UNIVERSIDADE ESTADUAL PAULISTA
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Tamara de Carvalho

Estudo da Ação de Nanoemulsões contendo Óleo de Copaíba na Inibição do Zika Vírus

São José do Rio Preto

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

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“Não importa o que aconteça, continue a nadar”

(WALTERS, GRAHAM ; PROCURANDO NEMO, 2003)

RESUMO

O vírus Zika (ZIKV), pertencente ao gênero dos *Flavivirus*, possui como principais vetores os mosquitos do gênero *Aedes*, e foi isolado pela primeira vez em 1947, na floresta Zika, na Uganda. Porém, a primeira epidemia da febre Zika foi registrada apenas em 2007, e a partir de então o número de casos proliferaram pelo mundo. Os sintomas mais comuns causados pelo ZIKV são febre, dor de cabeça, conjuntivite, dores articulares e musculares, e erupção cutânea. Ainda, o desenvolvimento de doenças neurológicas, como a Síndrome de Guillain-Barré e síndrome congênita do Zika, sendo que, já foram registrados diversos casos de microcefalia. Atualmente, não existem vacinas ou antivirais contra o ZIKV. Assim, faz-se de grande relevância estudos que agregam moléculas ativas como o óleo de copaíba, que já demonstrou diversas atividades antimicrobianas na literatura, associadas à nanoemulsões (NEs), potencializando a atividade e o modo de entrega em sistemas biológicos. Portanto, o objetivo deste estudo foi analisar a ação de NEs contendo óleo de copaíba na inibição do ZIKV. Inicialmente foi realizado o ensaio de viabilidade celular (MTT) em linhagens celulares VERO E6 e HuH-7 e a captação intracelular da droga. A inibição viral foi avaliada inicialmente por ensaio de formação de placas e na sequência foi realizado o ensaio de dose dependência que foi avaliado por qPCR. Por fim, foi realizado o ensaio de tempo de adição para identificar a etapa do ciclo viral que a droga está atuando, também avaliado por qPCR. Os resultados do ensaio de MTT demonstraram que a maior concentração não citotóxica, acima de 80% de viabilidade, 48 horas após o tratamento, foi de 180 µg/mL. As NEs internalizaram em todos os tempos analisados, em ambas as linhagens analisadas. Em sequência, observou-se uma redução relativa no número de focos de aproximadamente 70% e 80%, para a NE vazia (NEV) e contendo óleo de copaíba (NEC), respectivamente. A dose dependência em linhagem VERO E6 infectada com ZIKV, observou-se que para a NEV, a inibição do ZIKV foi similar em todas as concentrações analisadas e, para a NEC, a inibição foi dose dependente. Resultados preliminares do ensaio de tempo de adição demostraram que a NEC tem efeito virucida inibindo aproximadamente 92,5% da liberação do vírus. Ambos os tratamentos também demostraram efeito nas etapas pós-entrada, inibindo cerca de 99,1% do RNA intracelular do ZIKV. Até o momento, é possível concluir que, apesar de ambas NEs demonstrarem potencial em inibir o ZIKV, experimentos são necessários para confirmar os resultados e também para entender o modo de ação das NEs, de forma a esclarecer em qual etapa do ciclo viral os tratamentos estão inibindo o Zika.

Palavras chave: Antiviral, Nanoemulsão, Óleo de copaíba, Zika vírus.

ABSTRACT

The Zika virus (ZIKV), belongs to the genus *Flavivirus*, has as main vectors mosquitoes from *Aedes*genus and was first isolated in 1947 in the Zika forest in Uganda. However, the first epidemic of Zika fever was recorded only in 2007, and since then the number of cases has spread worldwide. The most common ZIKV symptoms are fever, headache, conjunctivitis, joint and muscle pain, and rash. Besides that, it can lead to the development of neurological diseases such as Guillain-Barre syndrome and congenital Zika, so that several cases of microcephaly have been reported. There are currently no vaccines or antivirals against ZIKV. For this reason, studies with different active molecules and drug delivery systems are of great importance. Thus, there is a great relevance of studies that combine active molecules such as copaiba oil, which has already shown different antimicrobial activities in the literature, and nanoemulsions (NEs), enhancing the activity and improving the mode of delivery in biological systems. Therefore, the aim of this study was to analyze the action of NEs containing copaiba oil on ZIKV inhibition. Initially, a cell viability assay (MTT) in VERO E6 and HuH-7 cell lines and intracellular uptake were performed. Viral inhibition was initially assessed by plaque assay and then dose dependence assay was evaluated by qPCR. Finally, the time of addition test was performed to identify the stage of the viral cycle that the drug is acting, also evaluated by qPCR. The results of the MTT assay showed that the highest non-cytotoxic concentration with cell viability above 80%, 48 hour post-treatment, was 180 µg/mL. The internalization of nanoemulsion in cells was confirmed in all times analyzed, in both cell lines. In the general inhibition assay, the relative foci number was reduced by approximately 70% and 80% for the empty nanoemulsion (ENE) and the containing copaiba oil nanoemulsion (CNE), respectively. In the nanoemulsion dose-dependency assay on ZIKV, it was observed that for the NEV inhibition was similar at all concentrations analyzed and for CNE the inhibition was dose dependent. Preliminary results of the time of addition assay show that the copaiba oil nanoemulsion has virucidal effect inhibiting 92.5% of virus release. Both treatments with nanoemulsions also have an effect on the post-entry stage, inhibiting about 99.1% of ZIKV intracellular RNA when compared to the control. Preliminarily, it can be concluded that, although both NEs show potential to inhibit ZIKV, further experiments are needed to confirm the results and also to understand the mode of action of NEs in order to clarify at which stage of the viral cycle treatments are inhibiting Zika.

Key words: Antiviral, Copaiba oil, Nanoemulsions, Zika virus.

LISTA DE ABREVIAÇÕES E SIGLAS

4-HPR: N- (4-hidroxifenil) retinamida

6 MMpr: 6-metilmercaptopurina ribosídeo

25 HC: 5-hidroxicolesterol

C: Proteína do capsídeo do Zika vírus, do Inglês *Core protein*

C: Controle, do Inglês Control

cDNA: DNA complementar, do inglês complementary DNA

CMC: carboximetilcelulose, do Inglês Carboxymethylcellulose

CNE: Nanoemulsão de óleo de copaíba, do Inglês Copaiba oil Nanoemulsion

CO_2: Dióxido de carbono

DENV: vírus da Dengue, do Inglês Dengue virus

DMEM: Meio Eagle Modificado por Dulbeco, do Inglês Dulbecco's Modified Eagle's Medium

ENE: Nanoemulsão vazia, do Inglês Empty Nanoemulsion

ENV: Proteína do envelope do Zika vírus

EGCG: epigalocatequina galato

FCO: Óleo de copaíba livre, do Inglês Free Copaiba Oil

FIOCRUZ: Fundação Oswaldo Cruz

H1N1: Gripe H1N1 ou suína

HIV: Vírus da imunodeficiência humana, do Inglês *Human Immunodeficiency Virus*

HuH: Hepatoma humano, do Inglês Human hepatoma (Referente à linhagem celular)

Kb: kilobase

MMPD: Merimepodib

MTT: Dimetil tiazolio 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NE: Nanoemulsão

NEC: Nanoemulsão contendo óleo de copaíba

NEs: Nanoemulsões

NEV: Nanoemulsão vazia

NPs: Nanopartículas

NS: Proteínas Não estruturais, do Inglês Non-Structural

OMS: Organização Mundial da Saúde

P/S: Referente ao antibiótico de penicilina e estreptomicina, do Inglês *Penicillin*/*Streptomycin*

PBS: Tampão fosfato salino, do Inglês Phospate buffered saline

PCR: Reação em cadeia da polimerase, do Inglês Polymerase Chain Reaction

PFU: Unidade formadora de placa, do Inglês Plaque Forming Unit

prM: Proteína precursora de membrana

RNA: Ácido Ribonucleico, do Inglês Ribonucleic Acid

RNA-C: Complexo de proteína viral

FBS: Soro Fetal Bovino, do Inglês Fetal Bovine Serum

WC: Controle de água, do Inglês Water Control

ZIKV: Vírus da Zika, do Inglês Zika virus

Sumário

CAPÍTULO I

Considerações gerais

1. INTRODUÇÃO

1.1. Arbovírus

Mais de 500 arbovírus, reconhecidos mundialmente, fazem parte de um grupo constituído por vírus que são transmitidos por vetores artrópodes, como por exemplo, mosquitos, carrapatos (CLETON et al., 2012; YOUNG, 2018), moscas e ácaros, dentre outros (SALIMI;CAIN; KLEIN, 2016). Sendo que desses arbovírus, 150 infectam humanos, causando doenças que podem resultar em epidemias (YOUNG, 2018). Estes vírus pertencem, majoritariamente, às famílias *Togaviridae*, *Bunyaviridae* e *Flaviviridae*, mas também, em menor número, às famílias *Reoviridae* e *Orthomyxoviridae* (CLETON et al., 2012).

Devido à rápida disseminação dos arbovírus, com possibilidade de resultar em surtos na população, estes são considerados um problema de saúde pública mundial (YOUNG, 2018), sendo que, mais de 30% da população do mundo vive em zonas de risco (MOURA-NETO et al., 2019). Na figura 1, podemos observar em rosa, as zonas no mapa com maior incidência de arboviroses.

Figura 1: Na coloração rosa, regiões com maior incidência de arboviroses. Mais de 30% da população vive em áreas de risco.

Fonte: (MOURA-NETO et al., 2019)

No Brasil, primeiramente, haviam relatos da circulação de dez arbovírus de importância clínica médica no país: os vírus Mayaro, Encefalite Equina Venezuelana, Encefalite equina Oriental e Chikungunya, pertencentes à família Togaviridae, os vírus

da Dengue (DENV), Febre amarela, vírus do complexo da encefalite japonesa, Encefalite de Saint Louis, West Nile, pertencentes à Flaviviridae, e finalmente, o vírus Oropouche, pertencente à Bunyaviridae (FIGUEIREDO, 2007). Somente depois, no ano de 2015, reportou-se o primeiro relato do décimo primeiro arbovírus circulante no Brasil, o Zika vírus (ZIKV) (ZANLUCA et al., 2015).

Os flavivírus são vírus que pertencem à família Flaviviridae e ao gênero *Flavivirus*, fazem parte das arboviroses que causam doenças humanas (DE ANDRADE et al., 2017; YOUNG, 2018). Os flavivírus: Zika, Dengue e Febre amarela, merecem destaque, em razão da expansão demográfica acelerada, de forma que, o número de casos de infecção proliferam pelo mundo, afligindo e preocupando a população mundial (MAYER;TESH; VASILAKIS, 2017).

A rápida disseminação desses arbovírus é resultante da propagação de seus vetores *Aedes aegypti e Aedes albopictus* pelo mundo, em razão dos processos de urbanização e desmatamento. A origem e a dispersão desses vetores diferem, de maneira que, o *Ae. aegypt* é nativo da África, enquanto o *Ae. albopictus* é da Ásia, porém, hoje em dia, ambos são encontrados nas Américas e Ásia (DE ANDRADE et al., 2017). Atualmente, as formas de controle para esses vetores são por meio do uso de inseticidas, e campanhas que incentivam a população ao cuidado para evitar o desenvolvimento de hábitats desses vetores, tais como: evitar água parada e o acúmulo de lixo (MOYES et al., 2017).

1.2. Febre do Zika

Em abril do ano de 1947, em uma floresta na Uganda, o ZIKV foi isolado pela primeira vez, da amostra de soro de um macaco rhesus (Rhesus 766). Posteriormente, em janeiro de 1948, o ZIKV foi isolado pela segunda vez, proveniente de um mosquito *Ae. africanus* (DICK;KITCHEN; HADDOW, 1952; YUN; LEE, 2017). Já o ano de 1952, foi marcado pelos primeiros casos de infecção desse vírus em humanos, na República Unida da Tanzânia e em Uganda, e ainda, pela confirmação da presença do vírus em humanos na Índia (KINDHAUSER et al., 2016).

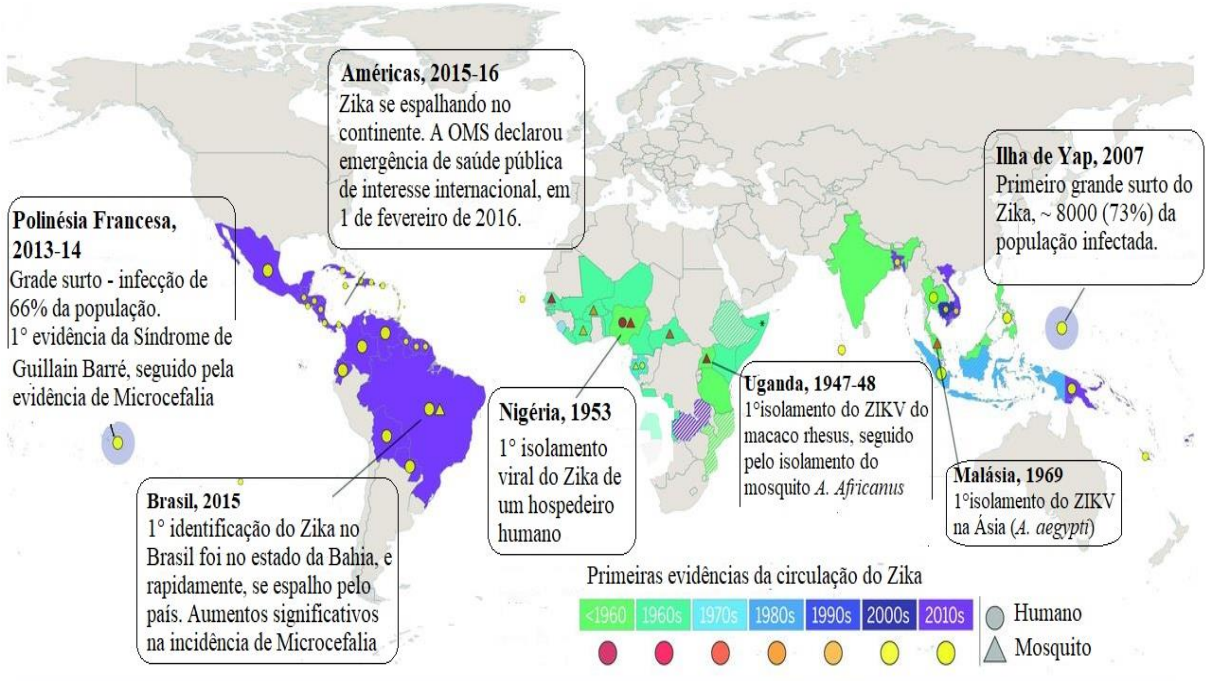
Porém, o primeiro surto causado por esse vírus, só ocorreu no ano de 2007, na Ilha de Yap, nos Estados Federados da Micronésia, em que foram observados os sintomas de febre, erupção cutânea, dor nas juntas e conjuntivite, sendo que, cerca de 70% da população foi infectada, em apenas 13 semanas. Os casos relacionando o ZIKV e o desenvolvimento de doenças neurológicas, a Síndrome de Guillain-Barré e microcefalia

em recém nascidos, acometendo diversas falhas neurológicas, como a paralisia cerebral, só ocorreu a partir do período de 2013-2014, durante um surto na Polinésia Francesa, que afetou 10% da população (KINDHAUSER et al., 2016; SAMPATHKUMAR; SANCHEZ, 2016; WATRIN et al., 2016). Outros surtos também foram registrados nas Ilhas de Páscoa e Cook e na Nova Caledônia (PLOURDE; BLOCH, 2016). Além dos sintomas apresentados, posteriormente, foi descrito na literatura casos de pacientes assintomáticos infectados pelo ZIKV (MUSSO et al., 2014; OLIVEIRA SOUTO et al., 2018)

O Brasil, um país com clima tropical, caracterizado pela sua biodiversidade e florestas tropicais, possui um grande potencial para o desenvolvimento de arboviroses. Dessa forma, em 2015, o Brasil teve seu primeiro caso de infecção por Zika vírus reportado, na cidade de Camaçari, no estado da Bahia. Esse marco foi seguido então, por outros casos de transmissão autóctone em diversos países do continente americano (FIGUEIREDO, 2007; PLOURDE; BLOCH, 2016; WAGGONER; PINSKY, 2016). É importante ressaltar que, nos anos de 2014 e 2015, coincidente com surgimento do ZIKV no Brasil, foi registrada uma grande epidemia de casos de microcefalia, o que levou ao início de investigações de uma possível correlação entre a infecção pelo ZIKV em mulheres grávidas e os casos de microcefalia (FAUCI; MORENS, 2016).

Na figura 2, podemos observar um mapa com os principais marcos históricos do Zika vírus, desde o primeiro isolamento do vírus na Uganda, o primeiro grande surto na Ilhas de Yap, até a chegada do vírus no Brasil.

Figura 2: Mapa com os principais marcos do Zika vírus, desde seu primeiro isolamento em Uganda, até a primeira identificação no Brasil.



Fonte: (LESSLER et al., 2016) – Adaptado.

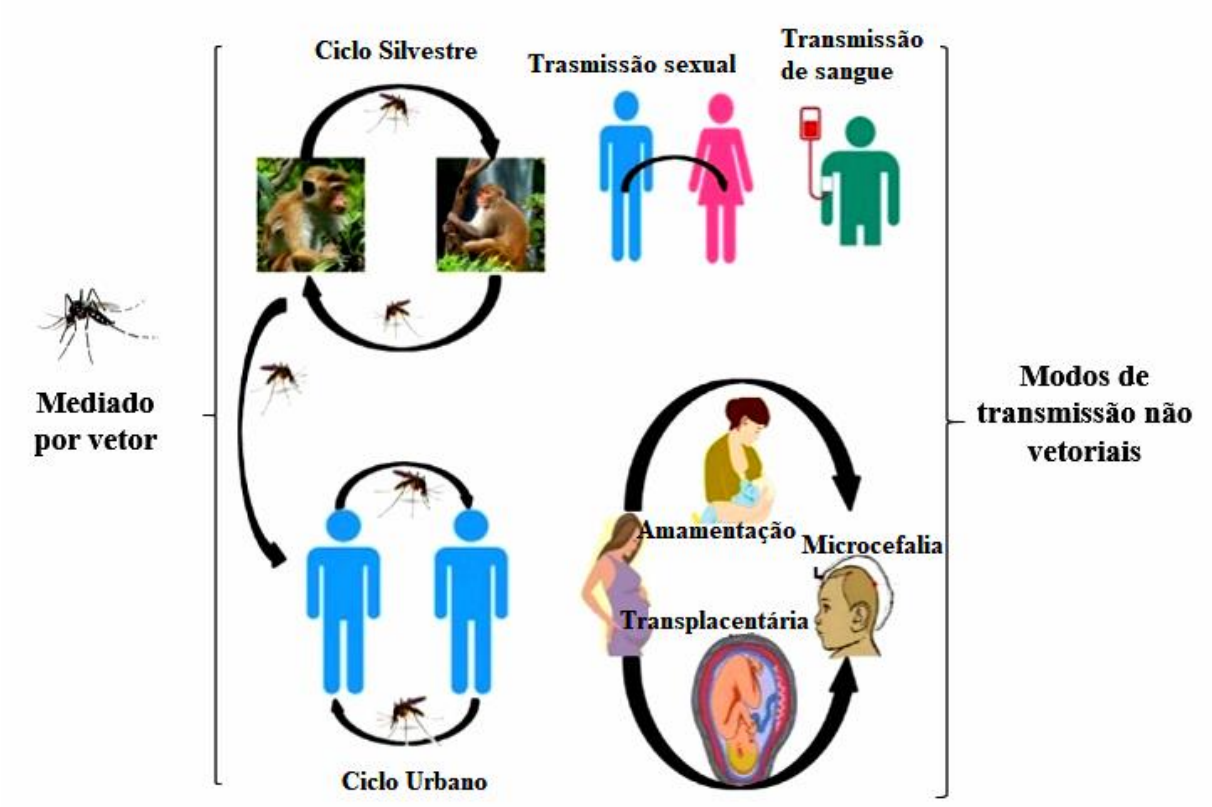
Os casos de infecção por Zika vírus já foram declarados pela Organização Mundial da Saúde (OMS) como um grande problema de saúde pública, comparando a gravidade da situação com os anos de 2009, durante a epidemia de H1N1, e de 2014, com os casos de ressurgimento da poliomielite do Paquistão e Síria, e ainda com as infecções por vírus Ebola (SAMPATHKUMAR; SANCHEZ, 2016).

O Zika vírus já foi isolado de diversos mosquitos *Aedes*, como o *Ae. africanus, Ae. aegypti, Ae. albopictus, Ae. hensilli, Ae. luteocephalus, Ae. furcifer* e *Ae. vittatus* (PAIXAO et al., 2016; GORSHKOV et al., 2018). No ano de 2016, a Fundação Oswaldo Cruz (FIOCRUZ), identificou o Zika vírus em mosquitos *Culex quinquefasciatus*, sendo então, um possível vetor do vírus (AGÊNCIA FIOCRUZ, 2016). A transmissão do ZIKV pode ser através de um vetor, em que temos dois ciclos de transmissão, o silvestre e o urbano. O ciclo silvestre compreende primatas não humanos e mosquitos em florestas. Já o ciclo urbano, está relacionado com a transmissão do vírus em humanos e mosquitos nas zonas urbanas (BAUD et al., 2017; SONG et al., 2017).

A transmissão do Zika vírus também pode não envolver necessariamente um vetor, em que, inicialmente, tem se a infecção de humanos pela picada do mosquito, e

posteriormente, a transmissão sexual, pela propagação do vírus em órgãos reprodutivos. Além disso, podemos observar a transmissão vertical do ZIKV, que seria a congênita ou perinatal, na qual o feto adquire o vírus da mãe durante a gestação (BASU; TUMBAN, 2016; PLOURDE; BLOCH, 2016), e diversos estudos na literatura demonstraram a análise da presença de RNA e proteínas virais em tecidos placentários e no líquido amniótico (MARTINES et al., 2016; OLIVEIRA MELO et al., 2016). Ainda, existe a possibilidade da transmissão do Zika vírus por meio de transfusões de sangue (BAUD et al., 2017; SONG et al., 2017). O esquema com todos esses modos de transmissão apresentados pode ser observado na figura 3.

Figura 3: Modos de transmissão do Zika vírus, mediado por vetor e os seus ciclos silvestre e urbano, ou não mediado por um vetor.



Fonte: (MISHRA; BEHERA, 2016) – Adaptado.

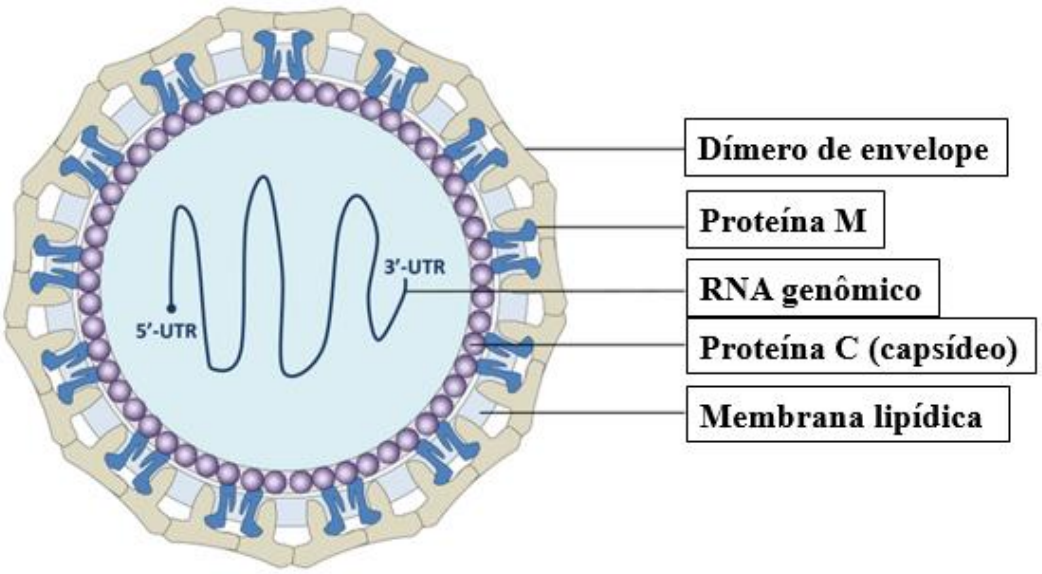
1.2.1. O vírus

O Zika vírus possui um genoma de RNA não segmentado, fita simples e polaridade positiva, e é um vírus envelopado. O genoma do ZIKV de 11kb codifica apenas uma poliproteína, e esta é clivada em várias proteínas individuais. Existem dois tipos de proteínas características do ZIKV: as proteínas estruturais, que são as proteínas

do Capsídeo (C), do envelope (E) e a precursora de membrana (prM), relacionadas a formação da partícula do vírus e aos processos de entrada, montagem e liberação dos vírions; e as não estruturais (NS), que são as proteínas NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5, e estão relacionadas à processos como replicação e controle da célula hospedeira (HAMEL et al., 2015; ABBINK;STEPHENSON; BAROUCH, 2018; BAZ; BOIVIN, 2019). Na figura 4, é possível observar a partícula do Zika vírus e suas proteínas estruturais e não estruturais.

Figura 4: Partícula do Zika vírus, suas proteínas estruturais: Capsídeo (C), do envelope (ENV) e a precursora de membrana (prM), o RNA genômico, e as proteínas não estruturais: NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5. (A) Estrutura do ZIKV (B) RNA genômico do ZIKV.

(A)



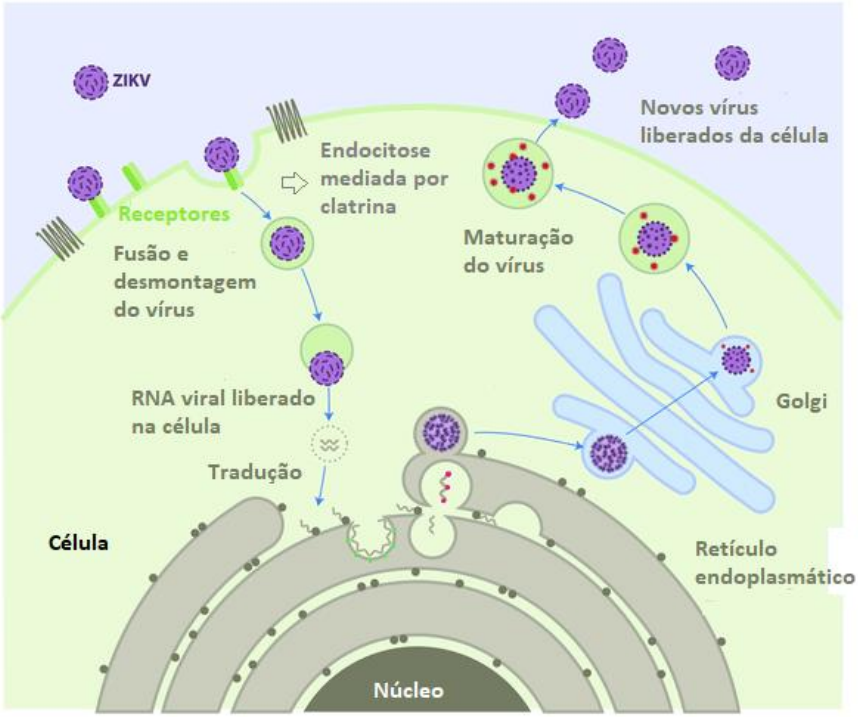
(B)

Fonte: (ABBINK;STEPHENSON; BAROUCH, 2018) – Adaptado.

As etapas de uma infecção viral por Zika (figura 5) são descritas a seguir: primeiramente, ocorrem interações entre glicoproteínas virais e receptores de superfície da célula e como resultado disso tem-se a ligação dos vírions na superfície da célula. Após, ocorre então a internalização na célula por endocitose, mediada por clatrinas

(WHITE et al., 2016). A liberação do genoma do vírus no citoplasma da célula, com a fusão da membrana do vírus e a membrana endossomal, ocorre devido a uma mudança na conformação do vírion, resultante da diminuição do pH do meio. Então, primeiramente tem-se a tradução do RNA de senso positivo em uma poliproteína, a qual as proteases da célula e do vírus, processam co-traducional e pós-traducionalmente. Essa poliproteína é composta pelas proteínas estruturais: C, prM, ENV, e não estruturais: NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5, conforme observado na figura 3 (SONG et al., 2017; ABBINK;STEPHENSON; BAROUCH, 2018). O complexo de replicação, que inclui o RNA viral e proteínas da célula e do vírus, é encontrado nas membranas intracelulares, que é o local de ocorrência da replicação. O processo de replicação envolve a síntese da fita de RNA de senso negativo, utilizada então como o molde para uma outra síntese de várias fitas de RNA de senso positivo. Tem-se então, a montagem dos vírions imaturos, que ocorre primeiro pelo brotamento de um complexo de proteína viral, o RNA-C, no retículo endoplasmático, em que se observa a incorporação da bicamada lipídica e das proteínas do vírus: ENV e prM. O próximo passo é maturação dos vírions imaturos, na via secretora trans-Golgi da célula hospedeira, que leva, finalmente, a liberação de vírions maduros, por exocitose (WHITE et al., 2016; SONG et al., 2017).

Figura 5: Esquema do ciclo replicativo do ZIKV.



Fonte: (ACOSTA-AMPUDIA et al., 2018) – Adaptado.

1.2.2. Prevenção, tratamento, vacina e estudos de antivirais

A infecção por Zika vírus pode ser prevenida de diversas formas. A primeira delas é a proteção de picadas de mosquitos transmissores do vírus, evitando, principalmente, o desenvolvimento de ambientes de proliferação do mosquito, como locais com água parada, e ainda por meio do uso de repelentes. Além disso, como o ZIKV também pode ser transmitido sexualmente, outro modo de prevenção seria por meio do uso de preservativos. Também é de grande importância a realização de uma triagem dos doadores antes de efetivamente iniciar as doações de sangue uma vez que esta pode ser outra forma de transmissão (RELICH; LOEFFELHOLZ, 2017).

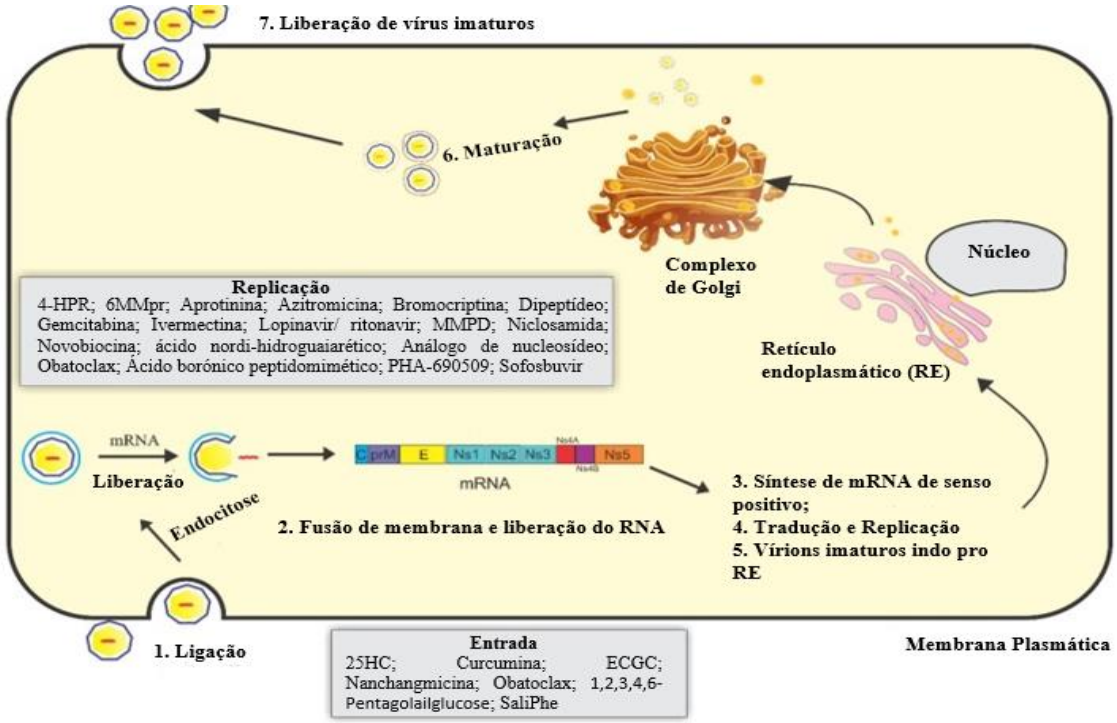
Até os dias atuais, o foco do tratamento para a infecção pelo ZIKV é no alívio dos sintomas básicos sofridos pelo paciente, de forma que, não há nenhuma vacina ou terapia com uso de antivirais ainda (BASARAB et al., 2016). Porém, existem vários estudos com o intuito do desenvolvimento de vacinas para o ZIKV, existindo mais de 50 candidatos em análise e grande probabilidade de futura aprovação de vacinas que são vírus atenuados ou purificados inativos, devido a outras aprovações de vacinas para outros flavivírus (LIN et al., 2018; MAO; CHAO, 2019). Há estudos de desenvolvimento de vacinas que englobam sistema de entrega de antígeno, como por exemplo, vacinas de ácidos nucleicos, e como mencionado anteriormente vírus atenuados e vírions inativos, tendo como foco acelerar o desenvolvimento de uma vacina para o ZIKV (PIERSON; GRAHAM, 2016).

Além da análise de futuras vacinas para o ZIKV, existem também diversos estudos na literatura que buscam moléculas ativas com ação contra o Zika vírus, principalmente englobando compostos naturais, por meio da extração de componentes ativos de plantas, raízes e flores (GACH et al., 2016). Um exemplo de composto natural com ação antiviral é a curcumina, que inibiu a etapa da replicação do ZIKV (MOUNCE et al., 2017), o EGCG, uma molécula do chá verde, que inibiu a etapa de entrada do ZIKV em células de rim de macaco verde africano (VERO E6) (CARNEIRO et al., 2016), e ainda estudos com a berberina e emodin, que demonstraram inativação do vírus Zika (BATISTA et al., 2019).

Na literatura, é possível observar outros estudos de antivirais com ação contra o ZIKV, como o Sofosbuvir, um fármaco que já tem ação comprovada em pacientes com infecção crônica pelo vírus da Hepatite C, demonstrou inibição na etapa de replicação do

ZIKV (SACRAMENTO et al., 2017). Diversos análogos de nucleosídeos já foram testados e também demonstraram ação inibitória contra o ZIKV (EYER et al., 2016). Portanto, diversas moléculas com ações em determinadas etapas do ciclo viral do Zika já foram identificados (Figura 6) (DA SILVA;OLIVEIRA SILVA MARTINS; JARDIM, 2018) e ainda há várias moléculas derivadas de plantas, que demonstraram ação inibitório contra o Zika vírus (ZHOU et al., 2017; CLAIN et al., 2019).

Figura 6: Compostos com ação nas etapas de entrada e replicação do ciclo viral do Zika.



Fonte: (DA SILVA;OLIVEIRA SILVA MARTINS; JARDIM, 2018) – Adaptado.

1.3. Óleo de copaíba

Visto o atual cenário apresentado, de estudos que buscam analisar compostos de origem natural e seus potenciais como possíveis antivirais, observa-se interesse no estudo do óleo de copaíba, que é, geralmente, extraído do tronco da árvore conhecida popularmente como Copaíba, pertencente à família Leguminosae e do gênero *Copaifera* (TOBOUTI et al., 2017), encontrada na floresta amazônica (CAMPOS-CARRARO et al., 2018). Na região brasileira, o óleo de copaíba pode ser obtido de diversas espécies de *Copaifera*, alguns exemplos são a *C. reticulata Ducke*, *C. offinalis L.*, *C. multijuga Hayne*, dentre outras. Sua composição de óleo puro, geralmente, engloba variações de porcentagens de concentrações dos diterpenos, sesquiterpenos e terpenos (TOBOUTI et

al., 2017; DIEFENBACH et al., 2018). Os principais componentes dos óleos essenciais da *Copaifera officinalis* utilizado nesse estudo foram β-cariofileno, *aloaromadendreno,* germacreno B, β-bisaboleno, δ-cadineno, α-cadineno (DIAS et al., 2014). Esses metabólitos secundários pertencem à classe dos sesquiterpenos e já possuem diversas atividades biológicas demonstradas na literatura, como pró-oxidante, antioxidante, anti-inflamatório, antitumoral, efeito antiparasitário, etc (BARTIKOVA et al., 2014).

As atividades do óleo de copaíba são observadas desde a antiguidade por índios nativos, que descreviam a possibilidade de cura de qualquer ferida por esse óleo. A população amazônica explorou outros usos do óleo, como tratamento de infecções de garganta, assim como também para estimular o apetite. As ações desse óleo sempre chamaram a atenção de diversos estudiosos para maior entendimento das potencialidades do óleo de copaíba. Além do uso medicinal, o óleo também foi utilizado por pintores do século 19, inclusive pelo pintor Van Gogh (TOBOUTI et al., 2017).

Esse óleo já demonstrou diversas propriedades, como atividade anti-inflamatória, antitumoral, antibacteriana, antioxidante, antifúngica, analgésica, anti-leishmania, além de demonstrar efeitos sobre a hipertensão arterial pulmonar (DOS SANTOS et al., 2012).

Em relação aos constituintes do óleo de copaíba, os metabólitos secundários de sesquiternos já possuem diversas atividades biológicas demonstradas na literatura, como efeito pró-oxidante, antioxidante, anti-inflamatório, antitumoral, antiparasitária, etc (BARTIKOVA et al., 2014). Nos diterpenos, existem diversos subgrupos que possuem atividades biológicas também, demonstrando ação antifúngica, citotóxica, fitotóxica, e ainda, atividade antiviral, dentre outros potenciais (REVEGLIA et al., 2018). Já para os terpenos, existem análises relacionando efeitos desse metabólito e de outros derivados contra bactérias patogênicas (MAHIZAN et al., 2019).

Ainda, as diversas ações antimicrobianas já descritas na literatura para o óleo de copaíba podem estar relacionadas com as características lipofílicas desse óleo, que podem acarretar em alterações na membrana celular, principalmente em relação às atividades antibacterianas já descritas (DIEFENBACH et al., 2018).

Portanto, a associação desse óleo a nanotecnologia faz-se de grande importância, em razão da ampla variedade de vantagens que esses sistemas trazem, como a melhora da entrega de drogas, principalmente as que possuem características lipofílicas, como é o caso de oleaginosas, ainda, também aumentam a biodisponibilidade de compostos, além

de melhorar a internalização nas células (SINGH et al., 2017). A nanotecnologia é um ramo da tecnologia que engloba diversas áreas, como segurança, tecnologia da informação, energia, agricultura, proteção ambiental, e saúde. O uso da nanotecnologia na medicina, com foco no aperfeiçoamento de técnicas para a saúde da população é a área de maior destaque, sendo considerada uma das pesquisas nanotecnológicas mais bem-sucedidas (GÖKÇAY; ARDA, 2015; GAO et al., 2016).

1.4 Nanotecnologia

A aplicação da nanotecnologia em terapias é resultante do estudo das diversas características dos nanomateriais, suas propriedades morfológicas de superfície e a possibilidade de modificações e associações químicas, com o objetivo de melhora dos sistemas de entrega de drogas, com alvos mais definidos e ainda, maior estabilidade. A nanomedicina utiliza então de estratégias da nanotecnologia, não só com o intuito aperfeiçoar terapias, mas também de sistematizar novas metodologias de tratamentos (FAROKHZAD; LANGER, 2006; KIM;AHN; KIM, 2019).

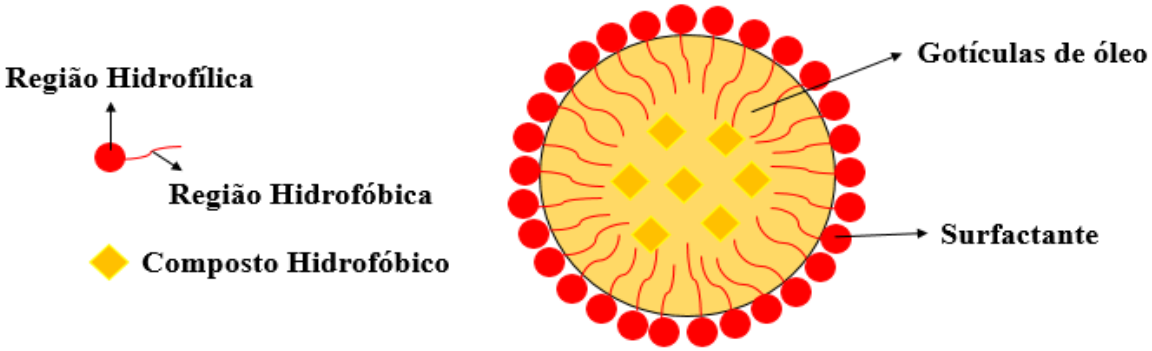
A nanomedicina requer conhecimentos interdisciplinares, de maneira a englobar estudos na química, física e biologia, resultando em um ramo com diversas aplicações, como desenvolvimento de nanomateriais para diagnóstico, a imaginologia e agentes teranósticos; estadiamento de doenças, por meio da amplificação de sinais no sangue ou ativação da detecção de DNA; uso na engenharia de tecido ósseo; carreadores de drogas, com diversas atividades antimicrobianas ou antitumorais, como as nanopartículas ou nanoemulsões (LIU;MIYOSHI; NAKAMURA, 2007; MOLINA et al., 2015; WALMSLEY et al., 2015; SHI; LAMMERS, 2019).

A utilização das nanopartículas (NP) na área médica iniciou-se na década de 1970, com o uso de nanobioconjugados, seguido então, pela diversidade de aplicações biológicas das nanoestruturas (BOISSELIER; ASTRUC, 2009). As nanopartículas possuem uma variedade de tipos de materiais utilizados na sua elaboração, como por exemplo, lipídios, metal, silício, sílica, polímeros, proteínas e carbono, e as nanoemulsões (NEs) possuem como principal característica a composição por uma fase de óleo interna e outra aquosa externa, com dimensões nanométricas (ASSIS et al., 2012; WOLFRAM et al., 2015). Dá se destaque a ampla aplicação médica de NPs de metais nobres, como prata, ouro e platina, seja no diagnóstico, como também no tratamento de doenças como

câncer, vírus da imunodeficiência humana (HIV), tuberculose e Parkinson (RAI et al., 2016).

Em função das limitações de algumas drogas atuais, como a baixa biodisponibilidade e solubilidade da maioria destas, é muito importante o uso de nanopartículas ou nanoemulsões, como sistemas de entrega, de forma que, o resultado é um aumento na eficácia do tratamento (BI et al., 2017; MIRHADI;REZAEE; MALAEKEH-NIKOUEI, 2018). As NPs ou NEs, possuem diversas características únicas, que podem acarretar na melhora da entrega de compostos, redução de efeitos adversos de drogas, como redução da toxicidade da droga e das doses terapêuticas, aumento da solubilidade e estabilidade em fluídos biológicos, dentre outros (WOLFRAM et al., 2015; DALCIN et al., 2019).

Figura 7: Nanoemulsão de óleo e água com um surfactante para estabilização, contendo um composto hidrofóbico**.**



Fonte: Autoria própria.

Como dito anteriormente, as NEs podem ser preparadas de forma a utilizar uma fase aquosa e outra de óleo, mas isso é feito juntamente com um surfactante, ou ainda, com um emulsificante, para proporcionar uma estabilização (Figura 7). O uso dessas nanoemulsões se aplica a um campo promissor, incluindo a indústria farmacêutica, alimentícia, cosméticos, dentre outras. As NEs tem proporcionado uma grande melhoria para a entrega de compostos ou drogas, principalmente quando esses possuem características hidrofóbicas (QIN et al., 2016; TAYEB; SAINSBURY, 2018; BONFERONI et al., 2019).

É importante ressaltar que existem diversos estudos na literatura associando nanopartículas ou nanoemulsões como potenciais antivirais, como é o caso do estudo de

Cagno et al (2018), que testou a ação de nanopartículas não tóxicas de amplo espectro em diversos vírus, inclusive no DENV, seus resultados demonstraram um potencial virucida, em nível nanomolar, de nanopartículas de ouro em dengue do tipo 2 (CAGNO et al., 2018). Já Huang et al (2019) obtiveram resultados da ação de uma nanopartícula de carbono derivado de monômero de benzoxazina, nos flavivírus Dengue, Encefalite japonesa e Zika, confirmando inclusive uma redução da infecciosidade viral (HUANG et al., 2019). Ainda, relatos de Raghavan (2016) demonstram um efeito inibitório no ZIKV e DENV, utilizando uma nanoemulsão lipídica, denominada Metadichol (RAGHAVAN, 2016a; b).

Dessa forma, faz-se de grande importância o uso de nanoemulsões associadas a moléculas ativas, com o intuito de analisar os efeitos e interações em células infectadas com o ZIKV. Esse trabalho teve como intuito o estudo da ação do óleo de copaíba, uma substância que já demonstrou diversas atividades antimicrobianos na literatura, associado à nanoemulsões surfactantes, em células infectadas com o Zika vírus.

2. Objetivo

2.1. Objetivo geral

Avaliar o potencial antiviral de nanoemulsões surfactantes vazia e contendo o óleo de copaíba contra o vírus Zika.

2.2. Objetivos específicos

- Analisar a citotoxicidade das nanoemulsões em 24, 48 e 96 horas, após a incubação, na linhagem celular VERO E6 e 24 e 48 horas na linhagem HuH-7;

- Analisar a captação intracelular das nanoemulsões nos tempos de 1, 2, 3 e 48 horas de tratamento, nas linhagens VERO E6 e HuH-7;

- Avaliar o potencial das nanoemulsões na inibição do ZIKV;

- Analisar a dose dependência das nanoemulsões na inibição do ZIKV.

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CAPÍTULO II

Artigo científico

Title: Zika virus inhibition by copaiba (*Copaifera officinalis*) oil nanoemulsion

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Abstract

Since the 2015 outbreak, Zika virus (ZIKV) has spread all over the world. It has become a major global health issue due to the neurological complications related to ZIKV infection such as Guillain–Barré Syndrome and Zika virus Congenital Syndrome. So far, there are no vaccines or specific treatments for ZIKV infection, which makes important to develop specific therapies for its treatment. Here we evaluated the ability of a copaiba oil nanoemulsion to inhibit ZIKV. For the *in vitro* assays, first, we defined the highest non-cytotoxic concentration of the copaiba-based nanoemulsion in VERO E6 and HuH-7 cells by MTT assay. A concentration of 180 µg/mL was chosen since it maintains 80% cell viability up to 96h after treatment. The intracellular uptake assay was performed and confirm the internalization of the nanoemulsions in VERO E6 and HuH-7 cells in all times analyzed. VERO E6 cells were infected and simultaneously treated with copaiba oil nanoemulsion (CNE) and empty nanoemulsion (ENE) at the highest non-toxic concentration. After 96h, results were evaluated by plaque assay revealing a viral inhibition of 80% for CNE and 70% for ENE. In order to understand in which steps of the viral life cycle the drug is acting on, we performed time-of-addition experiments and analyzed viral RNA by qPCR after 48h. Preliminary results show that the copaiba oil nanoemulsion has virucidal effect inhibiting 92.5% of virus release. Besides, both empty and copaiba oil nanoemulsions showed an effect in post-entry steps inhibiting 99.1% of ZIKV intracellular RNA, when compared to the control. Additional experiments are need to confirm the preliminary results and also to understand the mode of action of the copaiba oil nanoemulsion in inhibiting Zika virus infection.

Keywords: Antiviral, Nanoemulsion, Copaiba Oil, Zika Virus.

1. Introduction

The Zika virus (ZIKV), an arbovirus, that belongs to the genus *Flavivirus*, has as main vectors mosquitoes from *Aedes* genus, and was first isolated in 1947 in the Zika forest, in Uganda [1, 2]. The propagation of its vectors *Aedes aegypti* and *Aedes albopictus* worldwide due to the urbanization and deforestation processes, resulted in the rapid spread of this arbovirus [3, 4]. The first outbreak of this virus occurred only in 2007 on Yap Island in the Federated States of Micronesia, where symptoms of fever, rash, joint pain and conjunctivitis were observed [5-7]. Then, since the emergence of ZIKV in Brazil in 2015, ZIKV has spread all over the Americas and it has become a major global health issue due to the neurological complications related to ZIKV infection such as Guillain–Barré Syndrome and Zika virus Congenital Syndrome [8-11]. Besides the transmission by vectors, it can also be transmitted by blood transfusion and sexually which allows the transmission of the virus in vector-free environments [9, 12-14].

So far, there are no vaccines or specific treatments for ZIKV infection, which makes important to develop specific therapies for its treatment [15]. Thus, there are several studies with active compounds, such as copaiba oil, obtained from the trunk of various *Copaifera* species. These trees are native to the tropical regions of Latin American and Western Africa. In Brazil, there is more than twenty species of *Copaifera*, and the most abundant are *C. officinalis, C. guianensis Desf., C. reticulate Ducke, C. multijuga Hayne, C. confertif lora Bth., C. langsdorffii Desf., C. coriacea Mart., and C. cearensis Huber ex Ducke* [16]. The major components of the essential oils from *Copaifera officinalis* used in this study are β-caryophyllene, allo-aromadendrene, germacrene B, β-bisabolene,δ-cadinene and α-cadinene [17]. These secondary metabolites belong to the sesquiterpene class and already have several biological activities demonstrated in the literature, such as pro-oxidant, antioxidant, anti-inflammatory, antitumor, antiparasitic effect, etc [18].

This oil has already shown different activities as antitumor and analgesic actions, and also several antimicrobial activities, as antibacterial, antifungal and antiviral activity [18-20]. The various antimicrobial actions already described in the literature for copaiba oil may be related to the lipophilic characteristics of this oil, which may cause changes in the cell membrane, especially in relation to the antibacterial activities already described in studies [20].

Furthermore, there are several studies that associate active compounds with nanoemulsions, due to limitations such as low bioavailability and solubility, the association with these systems increases the effectiveness of treatment, leading to improved compound delivery, reduction of adverse drug effects such as toxicity and reduction of therapeutic doses, increase solubility and stability in biological fluids, among others effects [21-24]. The use of nanoemulsions is applied in several fields, including pharmaceutical, food, cosmetics, among others [25-27].

There are already several activities described for these nanoemulsion and copaiba oil systems, like a study that uses different types of nanoemulsion formulations containing copaiba oil to demonstrate its anti-inflammatory potential through the observation of vasodilation and cell accumulation in the epidermis [28]. Other studies that demonstrated an antimicrobial action of the association of copaiba oil with nanoemulsion systems, besides testing pure oil, such as the inhibitory action of the fungi *Paracoccidioides sp* [29] and genus *Candida* [30]. And yet against bacteria of the genus *Staphylococcus* [30]. In addition, antitumor activity has already been found in studies of an encapsulation containing copaiba oil and another medicine called Imiquimod [31].

It is of great importance that there are several studies in the literature associating nanoparticles or nanoemulsions as antiviral potentials, such as a study using a lipid nanoemulsion derived from natural components common in foods with inhibitory action against Zika and Dengue, also a *Flavivirus* [32, 33].

Thus, this study aimed to study the action of copaiba oil, a substance that has demonstrated several antimicrobial activities in the literature, associated with surfactant nanoemulsions, in cells infected with Zika virus.

2. Materials and Methods

2.1. Nanoemulsions

The copaiba oleoresin, extracted from the species *Copaifera officinalis*, was purchased from the Brazilian company FERQUIMA (São Paulo, Brazil). The surfactant used in the formulation of nanoemulsions (NE) was LIPOID E 80 ™ egg lecithin (Steinhausen, *Switzerland*) which comes from a natural source and is considered safe by the US Food and Drug Administration. It is composed mostly by the phospholipid phosphatidylcholine (80%).

Firstly, the surfactant and oil masses were measured respecting the 2: 1 (m:m) ratio of surfactant to oil phase. Then deionized water was added and the mixture was immediately subjected to ice bath sonication (Vibra-Cell ™, Sonics & Materials Inc.,

USA) to form the concentrated nanoemulsion. Subsequently, a 1:12.5 dilution of this concentrate was again subjected to ice bath sonication. To see if the vehicle itself already has any biological activity, formulations were made without oil, containing only the surfactant and water, in the same proportions as their respective nanoemulsion.

The nanoemulsions were characterized according to the physicochemical parameters: hydrodynamic diameter (HD), polydispersion index (PDI) and Zeta potencial (ZP) using the Dynamic Light Scattering (DLS) technique using Zetasizer equipment (ZetaSizer Nano ZS®, Malvern Instruments, Reino Unido).

2.2. Cell and virus

VERO E6 cell lines from African green monkey kidney (*Cercopithecus aethiops*), HuH-7, a hepatocyte derived human carcinoma cell line, and *Aedes albopictus* C6/36 cells were used. VERO E6 and HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Life Technologies, Califórnia, USA) supplemented with 10% fetal bovine serum (Cultilab, São Paulo, Brazil)100 IU/mL penicillin, 100 µg/mL streptomycin (P/S), 1% (v/v) non-essential amino acids (GIBCO-BRL, Life Technologies, Califórnia, USA) and maintained at 37 °C in a humidified 5% CO_2 incubator. *Aedes albopictus* cell line C6/36 was grown in Leibovitz L-15 (L-15) medium (Cultilab, São Paulo, Brazil), with the same suplementation as the other cell lines, maintained at 28ºC and without CO_2 enrichment incubator.

The Brazilian strain of Zika virus ($ZIKV^{BR}$) that was used in the experiments was isolated from a feverish case in Paraíba state, in northeastern Brazil, and was courtesy of Dr. Pedro Vasconcelos, Instituto Evandro Chagas. The access numbers deposited at Genbank for the ZIKV genome: KU321639, KU365777 to KU365780, KU729217 and KU729218.

2.3. Analysis of nanoemulsion cytotoxicity

The cytotoxicity profile of the empty nanoemulsion (ENE), nanoemulsion containing copaiba oil (CNE) and free copaiba oil (FCO) were measured by the MTT [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide] method (Sigma-Aldrich, Missouri, USA).

Twenty four hours before treatment, $5x10^3$ VERO E6 and HuH-7 cells were plated in a 96-multi-well plate. On the following day, CNE and FCO were added at concentrations ranging from 360, 180, 90, 45, 22.5, 11.25, 5.65 (µg/mL). ENE, since the concentration is based on the copaiba oil, treatment was performed with the same volume

used for each concentration of CNE. Cells were incubated for 24, 48 and 96 hours for VERO E6 and 24 and 48 hours for HuH-7 cells, in triplicate. After these times, the culture medium was removed from each well and exchanged for MTT solution (1 mg / mL in 100 µL of culture medium without supplementation) (Sigma-Aldrich, Missouri, USA). Cells were incubated for 30 minutes at 37°C. After the formation of the formazan crystals, the MTT solution was removed and 100 µL of DMSO (Synth, Sao Paulo, Brazil) per well were added. Then, the absorbance was measured at 572 nm, using a plate spectrophotometer (FLUOstar Omega/BMG LABTECH, Offenburg, BW, DE). The maximum non-cytotoxic concentration was determined considering a cut-off of 80% of cell viability.

2.4.Intracellular uptake

Approximately 10^5 VERO E6 and HuH-7 cells were plated in 6-well plates, and after 24 hours for complete cell adherence, the cells were incubated with nanoemulsion at the concentration previously established by MTT. For this assay, the nanoemulsion was labeled with the molecule aluminum-chloro-phthalocyanine (AlClPc), which has an excitation wavelength of 350 nm and the emission peak is 680 nm (range between 650-700 nm) to observe the fluorescence. 1, 2, 3 and 48 hours after incubation with the nanoemulsion, the medium was removed, the cells were washed with PBS, then viewed and photographed using a fluorescence microscope (Zeiss Axio Vert. A1) with a FITC filter (excitation spectra: 489 nm, blue laser, 415 ± 15 nm, FITC).

2.5. Viral inhibition assay

To perform ZIKV inhibition assay, initially $2x10^5$ VERO E6 cells were plated per well in a 12-well plate. Treatment groups were then prepared: Group A (control) = $ZIKV^{BR}$ + pure DMEM medium; Group B (CNE) = $ZIKV^{BR}$ + nanoemulsion containing copaiba oil; Group C (ENE) = $ZIKV^{BR}$ + empty nanoemulsion. For this assay, it was used the highest non-cytotoxic concentration. After 1 hour, fresh medium containing 1% FBS, 1% P/S and 1% Carboxymethylcellulose (CMC) was added to Group A and nanoemulsions (empty and containing copaiba oil) were added to group B and C at the proper concentration. Cells were incubated at 37°C and 5% CO_2 for 96 hours. After this time, cells were fixed with 10% formaldehyde for 30 min and stained with 1% crystal violet (Sigma-Aldrich, Missouri, USA) and the viral titer was quantified.

2.6. Analysis of the nanoemulsions dose dependence

For the dose dependence analysis of nanoemulsions, 10^5 VERO E6 cells were plated in a 12-well plate. After 24 h, the cells were incubated with nanoemulsions CNE and ENE at 360, 180, 90, 45, 22,5, 11,25 e 5,6 (µg/ml) and $1{,}16x10^4$ PFU/mL of $ZIKV^{BR}$. Cells were incubated for 1 hour at 37 °C and 5% CO_2. After 1 hour, complete medium was added to the cells along with the nanoemulsions at each concentration, except for the untreated control. Cell were incubated for 48 hours at 37 °C and 5% CO_2. The supernatant and intracellular samples were collected from each well for subsequent RNA extraction, cDNA synthesis and real time PCR was performed.

2.7. Virucidal effect of nanoemulsions

Initially, for the virucidal assay CNE e ENE at the highest non-cytotoxic concentration were placed together with $2{,}5x10^4$ PFU/mL of $ZIKV^{BR}$, and the contents were incubated at room temperature (RT) for 1 hour. For the untreated control, $ZIKV^{BR}$ was incubated with DMEM free of FBS and P/S. The mixture of each treatment was added to VERO E6 cells plated 24h prior to the experiment, in duplicates and incubated for 1 hour at 37 °C and 5% CO_2. Supplemented culture medium was added to the cells following incubation and cells were incubated at 37 °C and 5% CO_2 for 48 hours. Supernatant and intracellular samples were collected from each well for subsequent RNA extraction, cDNA synthesis and qPCR.

2.8.ZIKV entry inhibition assay

In order to analyze the early stages of viral infection, VERO E6 cells plated in a 12-well plate 24 hours prior, were incubated with CNE and ENE at 180 µg/mL for 1 hour at 37 °C and 5% CO_2. Subsequently, the cells were washed with Phosphate-Buffered Saline (PBS) solution and then, infected with $2{,}5x10^4$ PFU/mL of $ZIKV^{BR}$, and incubated again for 1 hour. Afterwards, fresh culture medium was added and cells were incubated for 48 hours at 37 °C and 5% CO_2. Thereafter, the supernatant and intracellular samples were collected from each well for subsequent RNA extraction, cDNA synthesis and qPCR.

2.9. ZIKV post-entry stages inhibition assay

In this assay, in order to study the action of NE in the steps following the entry of Zika virus into the cell, first, 10^5 VERO E6 cells were plated in a 12-well plate. After 24 hours, the cells were infected with $2{,}5x10^4$ PFU/mL $ZIKV^{BR}$. After 1 hour of incubation, ENE and CNE at 180 µg/mL were added to the cells and incubated for 48 hours at 37 °C

and 5% CO_2. The supernatant and intracellular samples were collected from each well for subsequent RNA extraction, cDNA synthesis and qPCR.

2.10. Viral RNA Extraction and cDNA Synthesis

RNA extraction was performed by the Trizol reagent (Invitrogen, Grand Island, EUA) method, per manufacturer's instructions. And after isolation, complementary DNA (cDNA) synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grans Island, EUA), according to manufacturer's instructions.

2.11. ZIKV RNA quantification real time PCR (qPCR)

In order to quantify the viral RNA in the assay qPCR was performed using *primers* ZIKV 1086, ZIKV 1162c and probe 1107-FAM described in Lanciotti et al (2008) [34] using The TaqMan® Universal PCR Master Mix in AmpErase UNG (Applied Byosistems) according to manufacturer's instructions. Reactions were read in the QuantStudioTM 12K Flex Real Time PCR System (Applied Biosystems Inc/Life Technologies, California, USA). Quantification of ZIKV RNA copies were obtained by the standard curve method.

2.12. Statistical analysis

For statistical analysis of the groups, comparisons were performed by analysis of variance (One-Way ANOVA) followed by Tukey test. *P* values <0.05 were considered significant. Statistical analyzes were performed using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA). Microsoft Excel was also used to aid in data processing.

3. Results

3.1. Analysis of nanoemulsion cytotoxicity

In order to investigate the cytotoxicity on VERO E6 and HuH-7 cell lines, were treated different concentrations (5,625; 11,25; 22,5; 45; 90, 180 and 360 µg/mL) of the nanoemulsions and free copaiba oil. After 24, 48 and 96 h of incubation results were analyzed by MTT assay. Both nanoemulsions were not cytotoxic at all the times evaluated, in any concentration tested (Figure 1A and 1B). However, free copaiba oil showed high levels of cytotoxicity after 48 hours (Figure 1C). The results also demonstrated that VERO E6 cells maintained a cell viability above 80% only up to the concentration of 180 µg/mL at all times analyzed, for the treatment with ENE and CNE.

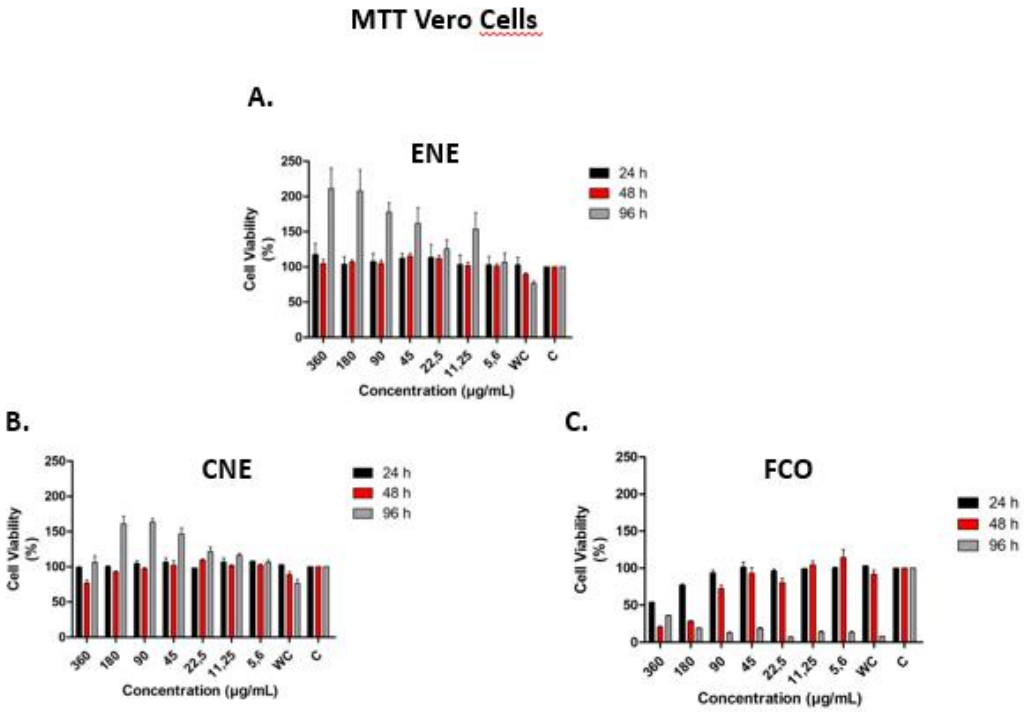


Figure 1: Cell viability of VERO E6 cells treated with empty nanoemulsion (ENE), copaiba oil nanoemulsion (CNE) and free copaiba oil (FCO), for 24, 48 and 96 hours in concentrations of 5,625; 11,25; 22,5; 45; 90, 180 and 360 (µg/mL). Control cells (C) and Water control (WC). (A) Treatment with ENE. (B) Treatment with CNE. (C) Treatment with free copaiba oil.

As it can be seen in figure 2 the ENE treatment were not cytotoxic for HuH-7 cells in any of the concentrations and times evaluated, while the CNE at 360 µg/mL showed cell viability of 67%, after 48 hours of treatment. Free copaiba oil treatment, showed high levels of cytotoxicity in both time points.

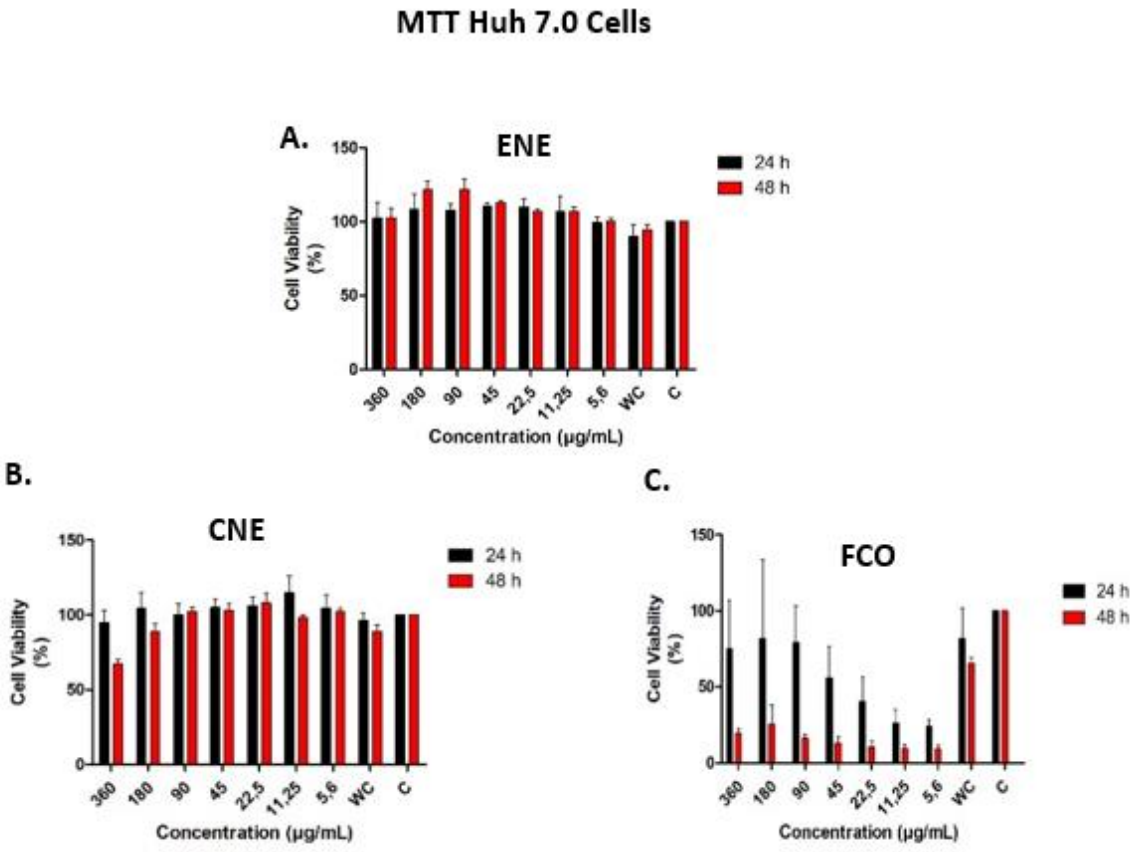


Figure 2: Cell viability of HuH-7 cells treated with empty nanoemulsion (ENE), copaiba oil nanoemulsion (CNE) and free copaiba oil (FCO), for 24, 48 and 96 hours in concentrations of 5,625; 11,25; 22,5; 45; 90, 180 and 360 (µg/mL). Control cells (C) and Water control (WC). (A) Treatment with ENE. (B) Treatment with CNE. (C) Treatment with free copaiba oil.

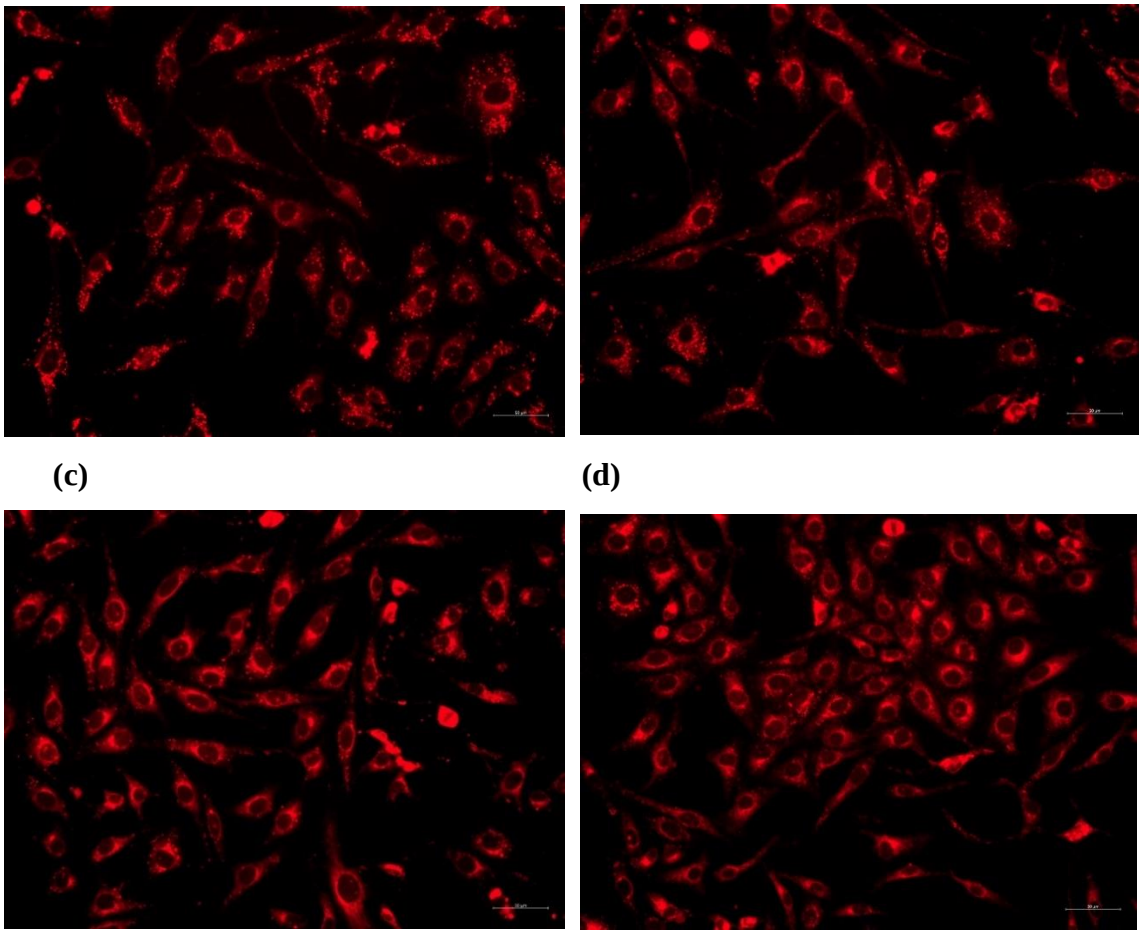
Based on MTT results, the maximum non-cytotoxic concentration (80% viability) selected for the following experiments was 180 µg/mL. It is also important to highlight that, as can be seen from the graphs above, treatment with free copaiba oil proved extremely toxic, which made it impossible to analyze the action of oil on ZIKV inhibition in later experiments.

3.2.Intracellular uptake

After performing cytotoxicity analysis of nanoemulsions in VERO E6 and HuH-7 cell lines, the next step was to confirm the cellular uptake of nanoemulsions at 1, 2, 3 and 48 hours of incubation. As copaiba oil is not a compound with fluorescent properties, aluminum chloro-phthalocyanine (AlClPc) was used for labeling the ENE , which has the same size and physical properties as the ones used in the following assays.

In VERO E6 cells, the nanoemulsion internalization can be observed at all times, especially in the cytoplasm of the cells (Figure 3). It can also be seen that fluorescence signal is stronger from 3 hours (Figure 3c), confirming better internalization at 3 and 48 hours in this cell line.

(a) **(b)**



(c) **(d)**

Figure 3: Fluorescent microscopy images of cellular uptake of nanoemulsions (180 µM) in VERO E6 after different periods of nanoemulsion incubation. The pictures were taken (a) 1 hour (b) 2 hours, (c) 3 hours and (d) 48 hours after nanoemulsion incubation. Scale bar: 50 µm.

For the HuH-7, the internalized nanoemulsions was also mainly in the cytoplasm of the cells (Figure 4), and a better internalization is observed after 2 hours of treatment (Figure 4b), as it can be seen by the stronger fluorescence, that remains up to 48 hours of treatment.

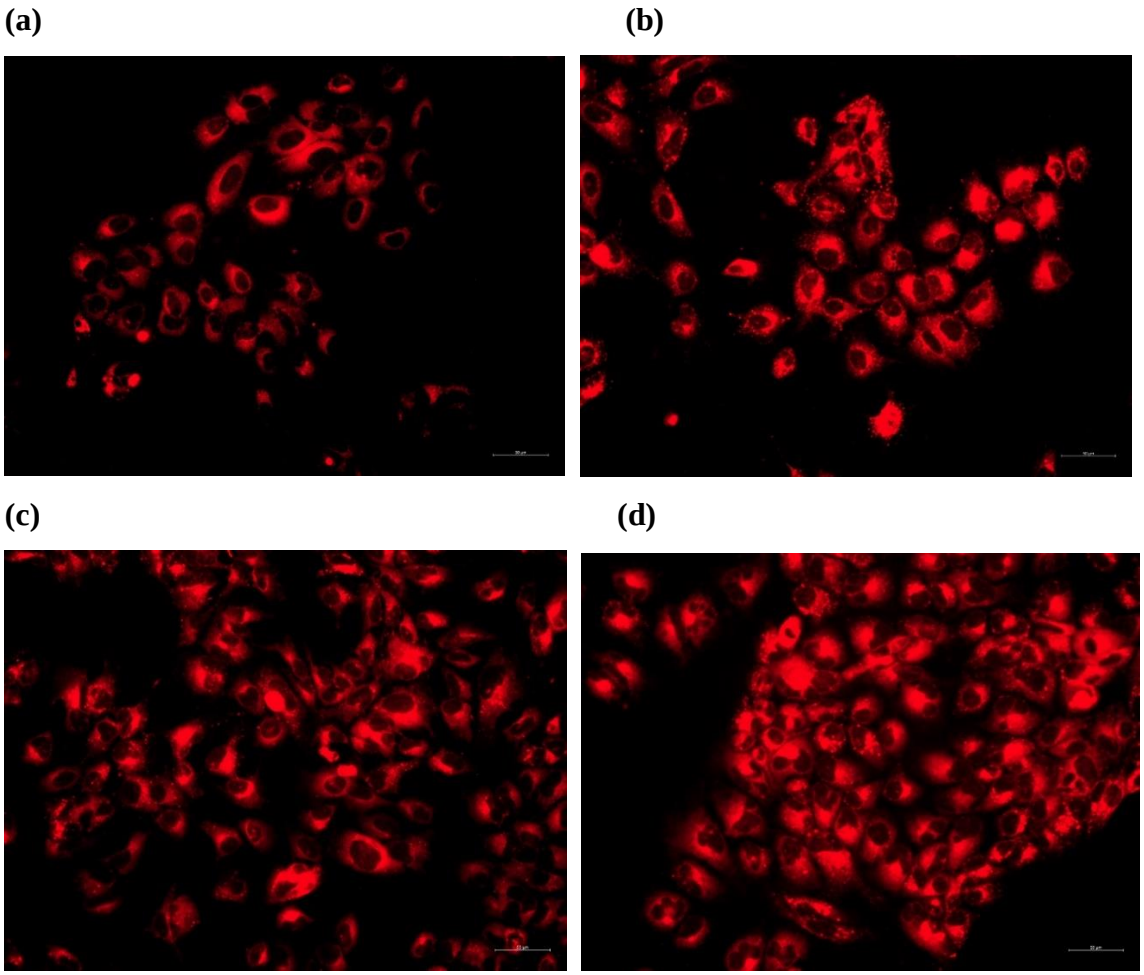


Figure 4: Fluorescent microscopy images of cellular uptake of nanoemulsions (180 µM) in HuH-7 after different periods of nanoemulsion incubation. The pictures were taken (a) 1 hour (b) 2 hours, (c) 3 hours and (d) 48 hours after nanoemulsion incubation. Scale bar: 50 µm.

3.3. Viral inhibition assay

A preliminary assay was performed to analyze the possible ZIKV inhibition effect of nanoemulsions. VERO E6 cells treated with ENE and CNE showed 70% e 80% of viral inhibition activity, respectively, when compared to control cells (***: $p < 0.001$ and **: $p < 0.01$).

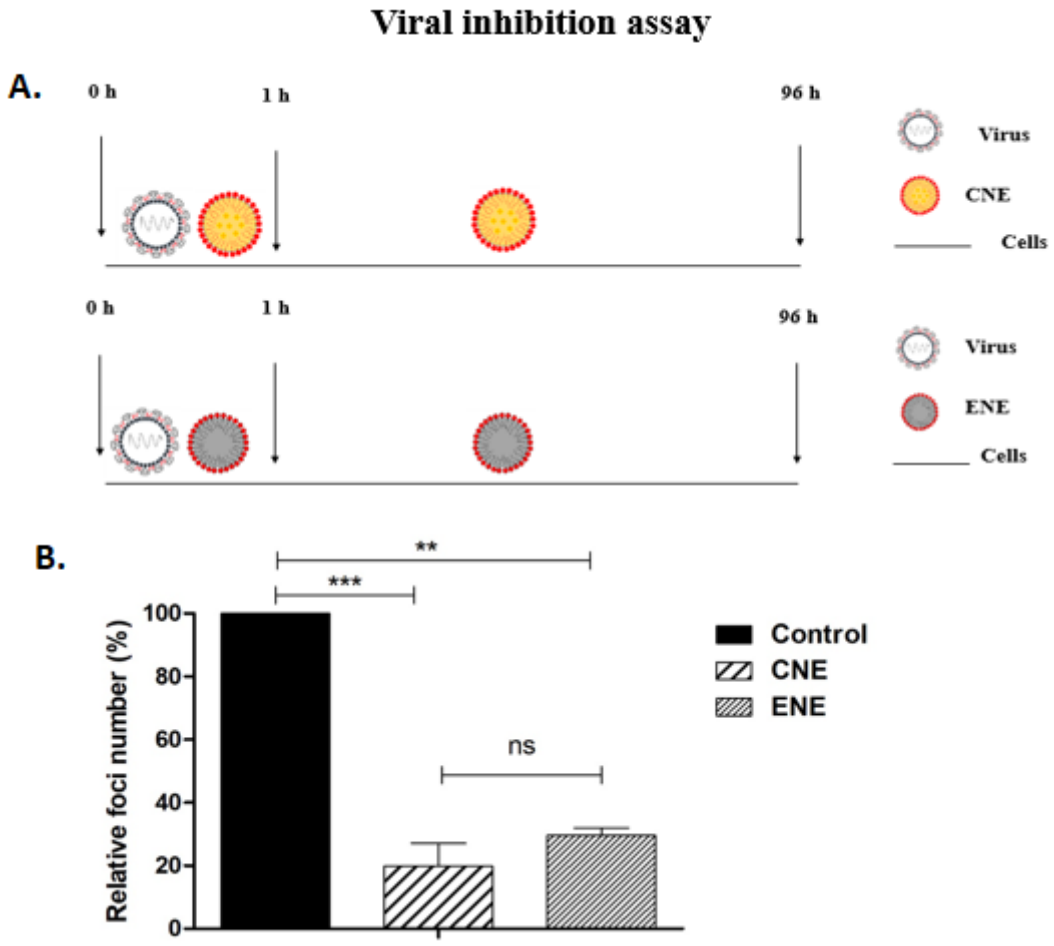


Figure 5: VERO E6 cells incubated with nanoemulsions presented viral activity inhibition. (A) Representative scheme of the viral inhibition assay. First, representing the test performed with the copaiba oil nanoemulsion, and, secondary, representing the test performed with the empty nanoemulsion. (B) Relative foci number (%) resulting of viral inhibition assay in VERO E6 cells infected with ZIKV. CNE represents treatment with copaiba oil nanoemulsion, ENE represents treatment with empty nanoemulsion and the control was performed with DMEM culture medium in place of the treatments. This result was performed in two independent events. Both treatments showed statistical difference in relation to control cells (**: $p < 0.01$ and ***: $p < 0.001$).

3.4. Analysis of the nanoemulsions dose dependence

In order to analyze the dose dependence of ENE and CNE, the dose dependence assay was performed. VERO E6 cells incubated with CNE demonstrated dose dependence response in the viral RNA levels. The ENE showed a decrease in the viral RNA in all volumes tested (Figure 6).

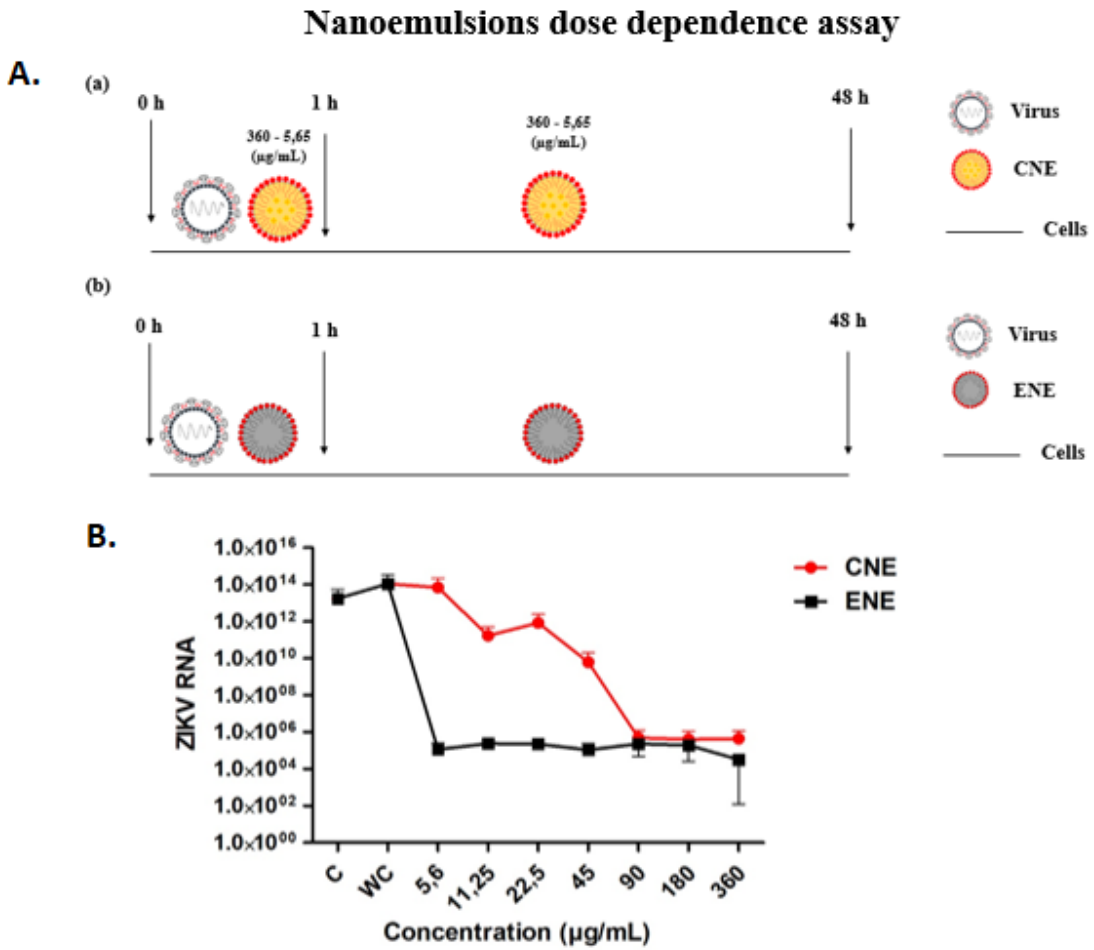


Figure 6: Nanoemulsions dose dependence assay. (A) Representative scheme of the nanoemulsions dose dependence assay. First, representing the test performed with CNE, and, secondary, representing the test perfomed with the ENE. (B) ZIKV RNA resulting of the dose dependence of CNE and ENE in VERO E6 cells infected with ZIKV. Control cells (C) and Water Control (WC).

3.5.Virucidal effect of nanoemulsions

A virucidal assay was performed to test the ability of these nanoemulsions to impair the virus particle infectivity. After 48 h, a considerable reduction of viral interacellular RNA levels was observed for both nanoemulsions (*: $p<0,05$ for CNE and **: $p<0,01$ for ENE) in VERO E6 cells. Besides, the copaiba oil nanoemulsion also presented a significant reduction in the extracellular viral RNA compared to control (*: $p<0,05$)

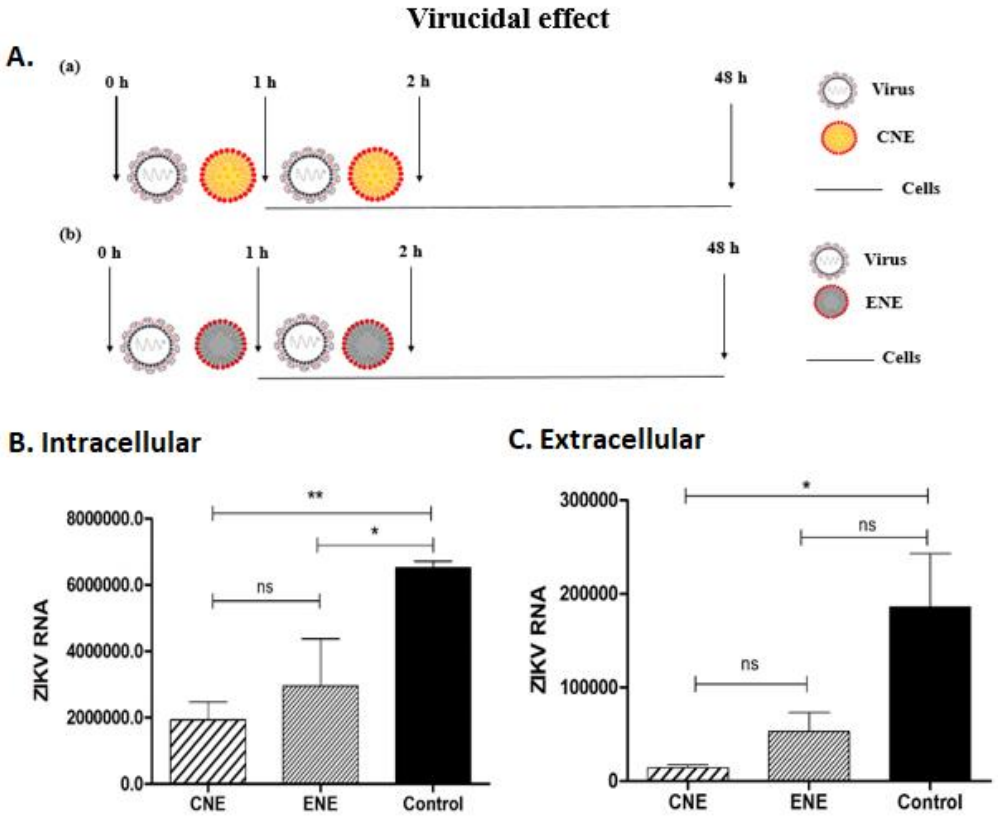


Figure 7: Virucidal assay. (A) Representative scheme of the virucidal effect of nanoemulsions. First, representing the assay performed with the copaiba oil nanoemulsion (CNE), and, secondary, representing the assay perfomed with the empty nanoemulsion (ENE). (B) ZIKV RNA resulting of intracellular of virucidal effect of nanoemulsions assay with the treatments with copaiba oil nanoemulsion (CNE) and empty nanoemulsion (ENE) in VERO E6 cells infected with ZIKV. Both treatments showed statistical difference in relation to control cells, but the two types of treatments did not present significant statistical difference among themselves (ns: not significant, *: $p<0,05$ and **: $p<0,01$). (C) represents the extracellular of assay.

3.6. ZIKV entry inhibition assay

The post-entry steps were also analyzed and there is a statistically significant decrease in intracellular ZIKV titer in cells treated with ENE and CNE when compared to the control. Also, there was a statistically significant decrease in the extracellular ZIKV RNA levels only in the treatment with CNE (*: $p<0,05$) (Figure 9B).

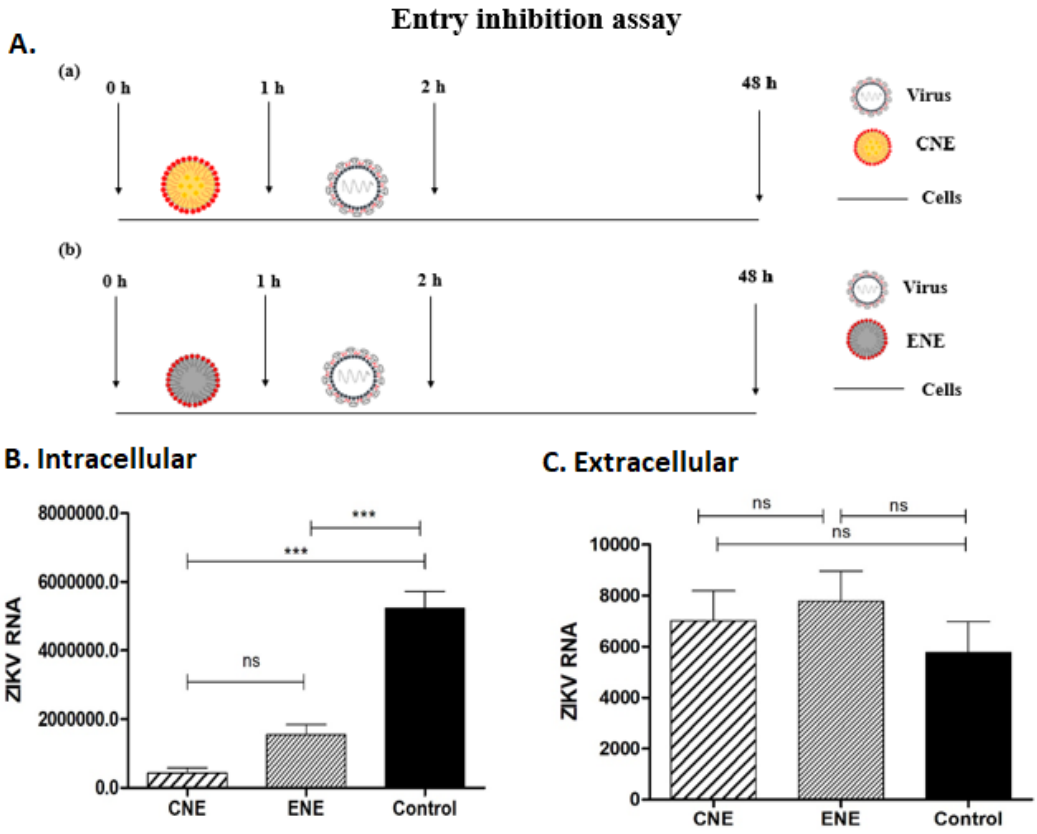


Figure 8: Entry inhibition assay. (A) Representative scheme of ZIKV entry inhibition assay. First, representing the test performed with the copaiba oil nanoemulsion, and, secondary, representing the test perfomed with the empty nanoemulsion. (B) ZIKV RNA levels resulting of intracellular of ZIKV entry inhibition assay. Both treatments showed statistical difference in relation to control cells, but the two types of treatments did not present significant statistical difference among themselves (ns: not significant, *: $p<0,05$ and ***: $p< 0,001$). (C) represents the extracellular ZIKV RNA levels of this assay. None of the treatments showed significant statistical difference compared to the control (ns: not significant).

3.7. ZIKV post-entry stages inhibition assay

In the last assay analyzed, post-entry steps, there is a statistically significant decrease in intracellular ZIKV titer in cells treated with empty and copaiba oil nanoemulsion compared to control group cells. Besides, there was a statistically significant decrease in the extracellular ZIKV RNA levels only in the treatment with copaiba oil-containing nanoemulsions (*: $p<0,05$) (Figure 9B).

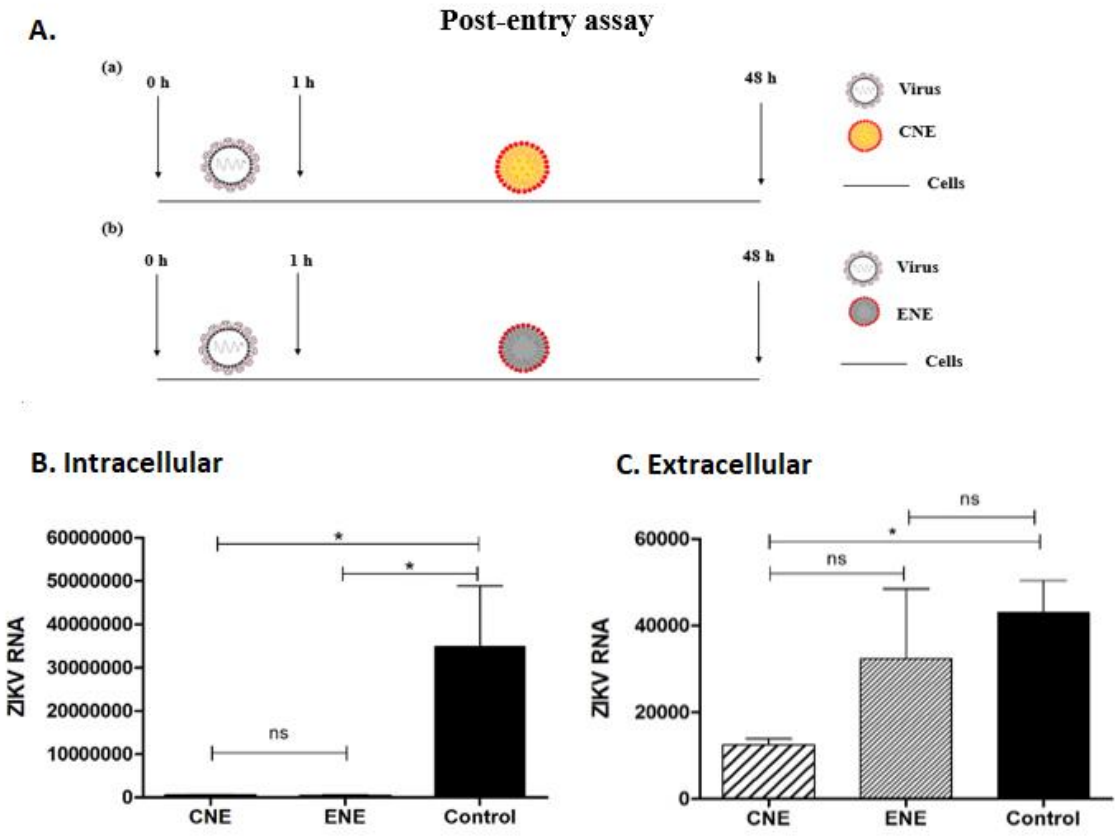


Figure 9: ZIKV post-entry stages inhibition assay. (A) Representative scheme of ZIKV post-entry stages inhibition assay. First, representing the assay performed with the copaiba oil nanoemulsion, and, secondary, representing the assay perfomed with the empty nanoemulsion. (B) ZIKV RNA resulting of intracellular of ZIKV post-entry stages inhibition assay. Both treatments showed statistical difference in relation to control cells, but the two types of treatments did not present significant statistical difference among themselves (ns: not significant and *: $p<0,05$). (C) represents the extracellular of this assay. Only the treatment with the copaiba oil nanoemulsion showed significant statistical difference (ns: not significant and *: $p<0,05$).

4. Discussion

Due to the current absence of vaccines and approved treatments against ZIKV infection, studies analyzing the action of antiviral compounds for this virus are of great importance [35]. Based on this and the various biological activities already proposed in the literature for copaiba oil, along with the use of nanoemulsions to potentiate the antimicrobial activities of natural oils, the present work studied the action of nanoemulsions containing copaiba oil as a possible antiviral against ZIKV [36, 37].

Surfactant stabilized nanoemulsions are systems that have as major advantage the reduction drug toxicity [38]. In the cell viability assay, free copaiba oil was analyzed, and was highly toxic for VERO E6 and HuH-7 cell lines. In contrast, empty nanoemulsion and when the copaiba oil was associated with nanoemulsions, no cytotoxicity was observed. These results are possibly due to a controlled release of copaiba oil into cells by the nanoemulsion, what could prevent cell damage representing an efficient delivery system [39, 40].

After analyzing cell viability, it was possible to confirm that the intracellular uptake of nanoemulsions was efficient at all times and in both cell lines analyzed. Due to the different morphologies and characteristics that nanoparticles can have, they can internalize in cells in different ways, being mainly related to the charge present in the nanoparticle and hydrophobicity [41]. The nanoemulsion location inside the cells is of great importance to confirm whether they are absorbed by the cells and their possible modes of action [42]. In the present study, it was observed that the nanoemulsion are present mainly in the cytoplasm of both cell lines, VERO E6 and HuH-7. The distribution of the nanoemulsion in the cell cytoplasm may be related to the mode of action of the NE structure with surfactant, since for this assay only ENE with AlClPc markers was used, although no studies of nanoemulsions with egg lecithin phospholipids surfactant could be found that corroborate with these internalization results. This cytoplasmic target can also be related to a greater delivery efficiency [43]. Internalizations with more efficient cytoplasmic targets are related to mechanisms of intracellular action, in order to improve the efficiency of treatment in this type of mechanism [44]. In this work, we observed an inhibitory effect on the intracellular content for all the analyzed stages of ZIKV cycle, which corroborates with the mechanism of intracellular action described.

The initial experiment to analyze the possible potential of the nanoemulsions in inhibiting ZIKV at a maximum non-toxic concentration of 180 μg/mL at any stage of its cycle demonstrated a reduction in relative foci number of approximately 70% and 80% for both ENE and CNE, respectively. The nanoemulsion structure of this study is composed only of water and surfactant (egg lecithin phospholipids), and this composition presented amphiphilic characteristics, being able to interact with the outside of cell membranes (hydrophilic) and have the hydrophobic active compound (copaiba oil) inside.

Subsequent trials revealed that viral inhibition by CNE is dose dependent. There are others studies demonstrating dose-dependent inhibition of natural compounds in

ZIKV, such as Mounce et al (2017) using curcumin, that discuss the reduction in dose-dependent infectivity in HeLa cells of the enveloped viruses Chikungunya, Zika and Vesicular Stomatitis virus by this compound and related these results to curcumin structure [45], and also, the study of Behrendt et al (2017), using the compound Pentagalloylglucose showed an extracellular inhibition in dose dependent manner, using Vero B4 cells [46]. Besides that, using the compounds Suramin and Floxuridine, studies showed a dose dependent inhibition of a stage of the ZIKV cycle, demonstrating an effect on the replication step [47, 48]. And, for the ENE, it was observed similar results for all concentrations analyzed, but this result could already be expected, since the variation in concentrations are related to the concentration of copaiba oil inside the nanoemulsion, which is not present in the empty nanoemulsion.

When analyzing the steps of viral life cycle separately, it was observed that ENE and CNE both have a virucidal effect. Since the empty nanoemulsion also has antiviral activity, part of this effect may be related to an interaction between the structure of the surfactant and the viral envelope that has lipoprotein composition. Studies in the literature have reported that surfactant nanoemulsions have shown action on other enveloped viruses by destabilizing the lipid envelope of Herpes simplex, Influenza, and Vaccinia viruses, while it was not observed in non-enveloped virus [49]. Chepurnov et al (2003) also demonstrated the antiviral potential of a surfactant nanoemulsion for Ebola virus [50]. According to these properties, studies by Ragnavan et al (2016) demonstrated the inhibitory activity of a lipid nanoemulsion derived from natural food components in the flavivirus ZIKV and DENV [32, 33]. Surfactants are known to have affinity with membranes, this can cause morphological changes in its integrity, which can explain the virucidal activity for enveloped virus [51, 52]. In contrast, this same characteristic can be problematic since it can cause cell disruption and hemolysis. However, preliminary analyzes performed by the Laboratory of Bioactive Compounds and Nanotechnology, indicate that the nanoemulsions used are not hemolytic (data not shown) and our cytotoxicity results show they are not cytotoxic in the tested concentration. And, the virucidal effect of CNE shows a direct action potential on the viral particle. Drugs that have this kind of effect are interesting because they act on the viral particle before it enters the cell, preventing cell damage from infected tissue. There are studies that show mainly the bactericidal action of copaiba oil on gram positive, which have walls with a thick layer of peptide glycan [36, 53, 54], which could be related to a possible action of the copaiba oil on ZIKV viral glycoproteins.

It was possible to observe that ENE and CNE also demonstrated inhibitory action post-entry steps which can be related to replication, maturation or virus release. Treatment with ENE decreased the intracellular viral RNA levels, while a reduction in both extracellular and intracellular viral RNA levels were observed for CNE treatment. It is not yet possible to determine in which of these later steps the nanoemulsions are acting, so further analysis are needed. Despite this, there is a study in the literature on the relation between lipid metabolism and the target of antiviral drugs, focusing on ZIKV and other flaviviruses, which describes the use of cholesterol derivatives as possible drugs with antiviral potential for flaviviruses, because they need lipids both in their envelope and also in replication [55]. There are several studies in the literature that demonstrate the action of drugs on ZIKV replication, such as the study by Sacramento et al (2017) showed that Sofosbuvir inhibited ZIKV replication in a dose-dependent manner, as did the analysis by Steuer et al (2011) with the also arbovirus DENV, demonstrated the inhibitory action of 32 compounds in the replication of this virus, in a dose dependent manner. However, no studies have been found, so far, that indicate the action of drugs in the stages of maturation and release [56, 57].

5. Conclusion

It was found that the nanoemulsion containing copaiba oil presents antiviral potential against ZIKV. The data indicate that both the structure of the nanoemulsion and its association with the oil have antiviral activity. The nanoemulsions showed low cytotoxicity, virucidal activity, entry inhibition and in post-entry stages activity. Although the results obtained so far demonstrate the potential of both NEs to inhibit ZIKV, further studies are needed to confirm the mechanism of action that these systems use to lead to these effects

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CAPÍTULO III

Conclusão

1. CONCLUSÃO

A realização do presente trabalho foi importante para analisar o potencial antiviral de nanoemulsões contendo óleo de copaíba. Primeiramente, análises da viabilidade celular com os tratamentos com as nanoemulsões vazia e contendo óleo de copaíba, e com o óleo puro, demonstraram a diminuição da citotoxicidade nas linhagens VERO E6 e HuH-7 resultante do encapsulamento do óleo de copaíba. Além disso, foi possível confirmar a internalização das nanoemulsões em todos os tempos analisados.

Foi possível verificar que a nanoemulsão contendo óleo de copaíba apresenta potencial antiviral contra o ZIKV. Os dados indicam que tanto a estrutura da nanoemulsão quanto a associação desta com o óleo apresentam atividade antiviral. Sendo a ação antiviral da nanoemulsão contendo óleo de copaíba dose dependente, enquanto a nanoemulsão vazia apresentou resultados semelhantes para todas as concentrações analisadas, em função da ausência do óleo. As nanoemulsões apresentaram atividade nas diversas etapas do ciclo de replicação do ZIKV, demonstrando atividade virucida, inibição na entrada e em etapas pós-entrada.

Apesar dos resultados obtidos até o momento demonstrarem o potencial de ambas nanoemulsões em inibir o ZIKV, são necessários mais estudos para confirmar o mecanismo de ação que esses sistemas utilizam para levarem a esses efeitos.