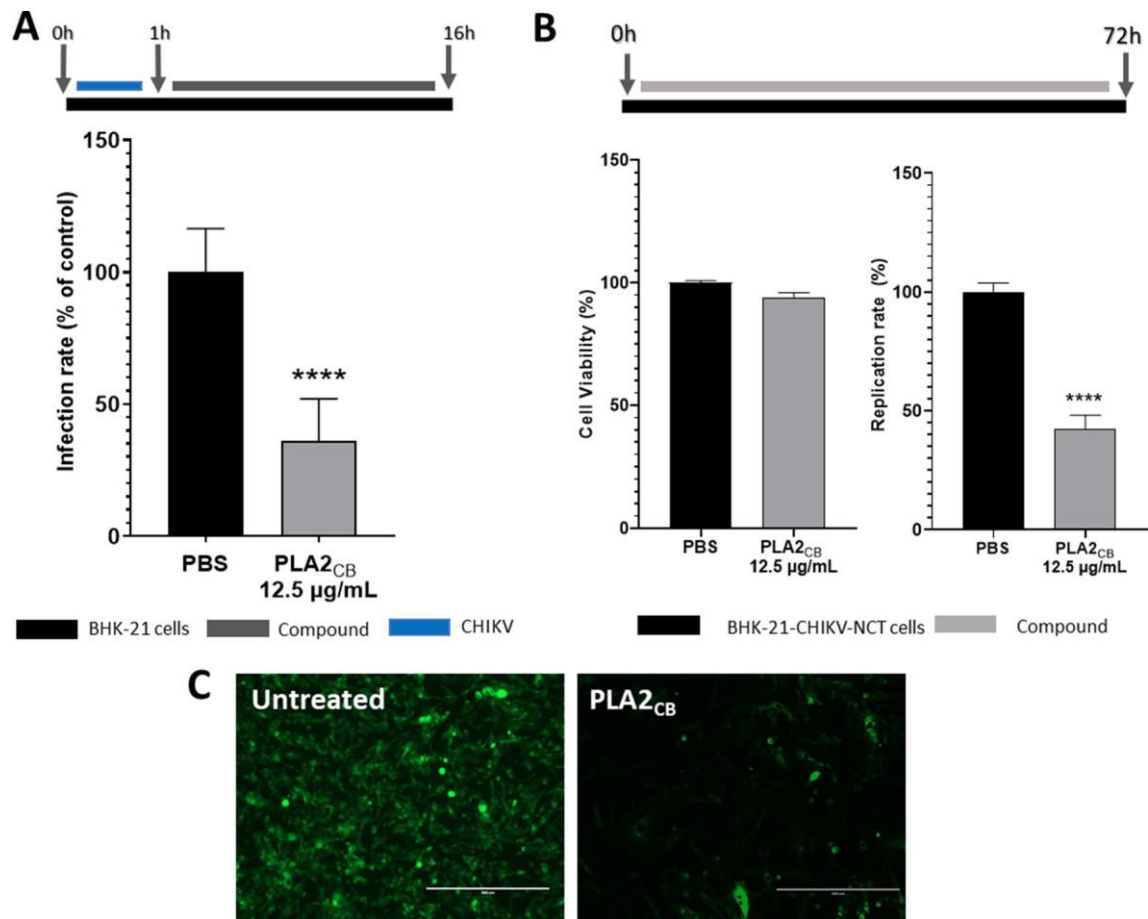


Figure 4. PLA2_{CB} inhibits post-entry stage of CHIKV. **(A)** BHK-21 cells were infected with CHIKV-*nanoluc* (MOI 0.1) for 1 h, washed to remove unbound virus and added of medium containing PLA2_{CB} at 12.5 µg/mL for 16 h. *Nanoluciferase* levels were measured to access CHIKV-*nanoluc* replication rates. **(B)** BHK-CHIKV- NCT cells were seed 24 h prior treatment and treated with PLA2_{CB} at 12,5 µg/mL for 72 h. *Renilla* luciferase activity and cellular viability measured. **(C)** Fluorescence of untreated control and PLA2_{CB} treatment in BHK-CHIKV-NCT, observed in fluorescence microscopy using 20× lens (scale bar 400 µm), in GFP filter. Schematic representation of each assay as indicated by BHK-21 cells or BHK-CHIKV-NCT (black bars), PLA2_{CB} (grey bars), and CHIKV-*nanoluc* (blue bars). Mean values ± SD of a minimum of three independent experiments each measured in triplicate. (****) $P < 0.0001$. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

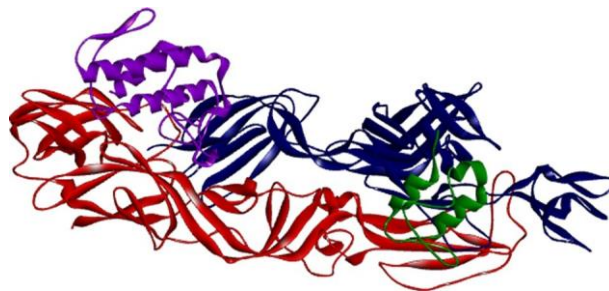


Figure 5. Molecular docking suggests possible interactions between PLA2_{CB} and CHIKV glycoproteins. The post-docking 3D image was generated in the DS Visualizer program (Dassault Systèmes BIOVIA, Discovery Studio Visualizer, 2016). The CHIKV envelope glycoproteins E1 (Red), E2 (Blue), E3 (green), complexed with PLA2_{CB} (purple) are shown. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

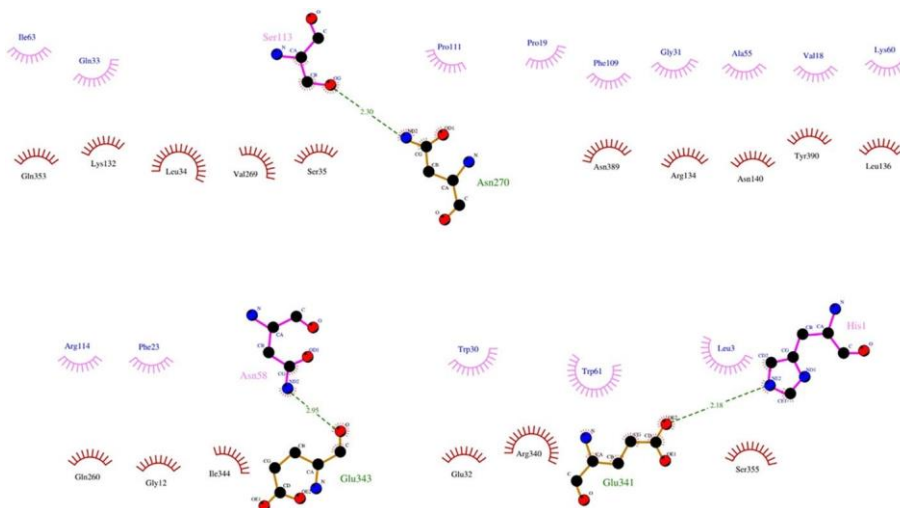


Figure 6. Molecular interactions between CHIKV E1 and PLA_{2CB}. 2D diagram of the interactions of the envelope glycoprotein E1 protein with PLA_{2CB}. The hydrogen bonds (green dashes) are shown between PLA_{2CB}(purple lines) and E1 glycoprotein (orange lines). Hydrophobic interactions are also shown between PLA_{2CB} (purple bows) and E1 glycoprotein (red bows). All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

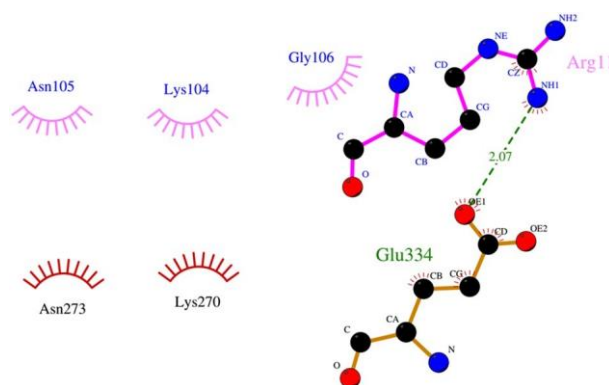


Figure 7. Molecular interactions between CHIKV E2 and PLA_{2CB}. 2D diagram of the interactions of the envelope glycoprotein E2 protein with PLA_{2CB}. The hydrogen bonds (green dashes) are shown between PLA_{2CB}(purple lines) and E2 glycoprotein (orange lines). Hydrophobic interactions are also shown between PLA_{2CB} (purple bows) and E2 glycoprotein (red bows). All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

analysis, the value heights indicate parallel changes in the intensity of each functional group. The binding inter-action between CHIKV virions and PLA_{2CB} was mainly revealed by the increase in the vibrational mode at 1068 cm⁻¹, which indicates detection of additional stretching of C-O ribose present in glycoprotein derived from the association CHIKV virions and PLA_{2CB} (Fig. 8B). Furthermore, the Stacked Walls (Fig. 9A) and split

heat map (Fig. 9B) reinforces the additional expression of vibrational mode at 1068 cm⁻¹ plus PLA_{2CB} under CHIKV virions association.

Discussion

Natural PLA_{2CB} has shown to have broad spectrum antiviral activity^{32–34}. Here, we assessed the antiviral activity of the PLA_{2CB} against CHIKV, as well as sought comprehension on its mechanism of action. Our results demonstrated that PLA_{2CB} strongly inhibited CHIKV infection, corroborating with Russo and colleagues work, which demonstrated that rPLA_{2CB} impaired CHIKV infection³⁵. Additionally, the results demonstrated that the pre-treatment of naïve cells with PLA_{2CB} protected host cells against CHIKV infection. In accordance with our study, Chen and coworkers have reported that phospholipase A2 isolated from the venom of the honeybee *Apis mellifera* was able to protect cells against Human immunodeficiency virus (HIV) and dengue virus (DENV) infections⁴¹. Fenard and colleagues also demonstrated that cells can be protected against HIV infection by different phospholipases A2 isolated from several mammalian species⁴². The PLA2s from snake venoms are classified

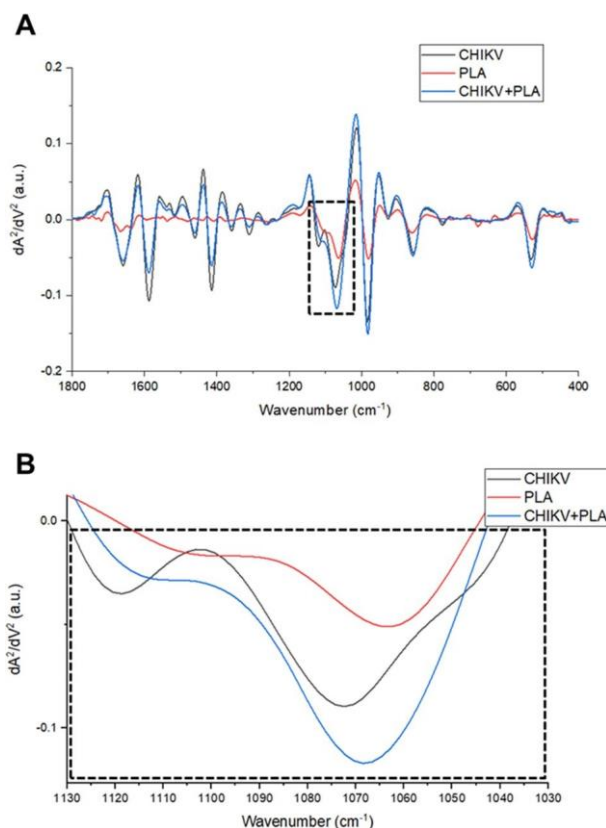


Figure 8. Infrared spectroscopy Spectral analysis indicates interactions between CHIKV virion and PLA_{2CB}. **(A)** Representative infrared average spectrum of second derivative analysis from PLA_{2CB} (red line), CHIKV virion (black line), and PLA_{2CB} plus CHIKV virion (blue line) employing an Fourier Transform Infrared (FTIR) methodology. **(B)** Second derivative analysis, which the value heights indicate the intensity of each functional group. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

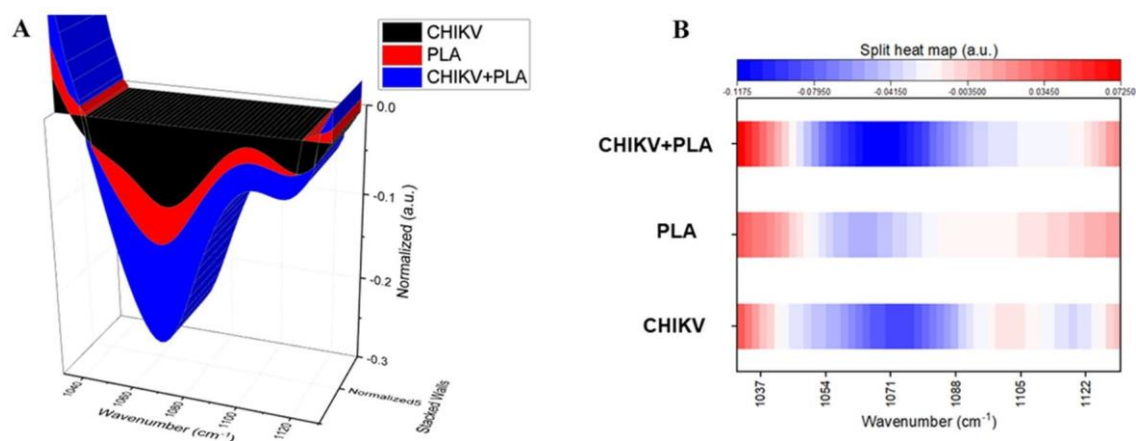


Figure 9. Infrared spectroscopy Spectral analysis between CHIKV virion and PLA_{2CB}. A representative StackedWalls **(A)** and split heat map **(B)** of the infrared average spectrum of second derivative analysis from PLA_{2CB} (red), CHIKV virions (black), and PLA_{2CB} plus CHIKV virions (blue). All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

in the group II of a secreted family phospholipases and show homology to the mammalian inflammatory PLA₂, which play different roles in the organism including in an immune response to infectious diseases^{43–46}. Therefore, our data might also suggest that PLA_{2CB} plays a role in host cell metabolism and as a result protects cells against viral infection, by the possible mimicking effect of phospholipases found in host cells.

Our findings that PLA2_{CB} has strong virucidal effect and interferes with viral entry to the host cells are consistent with previous findings made for two flaviviruses, DENV and yellow-fever (YFV)³⁴. The authors demonstrated that incubation of DENV or YFV virions with PLA2_{CB} results in inhibition of early steps of viral infection probably by disrupting virion envelope membrane and/or blocking virus adsorption^{33,34}. Additionally, Russo and coworkers described that incubation of rPLA2_{CB} with CHIKV prior to the infection of cells significantly impaired CHIKV infectivity³⁵. Therefore, our results are in agreement with previous data that suggested that the predominant activity of PLA2_{CB} is due to its virucidal effect, probably by acting on the virus particle. Several PLA2s isolated from snake venom have been described to possess antiviral activity against DENV, YFV, Herpes simplex types 1 and 2 and Influenza A (H3N2) by interacting with lipid membrane found in a pocket between glycoproteins and/or through attachment to the glycoproteins in the viral envelope surface^{33,34,41,47}. Based on this data, we performed a blind molecular docking using PLA2_{CB} and the CHIKV glycoproteins complex (E1, E2, and E3) to assess the possible interaction among them. The results demonstrated that PLA2_{CB} most likely bonded to both E1 and E2 with E1 being the main target. These results are also consistent with the virucidal effect described here and corroborate previously published data³⁵. The glycoproteins E1 and E2 are essential during the early stages of CHIKV infection. The CHIKV glycoprotein E2 is responsible for binding to cells receptors such as MXRA⁴⁸; the binding occurs in the “canyon” between protomers of CHIKV spike complex. In addition, E1 is a viral fusion protein that ensures the envelope fusion with host cells membranes^{49,50}. Thus, molecules that can interact with E1 and/or E2 attachment sites can prevent virus entry⁵¹. We propose that PLA2_{CB} might act by binding to E1 and/or E2 glycoproteins and preventing virion entry by impairing attachment to the cells and/or membrane fusion. PLA2_{CB}/glycoproteins interactions, characterized using an infrared spectrum assay, did indeed support the hypothesis of interaction between CHIKV virions and PLA2_{CB}. On the other hand, it is important to emphasize that alphavirus virions exist in two states with different characteristics, a normal infectious state and pre-fusion state when E1 is activated prior to attachment^{52–54}. In this context, the employment of cryogenic electron microscopy (cryo-EM) or atomic force microscopy presents as potential approaches to better characterize this interaction. Moreover, PLA2_{CB} also demonstrated a modest yet significant ability to inhibit post-entry steps of CHIKV infection. Using CHIKV subgenomic replicon it was found that both RNA replication and subgenomic RNA synthesis were inhibited. Shimizu and coworkers have previously reported that PLA2_{CB} also affected the post-entry steps of HCV infection³², corroborating with our findings. The mode how PLA2_{CB} affects RNA replication step remains currently unknown. However, it can be speculated that this may be associated with its effect on cell membranes that are required for formation of RNA replicase complexes. The exact mechanism of this as well as similarities and differences between anti-HCV and anti-CHIKV effects represent topics for additional studies. In summary, our study evidenced that PLA2_{CB} isolated from *Crotalus durissus terrificus* inhibited multiple steps of CHIKV infection. The enzyme was able to protect the target cells against CHIKV infection, impaired virus entry to the host cells, mainly by virucidal activity, and also disturbed post-entry steps of the CHIKV infection. Therefore, this data might be useful for further development of new antiviral approaches against CHIKV and provide the potential for treatment of Chikungunya fever.

Methods

Compound. The crude venom of *Crotalus durissus terrificus* was obtained from the “Animal Toxin Extraction Center” (CETA), duly registered and approved by the Ministry of the Environment under process number 3002678. The venom was collected from 28 specimens from the Morungaba—SP collection under the Brazilian Institute for the Environment and Renewing Natural Resources (IBAMA) authorization: 1/35/1998/000846–1, and extraction was performed by Jairo Marques do Vale (CETA). All experiments were performed in accordance with relevant named guidelines and regulations available in the federal universities, IBAMA and the Ministry of Environment. The isolation and purification of phospholipase PLA2_{CB} (Fig. 1A) from the venom of *Crotalus durissus terrificus* snakes were carried out at the Toxinology Laboratory of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, as previously described^{28,34}. The lyophilized protein was dissolved in PBS (phosphate buffer saline), filtered, and stored at -80°C. Dilutions of the stock solution containing the protein were made immediately prior to the experiments. For all the performed assays, PBS was used as the untreated control. All authors complied with the ARRIVE guidelines.

Cell culture. BHK-21 cells (fibroblasts derived from Syrian golden hamster kidney; ATCC CCL-10), purchased from The Global Bioresource Center (ATCC), were maintained in Dulbecco’s modified Eagle’s medium (DMEM, SIGMA-ALDRICH) supplemented with 100 U/mL of penicillin (HYCLONE LABORATORIES), 100 mg/mL of streptomycin (HYCLONE LABORATORIES), 1% dilution of stock of non-essential amino acids (Hyclone Laboratories) and 1% of fetal bovine serum (FBS, HYCLONE LABORATORIES) in a humidified 5% CO₂ incubator at 37 °C. Subgenomic replicon (SGR) harboring cell lines (BHK-CHIKV-NCT)³⁷ were maintained under the same conditions of BHK-21 cells (ATCC CCL-10), except for the addition of G418 (SIGMA-ALDRICH) at 5 mg/mL.

Rescue of CHIKV-*nanoluc* reporter virus. The CHIKV expressing *nanoluciferase* reporter (CHIKV-*nanoluc*) (Fig. 1B) used for the antiviral assays is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). The infectious cDNA of CHIKV-*nanoluc* was placed under control of the CMV promoter³⁶. To produce CHIKV-*nanoluc* virions, 2.3 × 10⁷ BHK-21 cells seeded in a T175 cm² flask were transfected with

1.5 µg of CHIKV-CMV-*nanoluc* plasmid using Lipofectamine 2000 and Opti-Mem medium. 48 h post-transfection (h.p.t.) the supernatant was collected and stored at -80 °C. To determine viral titers, 1 × 10⁵ BHK-21 cells were seeded in each of wells of 24 wells plate; 24 h later the cells were infected with tenfold serially dilutions of

CHIKV-*nanoluc*. Cells were incubated with virus for 1 h 37 °C; after this, the inoculums were removed, cells were washed with PBS to remove the unbound virus, and fresh medium supplemented with 1% dilution of stock of penicillin and streptomycin, 2% FBS and 1% carboxymethyl cellulose (CMC) was added. Infected cells were incubated for 2 days in a humidified 5% CO₂ incubator at 37 °C, followed by fixation with 4% formaldehyde and staining with 0.5% violet crystal. The viral foci were counted to determine viral titer which was presented in plaque forming units per milliliter (PFU/mL).

Cell viability assay. Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra-zolium bromide] (SIGMA-ALDRICH) assay as previously described⁵⁵. BHK-21 cells were plated to 48 well plates at a density of 5×10^4 cells per well and incubated overnight at 37 °C. Medium containing two-fold serial dilutions of PLA2CB (from 0.195 to 200 µg/mL) was added and cells were incubated for 16 h. After this, the medium was replaced with the MTT solution at 1 mg/mL, cells were incubated for 30 min, after which MTT solution was and replaced with 300 µL of DMSO (dimethyl sulfoxide) to solubilize the formazan crystals. The absorbance was measured at 490 nm on the Glomax microplate reader (PROMEGA). Cell viability was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean optical density of the treated and untreated control groups, respectively. The cytotoxic concentration of 50% (CC₅₀) was calculated using GraphPad Prism 8.

Determination of the effective concentration 50% (EC₅₀). To assess the antiviral activity of PLA2CB, BHK-21 cells were seeded at a density of 5×10^4 cells per well into 48 well plates for 24 h and infected with CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 PFU/cell as described by Oliveira and coworkers⁵⁵. The PLA2CB at concentrations ranging 0.195–200 µg/mL was added to growth media. Samples were harvested using Renilla-luciferase lysis buffer (PROMEGA) at 16 h post-infection (h.p.i.) and virus replication levels were quantified by measuring *nanoluciferase* activity using the Renilla luciferase Assay System (PROMEGA). The effective concentration of 50% inhibition (EC₅₀) was calculated using GraphPad Prism 8 software. The values of CC₅₀ and EC₅₀ were used to calculate the selectivity index (SI = CC₅₀/EC₅₀).

Time-of-addition assays. BHK-21 cells at the density of 5×10^4 cells per well were seeded in 48 well plates 24 h before infection and treatment. All infections were performed at MOI of 0.1 and efficiency of virus replication was assessed by measurement of *nanoluciferase* activity at 16 h.p.i. In pretreatment assay, cells were treated for 1 h with the compound prior to the CHIKV infection, extensively washed with PBS and added of CHIKV-*nanoluc* for 1 h. Then, cells were washed with PBS and incubated to remove unbound virus and added of fresh medium for 16 h (Fig. 2A). Alternatively, cells were treated for 1 h with the compound, washed with PBS and infected with CHIKV-*nanoluc* at the presence (Fig. 2B) of PLA2CB for 16 h. In entry inhibition assay, cells were infected using media containing the compound- and virus for 1 h, washed with PBS and incubated with fresh medium for 16 h (Fig. 3A). The virucidal activity was assessed using the same setting except inoculum containing compound and virus was incubated for 1 h before it was added to the cells (Fig. 3B). The impact of compound on attachment step was analyzed using the same setting as in entry inhibition assay except cells were incubated with virus and compound at 4 °C (Fig. 3C). A variant of this assay where the incubation at 4 °C was followed by incubation for 30 min at 37 °C was used to analyze the effect of compound on post-attachment steps of infection (Fig. 3D). In post-entry assay, cells were infected with CHIKV for 1 h, washed extensively with PBS, and the incubated in compound-containing medium for 16 h (Fig. 4A).

RNA replication assay using BHK-CHIKV-NCT cells. BHK-CHIKV-NCT cells that express CHIKV nonstructural proteins, a selection marker (puromycin acetyltransferase, Pac) and *Renilla* luciferase and EGFP reporters³⁷, were used to assess the activity of PLA2CB on CHIKV RNA replication. Cells were seeded at a density of 7×10^3 cells per well of a 96 well plate. After 24 h, cells were treated with the PLA2CB at 12.5 µg/mL for 72 h (Fig. 4B). The impact of compound on CHIKV RNA replication was estimated by quantification of *Renilla* luciferase expression. In addition, EGFP fluorescence was monitored by placing plates directly using an EVOS(THERMO-FISCHER) fluorescence microscope and using 20× lens and GFP filter.

Molecular docking analysis. The interaction between PLA2CB (PDB: 3R0L) and the envelope glycoproteins of the CHIKV (PDB: 3N42) was analyzed using blind docking performed in the PatchDock server⁵⁶, using the parameters predefined by the program and refined by the FireDock algorithm⁵⁷. The best docking positions were evaluated by the geometric complementarity score defined by PatchDock, with results refined and ranked by the global energy after refinement. The post-docking 3D image was generated in the DS Visualizer program, Dassault Systèmes BIOVIA, Discovery Studio Visualizer, version 17, San Diego: Dassault Systèmes, 2016, and a 2D diagram of the interactions interface between the molecules was generated with the aid of the LigPlot+ program⁵⁸.

Infrared spectroscopy spectral data analysis. An Fourier Transform Infrared (FTIR) spectrophotometer Vertex 70 (BRUKER OPTICS, REINSTETTEN, Germany) connected to a micro-attenuated total reflectance (ATR) platform was used to record sample signature at 1800 cm⁻¹ to 400 cm⁻¹ regions as described by Oliveira and coworkers⁵⁵. The ATR unit is composed of a diamond disc as an internal-reflection element. The sample dehydrated pellicle penetration depth ranges between 0.1 and 2 µm and depends on the wavelength, incidence angle of the beam, and the refractive index of ATR-crystal material. The infrared beam is reflected at the interface toward sample in the ATR-crystal. All samples (2 µL) were dried using airflow on ATR-crystal for

3 min before sample spectra recorded in triplicate. The air spectrum was used as a background in all ATR-FTIR analysis. Sample spectra and background were taken with 4 cm^{-1} of resolution and 32 scans were performed for analysis. The spectra were normalized employing the vector method and adjusted to rubber band baseline correction. The original data were plotted in the Origin Pro 9.0 (ORIGINLAB, Northampton, MA, USA) software to create the second derivative analysis. The second derivative was obtained by applying the Savitzky-Golay algorithm with polynomial order 5 and 20 points of the window. The value heights indicated the intensity of the functional group evaluated.

Statistical analysis. Individual experiments were performed in triplicate and all assays were performed a minimum of three times to confirm the reproducibility of the results. GraphPad Prism 8 software was used to assess statistical differences of means of readings using Student's unpaired t-test or Mann-Whitney tests. P values <0.01 were considered to be statistically significant.

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Competing interests

The authors declare no competing interests.

Additional information

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