Antiviral Activity of Rosemary Extract Against Zika Virus in Human Dermal Fibroblasts

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24 **Abbreviations**
25 C, Capsid
26 CC_{50}, 50% cytotoxic concentration
27 CA, Carnosic acid
28 CMC, Carboxymethylcellulose
29 CO, Carnosol
30 DENV, Dengue virus
31 DMEM, Dulbecco's modified Eagle's medium
32 DMSO, Dimethyl sulfoxide
33 E, Envelope
34 EV71, Enterovirus 71
35 F, Fusion
36 FBS, Fetal bovine serum
37 HFF-1, Human foreskin fibroblast cells
38 hRSV, Human respiratory syncytial virus
39 HSV, Herpes simplex virus
40 IC_{50}, 50% inhibitory concentration
41 JEV, Japanese encephalitis virus
42 MOI, Multiplicity of infection
43 NS, Non-structural
44 PBS, Phosphate-buffered saline
45 Pen/strep, penicillin/streptomycin
46 PFU, Plaque forming unit
47 PrM, Precursor membrane
48 RA, Rosmarinic acid
49 RdRp, RNA-dependent RNA polymerase
50 RE, Rosemary extract
51 SI, Selectivity index
52 WHO, World Health Organization
53 WNV, West Nile virus
54 YFV, Yellow fever virus
55 ZIKV, Zika virus

Abstract

Flaviviruses have increasingly emerged and re-emerged in recent decades, infecting millions of people annually. Zika virus is particularly concerning due to its associated pathological complications, including microcephaly in newborns and Guillain-Barré syndrome in adults, posing a significant threat to public health. Despite efforts made by the scientific community, no licensed drugs against flaviviruses have been developed. Medicinal plants show promise as a novel source of antiviral agents, as they possess a diverse array of biologically active secondary metabolites, making them potential candidates for therapeutic use. We sought to investigate the antiviral potential of rosemary extract (RE) against ZIKV in human dermal fibroblasts (HFF-1 cells), one of the earliest targets of infection. ZIKV was treated with various concentrations of RE or its individual major polyphenols, including rosmarinic acid (RA), carnosic acid (CA), and carnosol (CO), and the remaining infectivity of each sample was measured by plaque reduction assay. To evaluate the impact of RE on different stages of the ZIKV replication cycle, HFF-1 cells were treated before, during, and after infection, or the virus was treated before infection. RE exerted potent antiviral activity against ZIKV in both Vero and HFF-1 cells by directly acting on virus particles before infection. Importantly, RE significantly inhibited the later stages of the virus replication cycle by interfering with post-entry mechanisms within the host cell. Moreover, major RE-derived polyphenols CA and CO, but not RA, were shown to significantly reduce ZIKV infectivity. Overall, RE significantly impairs ZIKV infection *in vitro* by directly interacting with virus particles prior to adsorption and interfering with post-entry processes of the ZIKV replication cycle. This study provides rigorous evidence indicating the potential development of RE as an antiviral agent, warranting further investigation into the mechanisms

78 underlying its inhibitory activity against ZIKV and its effects on other medically important

79 flaviviruses.

Introduction

Zika virus (ZIKV) is a mosquito-borne virus that has received significant attention in recent years. ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family, which includes dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and other well-known human pathogens[1]. ZIKV was originally isolated from a Rhesus macaque monkey in Uganda when studying the ecology of YFV in 1947. After its discovery, ZIKV remained relatively unknown, sporadically causing minor outbreaks and mild febrile illness in few individuals[2,3]. The first major outbreak of ZIKV occurred on Yap Island in 2007, where close to 75% of the population was infected[4]. This was followed by an outbreak in French Polynesia from 2013 to 2014 and the largest recorded outbreak in Brazil in 2015, where approximately 1.3 million people were infected[3,5]. These outbreaks were linked to a significant increase in microcephaly in neonates of infected mothers and Guillain-Barré syndrome in adults, sparking a global panic[6]. Due to its rapid spread and associated neurological complications, ZIKV was declared a Public Health Emergency of International Concern by the World Health Organization (WHO) in February 2016[7].

ZIKV is a positive-sense single-stranded RNA virus with a genome of approximately 11 kb. The genome encodes a single polyprotein that is proteolytically cleaved into three structural proteins, including envelope (E), membrane (prM), and capsid (C) proteins, along with seven non-structural (NS) proteins, including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5[3,4]. ZIKV is primarily transmitted *Aedes aegypti* mosquitoes, which inject the virus into the skin of the host during a blood meal[8]. Throughout this process, ZIKV encounters cells in the epidermal and dermal layers of the skin such as keratinocytes, fibroblasts, Langerhans cells, dendritic cells, and mast cells, among others, representing the first line of defense against the virus[9,10].

103 Fibroblasts are the most abundant cell type found in the dermis and are responsible for producing
104 and maintaining the extracellular matrix[11]. It has been well characterized that fibroblasts are
105 highly permissive to multiple strains of ZIKV and serve as a primary target for viral replication
106 at the site of inoculation. Moreover, multiple pro-inflammatory genes are upregulated in
107 fibroblasts infected with ZIKV, suggesting they contribute to the local inflammatory response in
108 the early stages of infection[10,12]. Fibroblasts are thus a compelling *in vitro* model for studying
109 potential antiviral agents against ZIKV.

110 As of May 2024, 92 countries and territories throughout five of the six WHO regions
111 have recorded autochthonous mosquito-borne transmission of ZIKV. Additionally, competent
112 populations of *Aedes aegypti* mosquitoes have been established in 60 countries and territories
113 globally, raising concerns that autochthonous transmission may be introduced in these areas[3,12].
114 Despite an extensive worldwide research effort, there are currently no vaccines approved to
115 prevent ZIKV infection and no specific antivirals available for any flavivirus, leaving supportive
116 care as the only option to manage symptoms. Given the severe complications associated with
117 ZIKV infection and the continued spread of flaviviruses to new regions with changing climates,
118 novel antiviral agents are urgently needed to mitigate future outbreaks[13–15].

119 An attractive approach to developing therapeutics is the use of medicinal plants, as they
120 possess a diverse array of bioactive secondary metabolites[14]. These compounds have been
121 shaped by millions of years of evolution to provide plants selective advantages, including
122 protection against oxidative stress, ultraviolet radiation, predators, and infection[16,17]. Throughout
123 history, humans have used medicinal plants as a primary source of medicine for treating various
124 diseases, leading to the discovery of many drugs currently available on the market[18]. In fact,
125 approximately 11% of all essential drugs were derived from plants, with aspirin, quinine, and

126 morphine being among the most famous examples[19,20]. In the context of mosquito-borne
127 infections, quinine, a compound derived from the bark of the cinchona tree, is one of the most
128 important anti-malarial drugs in history and is listed on the WHO's Model List of Essential
129 Medicines for severe malaria[21]. Beyond their well-documented antioxidant and anti-
130 inflammatory properties, several plant-derived secondary metabolites have demonstrated
131 antiviral activity *in vitro* and *in vivo*, including quercetin and fisetin against DENV2, baicalein
132 against JEV and influenza virus, and epigallocatechin-3-gallate against herpes simplex virus
133 (HSV), Epstein Barr virus, and DENV, to name a few[22,23]. However, relatively few studies have
134 explored the antiviral potential of medicinal plants and their derivatives against ZIKV,
135 highlighting a gap in the field.

136 *Rosmarinus officinalis* L. (rosemary) is a medicinal herb native to the Mediterranean
137 region and belongs to the Lamiaceae family. Rosemary extract (RE) is widely used as a natural
138 preservative in food processing and has been extensively studied for its therapeutic properties,
139 including antioxidant, anti-inflammatory, antibacterial, antitumor, and neuroprotective effects,
140 among many others[24–26]. These biological activities can be attributed to its rich polyphenolic
141 composition, which includes phenolic acids like rosmarinic (RA), ursolic, and caffeic acids, as
142 well as phenolic diterpenes like carnosic acid (CA), carnosol (CO), and rosmanol[27]. To the best
143 of our knowledge, no studies have explored the antiviral activity of RE against flaviviruses,
144 although it has been shown to be effective in other viral models, including human respiratory
145 syncytial virus (hRSV)[28] and HSV[29–32]. That being said, RA, a major polyphenol in RE, was
146 identified as a potential antiviral agent against ZIKV and DENV *in silico*[33–35]. RA has also been
147 shown to inhibit all four serotypes of DENV *in vitro*[35] and significantly reduce mortality, viral
148 loads, and pro-inflammatory cytokine levels in JEV-infected mice[36]. In the current study, we

149 show that RE potently inhibits ZIKV infection by directly targeting virus particles prior to
150 infection and interfering with later stages of the virus life cycle. These findings suggest RE is a
151 promising therapeutic candidate for ZIKV, warranting further investigation into its antiviral
152 potential.

153 **Materials and Methods**
154
155 *Cells and Virus*

156 Human foreskin fibroblast cells (HFF-1: ATTC, SCRC-1041) and Vero E6 cells (African
157 green monkey kidney cells: ATTC, CRL-1586) were maintained in Dulbecco's modified Eagle's
158 medium (DMEM: Sigma, D6546) supplemented with 10% heat-inactivated fetal bovine serum
159 (FBS: Sigma, 14008C) and 1% penicillin/streptomycin (pen/strep: Gibco, 15140-122) at 37°C
160 with 5% CO_2. The PRAVBC59 strain of ZIKV (ATCC, VR1843) was propagated in Vero cells.
161 Briefly, Vero monolayers were infected with ZIKV at a multiplicity of infection (MOI) of 0.01
162 and incubated at 37°C and 5% CO_2 for 1 h. The viral inoculum was removed, and cells were
163 incubated for 5 days in culture medium containing 2% FBS and 1% pen/strep. Viral stocks were
164 quantified by plaque assay, aliquoted, and stored at -80°C.
165

166 *Reagent Preparation*

167 Rosemary extract was prepared as previously described[37]. Briefly, samples were prepared
168 by grinding and passing dried *Rosmarinus officinalis* L. leaves through a mesh sieve. Extraction
169 was performed following protocols established by the US National Cancer Institute. 5 g of
170 ground leaves were steeped overnight in 30 mL of dichloromethane-methanol (1:1). A slight
171 vacuum was used to collect the filtrate, and a methanol extraction was performed for 30 min. The
172 solvent was removed by rotary evaporation. RE was dissolved in dimethyl sulfoxide (DMSO) at
173 100 mg/mL, aliquoted, and stored protected from light at -20°C until further use. Based on
174 previous HPLC analysis, the RE used here is composed of 9.29 μM of RA and 1.59 μM of CA,
175 however the concentration of CO was not determined[38,39].
176

177 *Cell Viability Assay*

178 To determine cell viability, HFF-1 and Vero cells were seeded in 96-well tissue culture
179 plates and incubated at 37°C with 5% CO_2 until 90% confluent. HFF-1 and Vero cells were
180 treated with DMEM containing various concentrations of RE in triplicate for 1 h at 37°C with
181 5% CO_2, using 1% DMSO as a vehicle control. Subsequently, the medium was replaced with
182 fresh culture medium containing 10% FBS and 1% pen/strep, and cells were incubated for either
183 96 h (HFF-1) or 120 h (Vero) at 37°C with 5% CO_2. To best represent the different treatment
184 conditions in the time-of-addition analysis, HFF-1 cells were also incubated with various
185 concentrations of RE in DMEM with 10% FBS and 1% pen/strep for 96 h. Following incubation,
186 culture medium was replaced with 100 µL of fresh medium and 10 µL of WST-1 cell
187 proliferation reagent (Roche, 5015944001). After 2 h, absorbance was measured at 460 nm using
188 a spectrophotometer (Bio-Tek, Synergy HT-1, 191356) and triplicates were averaged. The
189 cytotoxic concentration of RE that reduced viability by 50% (CC_{50}) was calculated by non-linear
190 regression analysis (GraphPad Prism).

191

192 *Plaque Reduction Assay*

193 Plaque reduction assay was used to screen the antiviral activity of RE and its major
194 polyphenol constituents, including RA, CA, and CO. 100 µL of ZIKV (290,000 PFU) was
195 treated with increasing concentrations of each compound for 1 h at 37°C with 5% CO_2, using 1%
196 DMSO as a vehicle control. The remaining infectivity of each sample was then quantified by
197 plaque assay. The inhibitory concentration that reduced plaque numbers by 50% (IC_{50}) was
198 calculated by non-linear regression analysis (GraphPad Prism).

199

200 *Time-of-Addition Analysis in HFF-1 Cells*

201 To determine the mode of antiviral activity, HFF-1 cells and ZIKV were incubated with
202 RE at different stages of virus infection. Briefly, HFF-1 cells were seeded on 24-well plates and
203 incubated at 37°C with 5% CO_2 until 90% confluent. Cells were infected with ZIKV (MOI of 1)
204 under four different treatment conditions, using 1% DMSO as a vehicle control. 1) Cells were
205 pretreated with various concentrations of RE for 1 h at 37°C. The supernatant was removed, and
206 wells were washed once with 1X phosphate-buffered saline (PBS: Gibco, 10010023) before viral
207 inoculation. 2) Cells were infected with ZIKV and simultaneously treated with various
208 concentrations of RE for 1 h at 37°C. 3) Cells were infected with ZIKV for 1 h at 37°C, the
209 inoculum was removed, and culture medium containing various concentrations of RE was added
210 to each well for the entire post-infection period. 4) ZIKV was incubated with various
211 concentrations of RE for 1 h at 37°C before being added to the cells. In all treatment conditions,
212 wells were washed once with PBS after the 1 h adsorption period to remove unbound ZIKV.
213 Next, 500 µL of culture medium with 10% FBS and 1% pen/strep was added to each well and
214 cells were incubated at 37°C and 5% CO_2. Supernatant was collected, aliquoted, and stored at -
215 80°C after 0 and 96 h post-infection. Viral titers were quantified by plaque assay. The inhibitory
216 concentration that reduced viral titers by 50% (IC_{50}) was calculated by non-linear regression
217 analysis (GraphPad Prism).

218

219 *Plaque Assay*

220 Vero E6 cells were seeded in 12-well and 24-well plates and incubated at 37°C with 5%
221 CO_2 until 90% confluent. Serial 1:10 dilutions were prepared for pre-treated ZIKV stocks or
222 supernatant harvested from HFF-1 cells 96 h post-infection, whereas a 1:2 dilution was prepared

223 for samples collected 0 h post-infection. Cells were infected in duplicate with 200 µL (1:10
224 dilutions) or 100 µL (1:2 dilutions) of each dilution in 12- or 24-well plates, respectively.
225 Following a 1 h adsorption period at 37°C, virus inoculum was removed, and cells were overlaid
226 with 1 mL (12-well) or 0.5 mL (24-well) of a 1:1 mixture of carboxymethylcellulose (CMC) and
227 DMEM with 2% FBS and 1% pen/strep. Plates were incubated at 37°C with 5% CO_2 for 5 days
228 for plaque development, the overlay was discarded, and wells were washed twice with PBS.
229 Cells were simultaneously fixed and stained with 1% crystal violet dissolved in 8%
230 formaldehyde and 30% ethanol for 30 min at room temperature. Excess stain was removed using
231 tap water and plaques were counted. Plaque forming units per mL (PFU/mL) was calculated for
232 each sample using the following equation:

233 $$\frac{PFU}{mL} = \frac{Average\ Number\ of\ Plaques}{(Dilution\ Factor)(Volume\ of\ Inoculum)}$$

234

235 *Statistical Analysis*

236 One-way ANOVA analysis was used to evaluate WST-1 viability assay data and two-
237 way ANOVA analysis was used to evaluate plaque assay data. Multiple comparisons were
238 performed using Dunnett's *post-hoc* test where appropriate. The CC_{50} and IC_{50} were determined
239 by analyzing normalized data (% control) using non-linear regression (variable slope). Results
240 from all experiments were expressed as mean ± SEM and considered significant if $p<0.05$
241 between the vehicle control (DMSO) and RE or polyphenol treated groups. Statistical analyses
242 were conducted using GraphPad Prism.

243 **Results**

244 *Cytotoxicity of RE in cell culture*

245 WST-1 assay was used to assess the impact of RE treatment on HFF-1 and Vero cells.
246 Both cell lines were treated with increasing concentrations of RE ranging from 20 to 200 µg/mL
247 or an equivalent volume of DMSO. Incubation periods were chosen to best represent the
248 conditions of the different functional assays. WST-1 assay values are presented as % control for
249 each independent experiment compared to vehicle control DMSO treated cells (100% cell
250 viability). No difference in viability was observed in HFF-1 cells treated with 20 to 80 µg/mL of
251 RE for 1 h followed by a 96 h incubation ($p>0.05$). However, there was a significant decrease in
252 viability beginning at 100 µg/mL, with a CC_{50} of 120.2 µg/mL (Fig. 1a and 1b). Comparatively,
253 HFF-1 cells treated for the entire 96 h incubation period showed a 7.5% increase in cell viability
254 at 40 µg/mL ($p<0.01$). There was a significant decrease in viability beginning at 80 µg/mL, with
255 a CC_{50} of 107.3 µg/mL (Fig. 1c and 1d). Vero were unaffected by treatment with 20 µg/mL of
256 RE for 1 h followed by a 120 h incubation, representing the maximum concentration cells were
257 exposed to in the plaque reduction assay ($p>0.05$, Fig. 2a).

258

259 *RE potently inhibits ZIKV replication in a dose-dependent manner in Vero*

260 To test the potential antiviral activity of RE, ZIKV was pretreated with increasing
261 concentrations of RE from 25 to 200 µg/mL or an equivalent volume of DMSO for 1 h. The viral
262 titer of each sample was then quantified by plaque assay. Results demonstrated a significant
263 dose-dependent decrease in infectious ZIKV particles for all tested concentrations (Fig. 2b,
264 $p<0.0001$). Concentrations of 25, 50, 75, 100, 125, 150, 175, and 200 µg/mL caused a 53.6, 69.5,
265 85.6, 92.7, 93.2, 98.9, and 99.6% reduction in ZIKV titers, respectively (Fig. 2c, $IC_{50} = 24.95$
266 µg/mL). Due to the nature of this assay, the selectivity index (SI) was not calculated. The highest

concentration ZIKV was pre-treated with was 200 µg/mL, but this was serially diluted ten-fold before being added to Vero. As a result, Vero inoculated with the 10^1 dilution were exposed to 20 µg/mL of RE, which was not cytotoxic (Fig. 2a).

RE directly interacts with virus particles and inhibits post-entry mechanisms in fibroblasts

To determine the antiviral potential of RE against ZIKV infection in HFF-1 cells and dissect the mechanism of action, HFF-1 cells were treated with RE before, concurrent with, or after viral inoculation, or ZIKV was treated directly with RE prior to infection. Increasing concentrations of RE ranging from 20 to 100 µg/mL or an equivalent volume of DMSO was used in all experiments. Based on what is frequently used in the literature[1,10,40–42], cells were infected at an MOI of 1 and cell-free supernatant was collected 0 and 96 h post-infection, which is when ZIKV titers peak in HFF-1 cells (Supplemental Fig. 1). The percent reduction was calculated relative to the amount of virus produced in control DMSO treatment groups. No antiviral effect was observed in HFF-1 cells treated with RE for 1 h prior to infection (Fig. 3). Treatment of HFF-1 cells during the adsorption period significantly enhanced ZIKV replication, with viral titers increasing by 195.5, 104.5, 98.7, 195.9, and 88.6% at concentrations of 20 ($p<0.0001$), 40 ($p<0.01$), 60 ($p<0.01$), 80 ($p<0.0001$), and 100 µg/mL ($p<0.05$), respectively, relative to the control. However, these changes did not follow a consistent trend across increasing concentrations of RE, as there was no clear dose-response relationship (Fig. 4). RE demonstrated the most potent antiviral activity in cells treated after the 1 h infection period, as virus production was significantly inhibited by every concentration tested (Fig. 5a, $p<0.0001$). There was a 73.0 and 99.9% reduction in viral titers in cells treated with 20 and 40 µg/mL of RE and a 100% reduction in viral titers below the detectable limit in cells treated with 60, 80, and

290 100 µg/mL (Fig. 5b, IC_{50} = 17.92 µg/mL, SI = 6.00). However, concentrations of 80 µg/mL and
291 above were cytotoxic in HFF-1 cells treated with RE for 96 h (Fig. 1c). Lastly, pre-treatment of
292 ZIKV with RE for 1 h prior to infection increased virus production by 46.7% at 20 µg/mL
293 ($p<0.05$) and significantly reduced virus production by 99.6% at 60 µg/mL and 100% at 80 and
294 100 µg/mL (Fig. 6, IC_{50} = 56.25 µg/mL, SI = 2.14). However, 100 µg/mL was cytotoxic when
295 HFF-1 cells were treated for 1 h followed by a 96 h incubation (Fig. 1a).
296
297 *CA and CO, but not RA, exhibit virucidal activity against ZIKV in Vero*
298 To determine if any of the three main polyphenols in RE could exert similar antiviral
299 effects, ZIKV was pretreated with increasing concentrations of each compound from 25 to 100
300 µM for 1 h at 37°C. The remaining infectivity of each sample was quantified by plaque assay.
301 No effect on viral replication was observed when ZIKV was pretreated with RA (Fig. 7, $p>0.05$).
302 However, CA and CO both resulted in a significant dose-dependent decrease in infectivity for all
303 tested concentrations, with CA causing a 30.5 ($p<0.01$), 69.7 ($p<0.0001$), and 99.2% ($p<0.0001$)
304 reduction (Fig. 8) and CO causing a 67.4 ($p<0.001$), 94.2 ($p<0.0001$), and 99.0% ($p<0.0001$)
305 reduction (Fig. 9) in viral titers at concentrations of 25, 50, and 100 µM, respectively, relative to
306 an equivalent volume of DMSO.

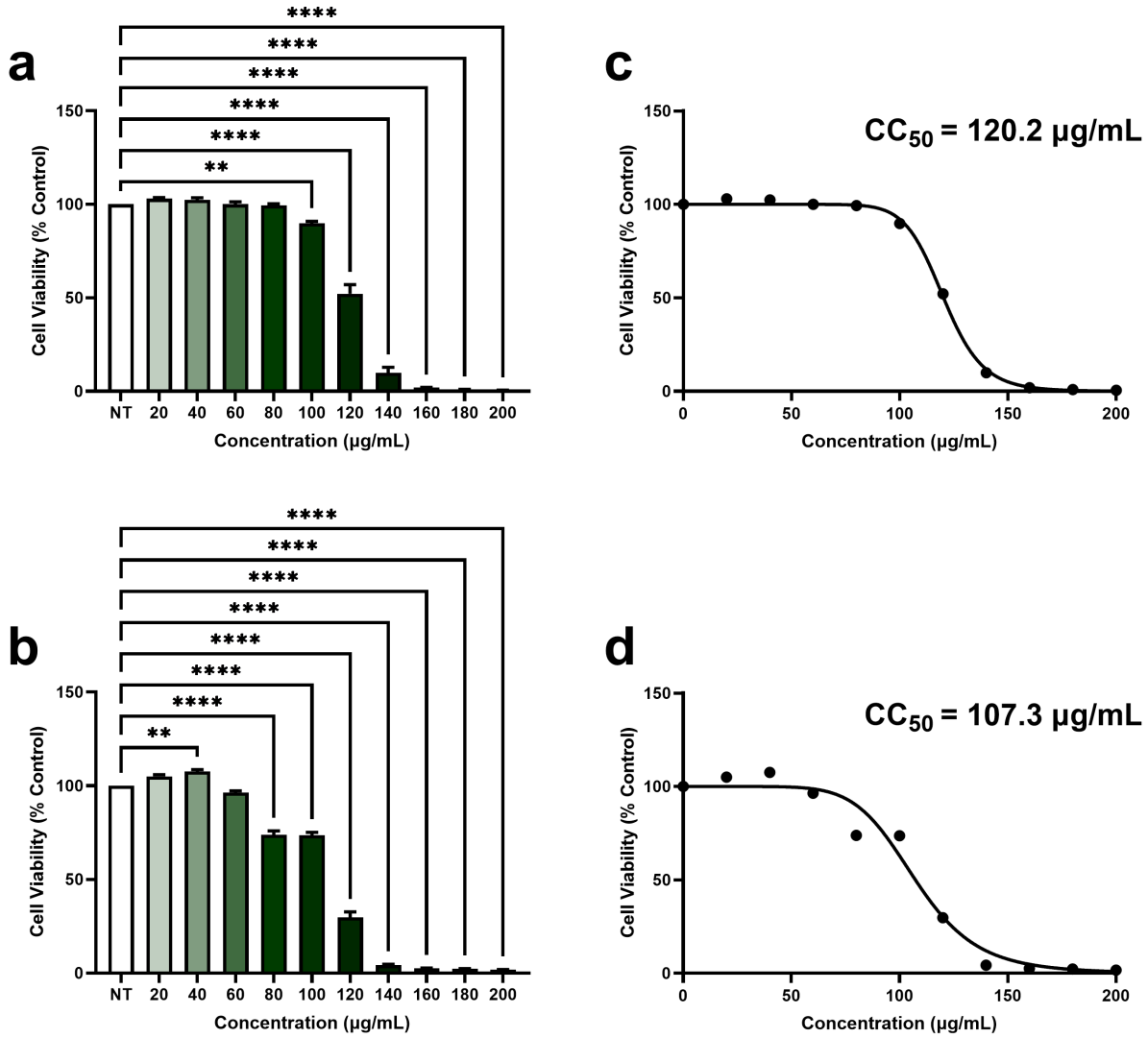

Figure 1: Cell viability of HFF-1 cells treated with increasing concentrations of RE. (a) HFF-1 cells were incubated with increasing concentrations of RE or a vehicle control (DMSO) for 1 h followed by 96 h with fresh medium. (b) HFF-1 cells were incubated with increasing concentrations of RE or a vehicle control (DMSO) for 96 h. Cell viability was determined by a WST-1 assay. Data are expressed as mean % of control ±SEM for n = 3 independent experiments. A one-way ANOVA and Dunnett's multiple comparisons were used to determine differences in cell viability following RE incubation. **$p<0.01$, ****$p<0.0001$ relative to HFF-1 cells incubated with DMSO (NT). CC_{50} values for HFF-1 cells treated for (c) 1 h or (d) 96 h were calculated by non-linear regression analysis (inhibitor vs normalized response – variable slope).

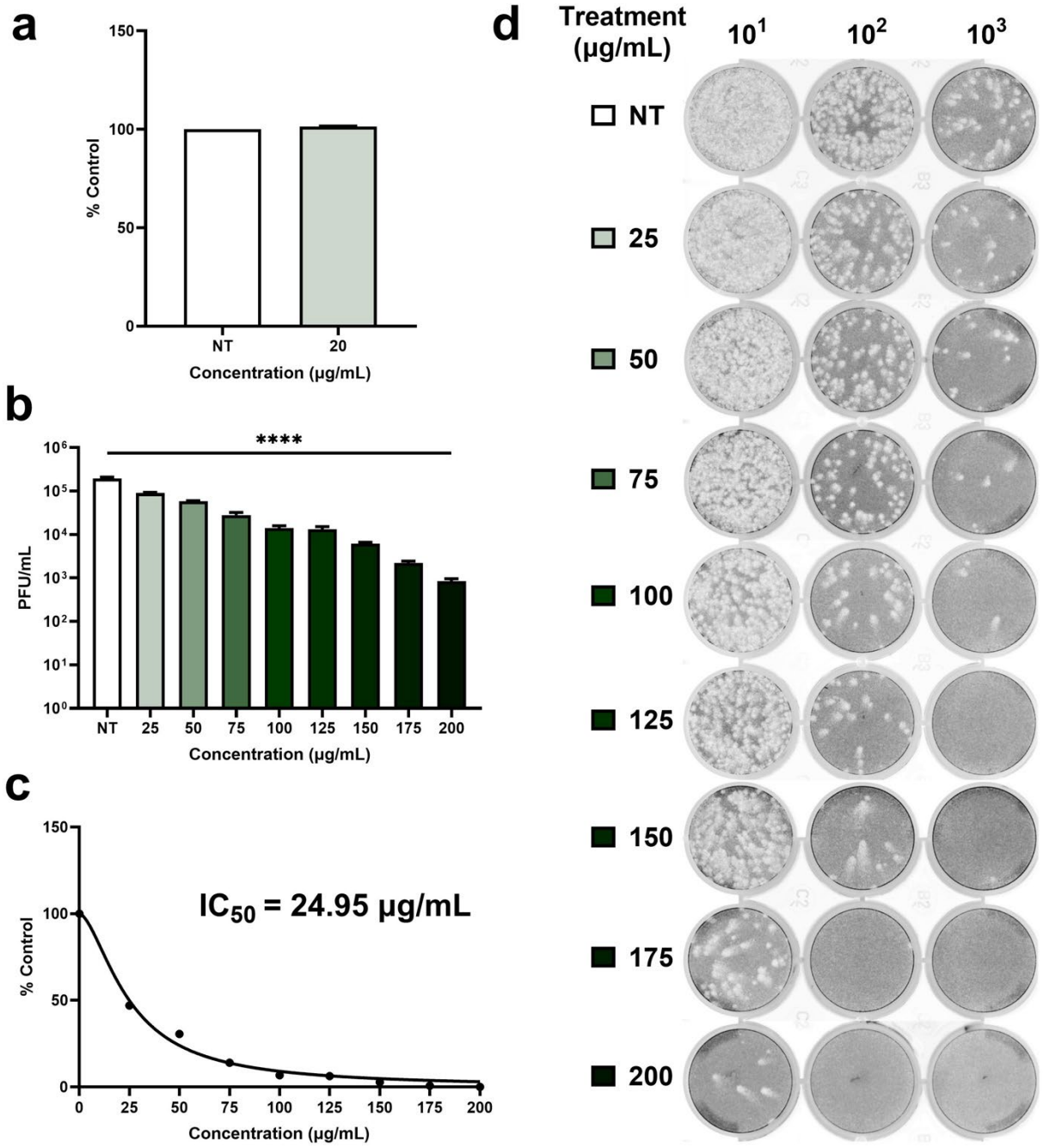

Figure 2: RE inhibits ZIKV replication in Vero in a dose-dependent manner. (a) Vero were incubated with increasing concentrations of RE or a vehicle control (DMSO) for 1 h followed by 120 h with fresh medium. Cell viability was determined by a WST-1 assay. Data are expressed as % of control ±SEM for n = 3 independent experiments. Vero were exposed to a maximum concentration of 20 µg/mL in the plaque reduction assay; thus, the corresponding representative data is shown. A one-way ANOVA and Dunnett's multiple comparisons were used to determine differences in cell viability following RE incubation. (b) As an initial screen**,** the virucidal activity of RE was evaluated by plaque reduction assay. ZIKV (290,000 PFU) was incubated with increasing concentrations of RE or an equal volume of a vehicle control (DMSO) for 1 h.

329 The viral titer of each sample was then determined by plaque assay. Data are expressed as mean
330 PFU/mL ±SEM for n = 3 independent experiments, each replicated in duplicate. A one-way
331 ANOVA and Dunnett's multiple comparisons were used to determine differences in viral titers
332 following RE treatment. ****$p<0.0001$ relative to ZIKV incubated with DMSO (NT) for all
333 concentrations tested. (c) The IC_{50} value was calculated by non-linear regression analysis
334 (inhibitor vs normalized response – variable slope). (d) Plaque assay images taken using an
335 Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment
336 in its entirety.

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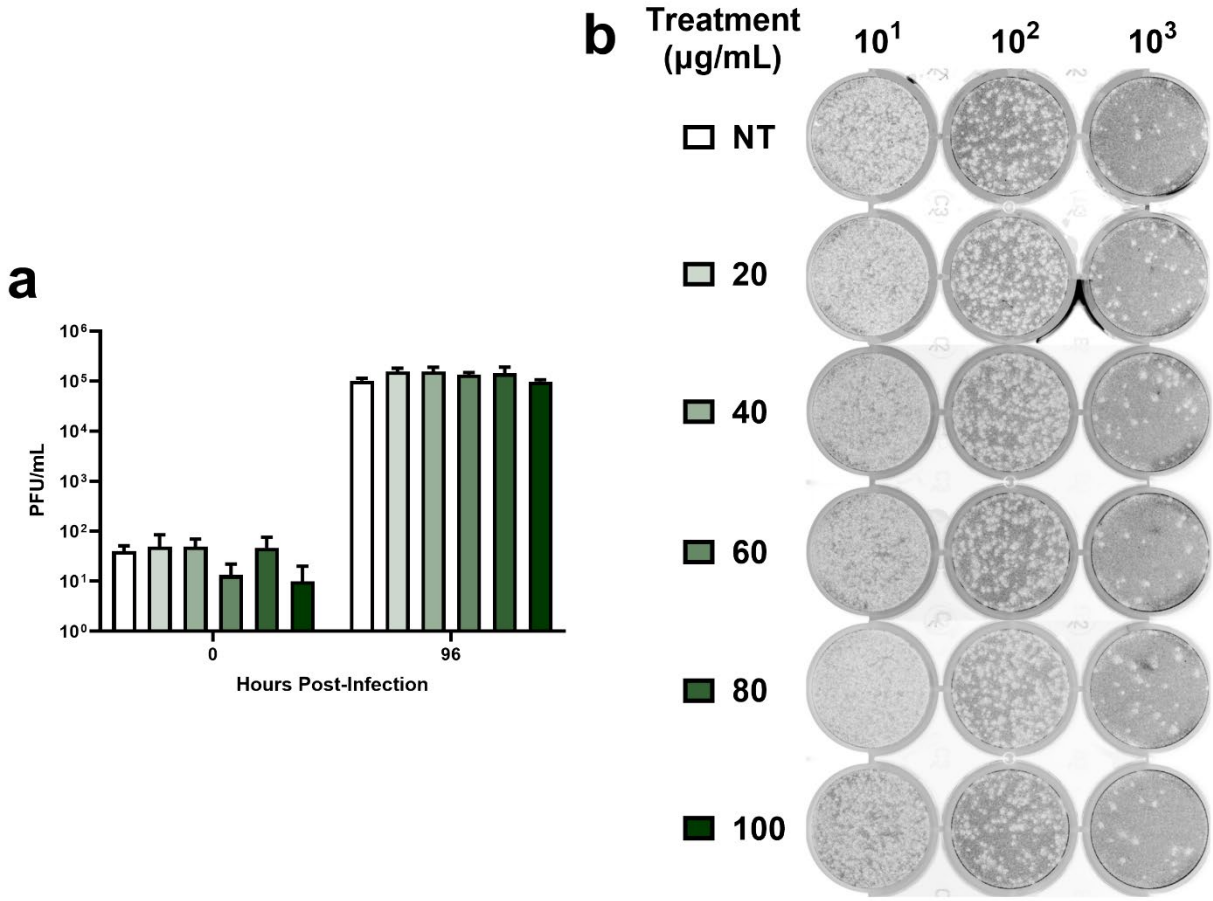

338
339 **Figure 3: Treatment of HFF-1 cells with RE before infection does not impact ZIKV**
340 **replication**. (a) HFF-1 cells were treated with increasing concentrations of RE or a vehicle
341 control (DMSO) for 1 h. Following incubation, cells were infected with ZIKV (MOI = 1), and
342 cell-free supernatants were collected 0 and 96 h post-infection. Viral titers were quantified by
343 plaque assay. Data are expressed as mean PFU/mL ±SEM for n = 3 independent experiments,
344 each replicated in duplicate. A two-way ANOVA and Dunnett's multiple comparisons were used
345 to determine differences in viral titers following RE treatment relative to HFF-1 cells treated
346 with DMSO (NT). (b) Plaque assay images taken using an Amersham Typhoon Biomolecular
347 Imager and represent wells from one independent experiment in its entirety.

348

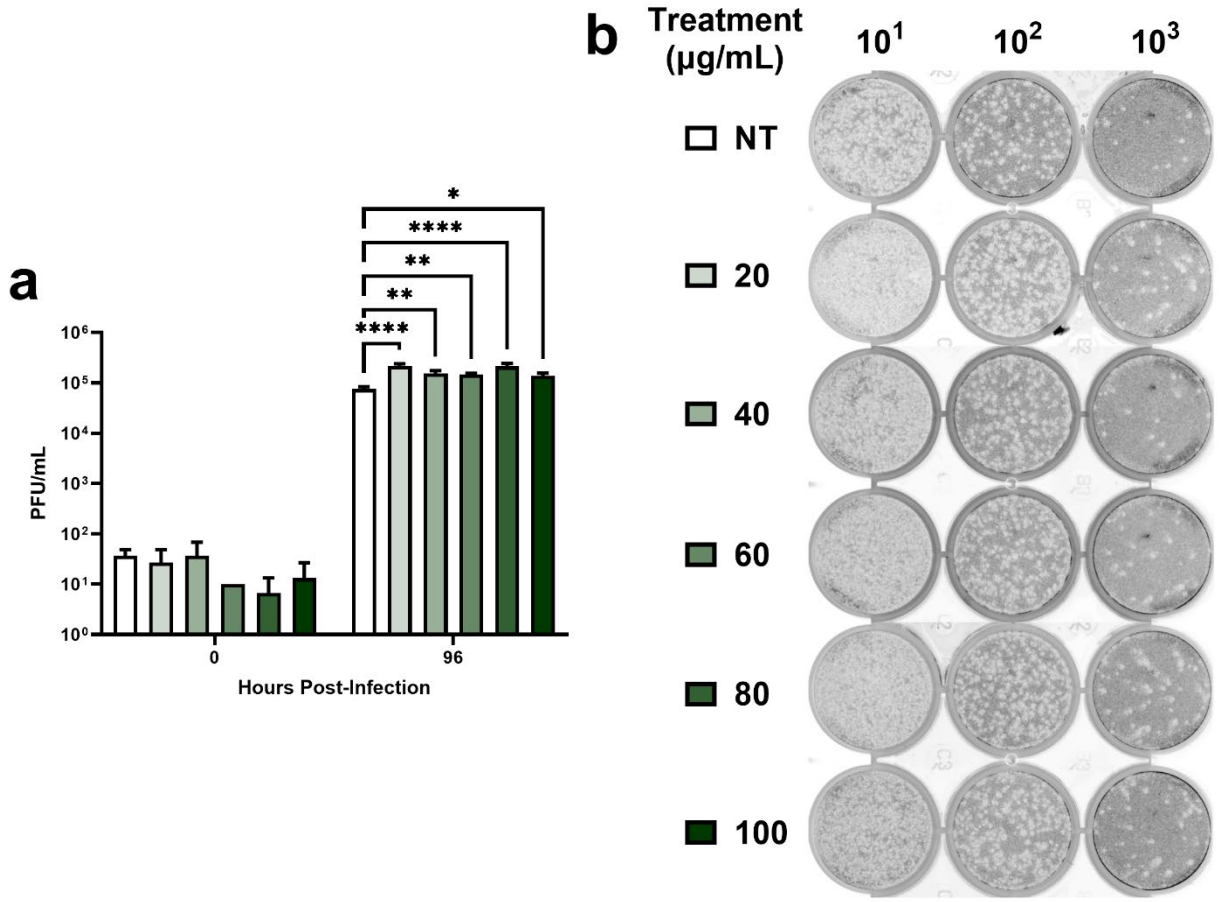

Figure 4: Treatment of HFF-1 cells with RE during infection enhances ZIKV replication. (a) HFF-1 cells were infected with ZIKV (MOI = 1) and simultaneously treated with increasing concentrations of RE or a vehicle control (DMSO) for 1 h. Cell-free supernatants were collected 0 and 96 h post-infection, and the viral titer of each sample was quantified by plaque assay. Data are expressed as mean PFU/mL ±SEM for n = 3 independent experiments, each replicated in duplicate. A two-way ANOVA and Dunnett's multiple comparisons were used to determine differences in viral titers following RE treatment. *$p<0.05$, **$p<0.01$, ****$p<0.0001$ relative to HFF-1 cells treated with DMSO (NT). (b) Plaque assay images taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

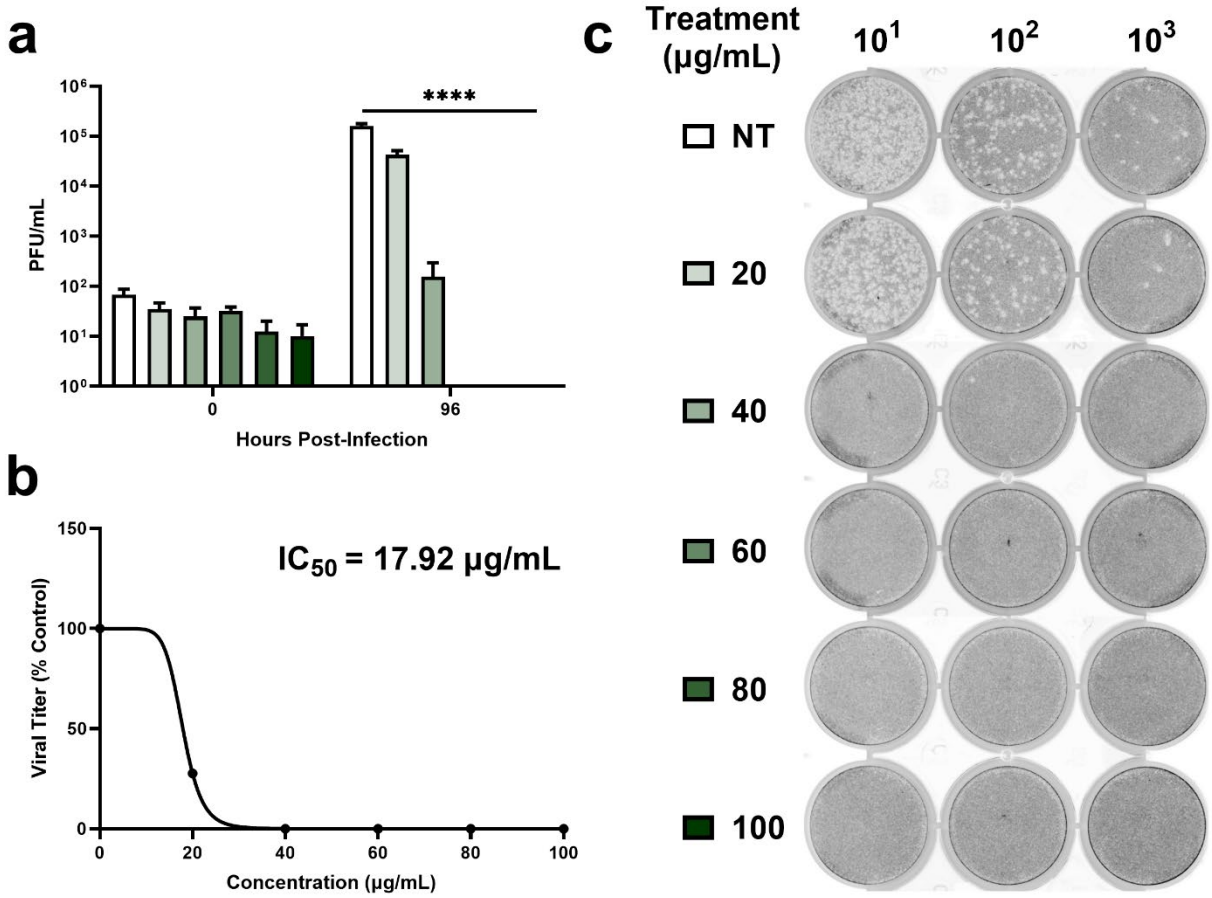

Figure 5: Treatment of HFF-1 cells with RE after infection inhibits ZIKV replication. (a) HFF-1 cells were infected with ZIKV (MOI = 1) for 1 h and subsequently treated with increasing concentrations of RE or a vehicle control (DMSO) for the entire post-infection period. Cell-free supernatants were collected 0 and 96 h post-infection and viral titers were quantified by plaque assay. Data are expressed as mean PFU/mL ±SEM for n = 4 independent experiments, each replicated in duplicate. A two-way ANOVA and Dunnett's multiple comparisons were used to determine differences in viral titers following RE treatment. ****$p<0.0001$ relative to HFF-1 cells treated with DMSO (NT). (b) The IC_{50} value was calculated by non-linear regression analysis (inhibitor vs normalized response – variable slope). (c) Plaque assay images taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

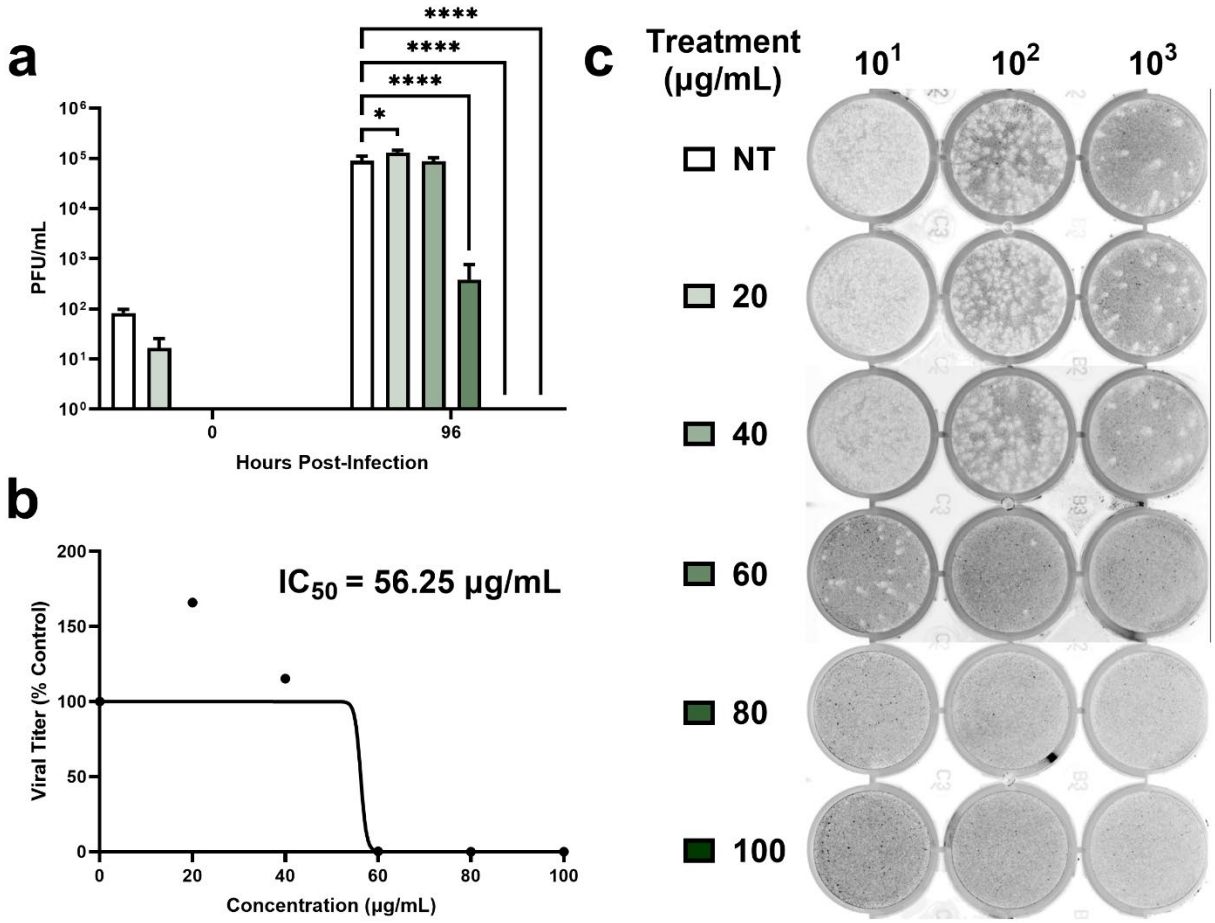

Figure 6: RE exhibits virucidal activity against ZIKV in HFF-1 cells. (a) ZIKV was incubated with increasing concentrations of RE or an equal volume of a vehicle control (DMSO) for 1 h. Subsequently, HFF-1 cells were infected with each sample (MOI = 1) and cell-free supernatants were collected 0 and 96 h post-infection. Viral titers were quantified by plaque assay. Data are expressed as mean PFU/mL ±SEM for n = 3 independent experiments, each replicated in duplicate. A two-way ANOVA and Dunnett's multiple comparisons were used to determine differences in viral titers following RE treatment. *$p<0.05$, ****$p<0.0001$ relative to HFF-1 cells infected with DMSO treated ZIKV (NT). (b) The IC_{50} value was calculated by non-linear regression analysis (inhibitor vs normalized response – variable slope). (c) Plaque assay images taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

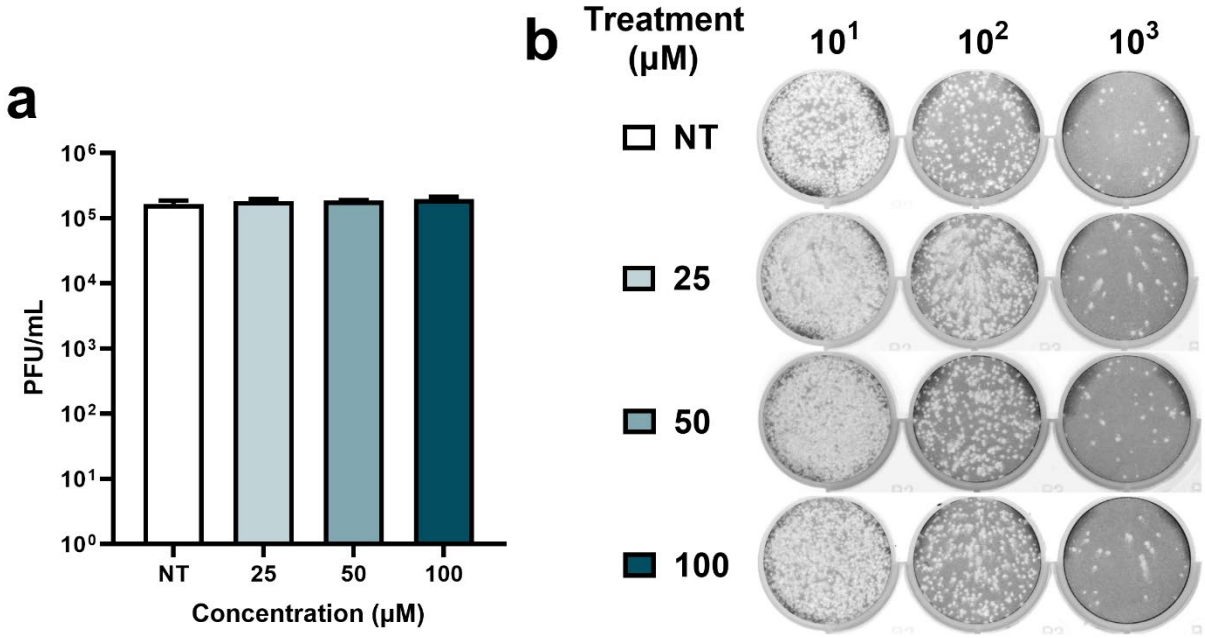

Figure 7: RA does not affect ZIKV infection. (a) The virucidal activity of RA was evaluated by plaque reduction assay. ZIKV (290,000 PFU) was incubated with increasing concentrations of RA or an equal volume of a vehicle control (DMSO) for 1 h. Viral titers in each sample were then determined by plaque assay. Data are expressed as mean PFU/mL ±SEM for n = 3 independent experiments, each replicated in duplicate. A one-way ANOVA and Dunnett's multiple comparisons were used to determine differences in viral titers following RA treatment relative to DMSO (NT). (b) Plaque assay images taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

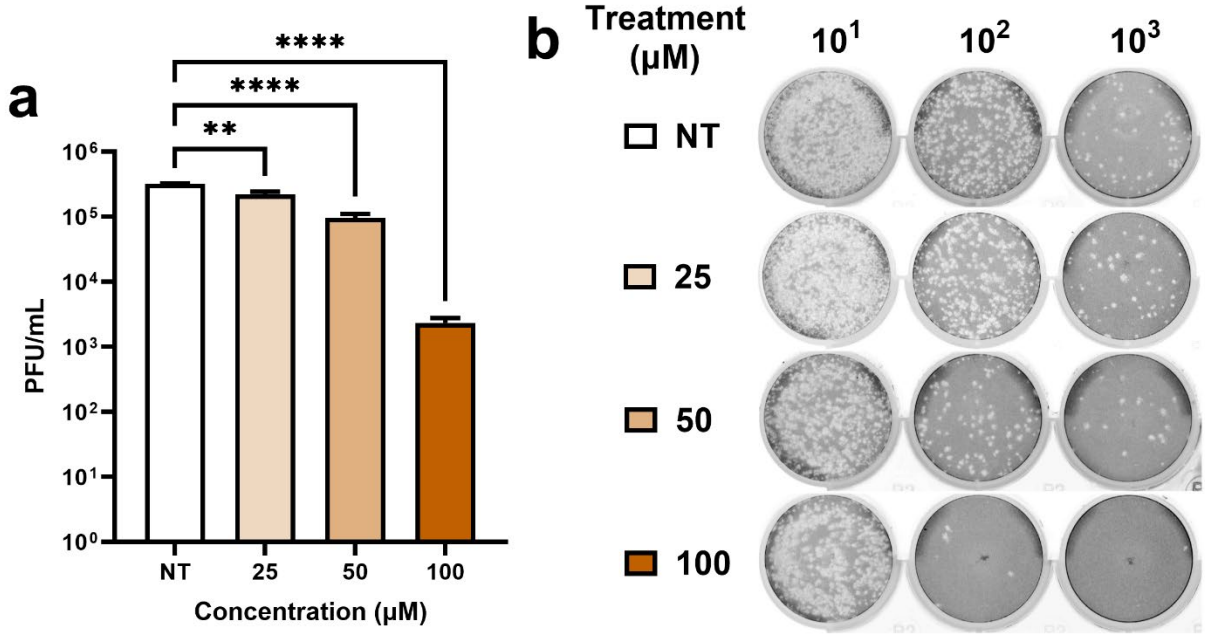

Figure 8: CA inhibits ZIKV infection in a dose-dependent manner. (a) The virucidal activity of CA was evaluated by plaque reduction assay. ZIKV (290,000 PFU) was incubated with increasing concentrations of CA or an equal volume of a vehicle control (DMSO) for 1 h. Viral titers in each sample were then determined by plaque assay. Data are expressed as mean PFU/mL ±SEM for n = 3 independent experiments, each replicated in duplicate. A one-way ANOVA and Dunnett's multiple comparisons were used to determine differences in viral titers following CA treatment. **$p<0.01$, ****$p<0.0001$ relative to ZIKV incubated with DMSO (NT). (b) Plaque assay images taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

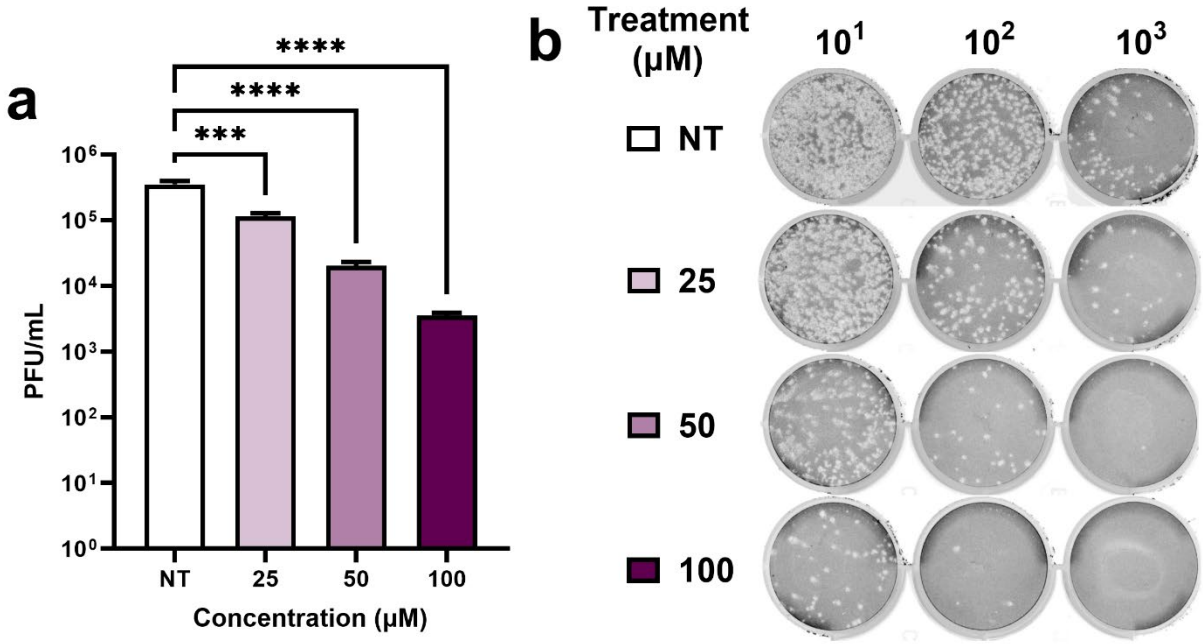

Figure 9: CO inhibits ZIKV infection in a dose-dependent manner. (a) The virucidal activity of CO was evaluated by plaque reduction assay. ZIKV (290,000 PFU) was incubated with increasing concentrations of CO or an equal volume of a vehicle control (DMSO) for 1 h. Viral titers in each sample were then determined by plaque assay. Data are expressed as mean PFU/mL ±SEM for n = 3 independent experiments, each replicated in duplicate. A one-way ANOVA and Dunnett's multiple comparisons were used to determine differences in viral titers following CO treatment. ***$p<0.001$, ****$p<0.0001$ relative to ZIKV incubated with DMSO (NT). (b) Plaque assay images taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

419 **Discussion**

420 Although cases of ZIKV have significantly declined since reaching their peak in 2016,
421 the virus continues to circulate at low levels in tropical and subtropical regions, posing an
422 ongoing threat to human health[15]. Despite extensive efforts by the global scientific community,
423 there are no vaccines or antiviral treatments against ZIKV currently available, leaving us
424 unprepared for potential future outbreaks[3]. This study aimed to evaluate the antiviral potential of
425 RE against ZIKV in human dermal fibroblasts, one of the earliest targets of infection. Here, we
426 provide novel evidence demonstrating that RE exerts potent *in vitro* antiviral effects against
427 ZIKV. RE was found to significantly impair ZIKV infection in Vero cells when the virus was
428 treated before infection (Fig. 2b). To assess the effects of RE on the ZIKV replication cycle,
429 HFF-1 cells were treated before, during, and after infection or ZIKV was treated before
430 infection. No antiviral activity was observed when HFF-1 cells were treated before (Fig. 3a) or
431 during infection (Fig. 4a), however, ZIKV replication unexpectedly increased in the latter
432 condition. There was a significant decrease in viral titers when ZIKV was pre-treated with RE,
433 indicating RE may target the initial stages of the viral life cycle in HFF-1 cells (Fig. 6a). RE was
434 most effective when HFF-1 cells were treated post-infection, indicating RE may also targets the
435 later stages of the viral life cycle (Fig. 5a). Of the three main polyphenols found in RE, CA (Fig.
436 8a) and CO (Fig. 9a), but not RA (Fig. 7a), are potent antiviral compounds against ZIKV, and
437 thus could be used as potential stand-alone antiviral compounds. To the best of our knowledge,
438 this is the first study describing the antiviral activity of RE in the context of flavivirus infection,
439 specifically ZIKV.

440 To determine the antiviral potential of RE against ZIKV, we employed a plaque reduction
441 assay. This approach is commonly used in the literature[35,43–45] to screen different compounds for
442 antiviral activity and involves directly treating the virus before infecting a susceptible cell line,

443 such as Vero. Here, we observed a significant dose-dependent decrease in viral replication with
444 all concentrations of RE, with the lowest concentration causing a 53.6 % reduction in viral titers
445 (Fig. 2b and 2c, IC_{50} = 24.95 µg/mL). Similar results have been shown in other viral models, as
446 RE inhibits the replication of HSV-1, HSV-2[29,31], and human immunodeficiency virus 1[30] in
447 Vero. However, Nolkemper et al. reported IC_{50} values of 0.646 µg/mL against HSV-1 and 1.055
448 µg/mL and against HSV-2, which are much lower than those observed in the current study[29].
449 This discrepancy could be attributed to differences between viral models, procedural differences
450 (treatment of 200 PFU of HSV with no dilution prior to inoculation vs. 290,000 PFU of ZIKV
451 followed by serial dilutions in our model), and the RE extraction method (aqueous vs. methanol
452 in our model). Overall, our results are in general agreement with the literature, as several classes
453 of natural products reported to possess anti-ZIKV properties exhibit biological effects during
454 viral adsorption and internalization or exert a virucidal effect by directly interacting with virus
455 particles[22].
456 To identify which stage(s) of the ZIKV replication cycle are affected by RE, we
457 performed four experiments using HFF-1 cells: 1) treatment of cells before infection, 2)
458 simultaneous treatment and infection of cells, 3) treatment of cells after infection, and 4)
459 treatment of ZIKV before infection. Results show that treating the cells before or during the
460 adsorption phase does not reduce ZIKV replication (Fig. 3a and 4a). Nolkemper et al. found
461 similar results, with no effect on HSV-2 and a minimal 24% reduction in HSV-1 replication
462 when RC-37 cells (African green monkey kidney cells) were treated with a non-cytotoxic
463 concentration of RE before infection. However, treatment of RC-37 cells during infection caused
464 a 36% and 67% reduction in HSV-1 and HSV-2 replication, respectively[29]. In another study, 20
465 µg/mL RE caused a 66-fold reduction in hRSV production in A549 cells treated before infection,

466 conflicting with our results, though a different virus and cell type[28]. Moreover, Lin et al.
467 demonstrated a significant 21.9% reduction in viral VP1 mRNA and an 88.6% reduction in
468 EV71 replication in RD cells treated with 10 µM of RA, one of the main constituents found in
469 RE, during infection. Similar to our findings, RA minimally inhibited EV71 by 5.54% in RD
470 cells treated before infection[46]. Surprisingly, we observed a statistically significant increase in
471 ZIKV production in cells treated during infection with every concentration of RE (Fig. 4a) as
472 well as in cells infected with ZIKV pretreated with 20 µg/mL (Fig. 5a). As far as we know, this
473 effect has not been reported in the literature. Considering that pH can have a significant impact
474 on ZIKV infectivity[47], we measured the pH of various concentrations of RE prepared in DMEM.
475 No major changes in pH were observed in comparison to DMSO, with all values falling within a
476 range of approximately 8.2 to 8.5 (data not shown). Therefore, the increase in virus production
477 may have occurred due to an experimental artefact, but further investigation is required to
478 determine the mechanisms at play following RE treatment during ZIKV infection.
479 Unlike the results above, RE was shown to exert potent antiviral activity when cells were
480 treated post-infection. There was a considerable 53.6% decrease in ZIKV replication at the
481 lowest concentration tested (20 µg/mL), with viral titers falling below the detectable limit at 60
482 µg/mL and beyond (Fig. 5, IC_{50} = 17.92 µg/mL, SI = 6.00). This is in agreement with Horvath et
483 al., who found a 90.46% reduction in HSV-2 genome concentrations in Vero cells treated with
484 0.47 mg/mL of RE after infection[32]. Contrastingly, RE had no effect on HSV-1 or HSV-2 plaque
485 formation in RC-37 cells[29]. Of the main polyphenols found in RE, CA has been shown to reduce
486 HSV-2[32] and hRSV[28] genome concentrations in cells treated post-infection, whereas RA impairs
487 EV71[46] replication, further supporting our results. Based on an *in-silico* study conducted by
488 Srinivasan et al., RE may interfere with the NS5 RNA-dependent RNA polymerase (RdRp),

489 which is essential for replicating the ZIKV genome. This is due to inhibitory potential of CA (-
490 6.8 kcal/mol), CO (-7.0 kcal/mol), RA (6.9 kcal/mol), and several other derivatives, which were
491 predicted to have high binding energy against the RdRp, although *in vitro* studies are required to
492 validate this data[26]. To further explore RE's effects post-infection, we conducted preliminary
493 experiments to visualize the impact of RE treatment on ZIKV M protein expression by an
494 immunofluorescence assay. Here, we show a visible reduction in the number of HFF-1 cells
495 expressing the M viral protein when treated with 50 µg/mL of RE, indicating ZIKV replication
496 was impaired (Supplemental Fig. 2). Collectively, this data suggests that RE acts on the later
497 stages of the viral life cycle by potentially regulating genome translation and replication and/or
498 virion assembly and release.

499 As seen with the plaque reduction assay, pre-treatment of ZIKV with RE significantly
500 inhibited infection in HFF-1 cells, causing a 99.6% reduction in progeny virus at a concentration
501 of 60 µg/mL and complete inhibition at 80 and 100 µg/mL (Fig. 6, IC_{50} = 56.25 µg/mL, SI =
502 2.14). Similar results were seen following pretreatment of HSV at the maximum non-cytotoxic
503 concentration of RE (actual concentration not reported) in RC-37 cells, resulting in a 97% and
504 99% reduction in HSV-1 and HSV-2, respectively[29]. Unlike our plaque reduction assay results,
505 no antiviral effect was seen at concentrations below 60 µg/mL in HFF-1 cells, which may be due
506 to the distinct species and tissue origin of each cell line as well as fundamental differences
507 between each assay. To illustrate this, ZIKV was treated with RE for 1 h and the remaining
508 infectivity in each sample was quantified by plaque reduction assay, whereas in the HFF-1
509 experiment, a plaque assay was used to quantify the production of infectious virions in HFF-1
510 cells following infection with pre-treated ZIKV. Since no antiviral effects were observed in HFF-
511 1 cells treated during infection, these findings indicate that RE directly interacts with

512 extracellular ZIKV particles by potentially binding to viral proteins involved in mediating viral
513 entry or damaging virions, thereby impairing the ability to infect host cells.
514 To determine if the major polyphenols found in RE exert similar effects against ZIKV,
515 each compound was screened for antiviral activity using a plaque reduction assay. Here, we
516 show that pre-treatment of ZIKV with CA and CO significantly reduces ZIKV plaque formation
517 in a dose-dependent manner, with CA causing a 99.2% reduction and CO causing a 99.0%
518 reduction at 50 μM (Fig. 8a and 9a). Considering that CO is the major oxidation product of
519 CA[48], they likely have very similar biological properties. In comparison, RA had no effect on
520 ZIKV infection with any of the concentrations tested (Fig. 7a). These results were somewhat
521 unexpected, as RA is the only compound with existing evidence of its antiviral activity against
522 flaviviruses. Pre-treatment of DENV with RA was shown to inhibit all four serotypes *in vitro*[35]
523 as well as significantly reduced mortality, viral mRNA, and proinflammatory cytokine levels in
524 mice treated peritoneally with RA and infected with a lethal dose of JEV[36]. There is also *in vitro*
525 evidence demonstrating the efficacy of RA against the H1N1 subtype of influenza A[43],
526 EV71[46,49], and hepatitis B virus[50] as well as *in vivo* evidence against a lethal dose of EV71 in
527 mice[51]. This is especially surprising due to the structural similarities between flaviviruses,
528 indicating its effects may be DENV and JEV specific. The antiviral effects of CA have also been
529 well characterized in the literature, as it was shown to be effective against HSV-2[32], hRSV[28], and
530 H1N1[43], although this conflicts with another report suggesting that it has no effect on influenza
531 A virus[28]. Interestingly, we were not expecting CO to have such a profound effect, as only two
532 groups have analyzed its antiviral properties with conflicting results[28,52].
533 There are several limitations that are worth considering. Many antiviral studies use a
534 positive control to confirm the performance of each assay and ensure the results are interpreted

535 properly. Considering no antiviral therapeutic is available against ZIKV, this could unfortunately
536 not be included. Although plants are a promising source of compounds, using whole extracts also
537 presents limitations. Depending on the soil characteristics, climate, radiation, light exposure, and
538 several other factors, the composition of secondary metabolites within the extract may vary,
539 limiting its comparability and reproducibility. To mitigate this, studies should report the
540 composition of each extract so comparisons can be made throughout the literature. Lastly, while
541 *in vitro* studies provide valuable insights and are necessary during the early stages of
542 investigation, the effects seen may not directly translate to humans, representing a major
543 challenge in the field. The human body consists of many diverse cell types that interact within
544 complex tissues and organ systems, and antiviral efficacy can be influenced by metabolism,
545 immune responses, bioavailability, and other factors. Therefore, it is important that antivirals are
546 evaluated in small animal models, as this is a more physiologically relevant system. However,
547 there is a lack of an immunocompetent animal model for ZIKV and most flaviviruses, which
548 needs to be addressed moving forward.

549 As mosquitoes and flaviviruses continue to spread to new regions and threaten human
550 health, new antiviral agents are urgently needed. In the present study, we demonstrated that RE is
551 a potent antiviral agent against ZIKV. Importantly, RE inhibited ZIKV infection by directly
552 interacting with virus particles before infection in Vero and HFF-1 cells and interfering with
553 post-entry processes of the ZIKV replication cycle in HFF-1 cells. After screening the three most
554 abundant polyphenols found in RE, we determined that CA and CO are potential contributors to
555 the antiviral activity of RE and promising candidates on their own worth following up. Whether
556 or not RE can successfully impede other medically important flaviviruses, such as DENV and
557 WNV, remains to be explored. Overall, our results provide substantial evidence highlighting the

558 antiviral activity of RE, pointing to its potential use as a novel therapeutic against ZIKV and

559 warranting further investigation into its effects in other cell and viral models.

Authorship Contributions

JTM, RWEC, JMC, EIP, and AJM conceived and designed the study. JTM was assisted by RWEC and NJH in carrying out experiments. JTM and AJM analyzed the data and wrote the manuscript. All authors contributed to discussion and editing of the manuscript.

Funding

This research has been supported and funded by grants and awards to AJM from the Natural Sciences and Research Council of Canada (NSERC Discovery), Canada Foundation for Innovation (CFI), the Ontario Research Fund (ORF) and Brock University. JTM was supported by an NSERC Master's Canada Graduate Scholarship (CGS-M) and Queen Elizabeth II Graduate Scholarship in Science and Technology (QEII-GSST); RWEC and JMC were supported by an NSERC Doctoral Canada Graduate Scholarship (CGS-D) and NJH was supported by an NSERC Doctoral Postgraduate Scholarship (PGS-D) and a QEII-GSST.

Conflicts of interests and disclosures

The authors declare no conflicts of interest.

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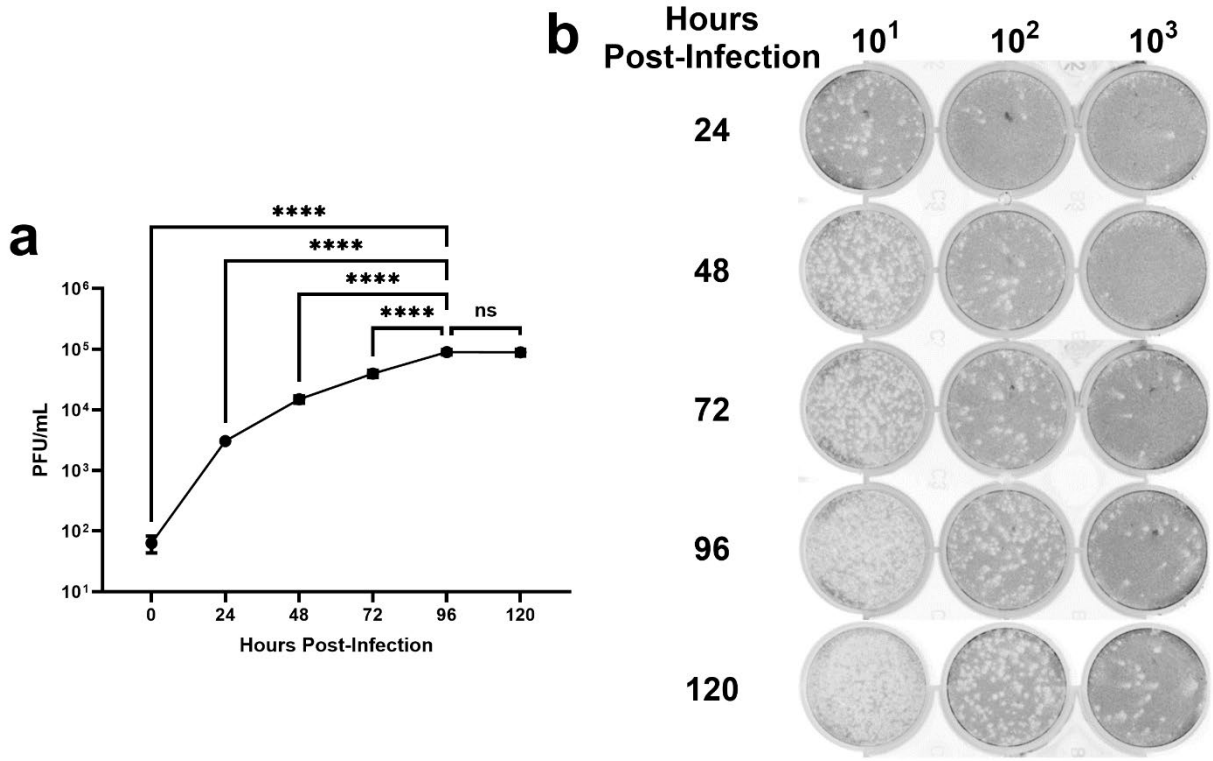
705 **Supplemental Material**



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707 **Supplemental Figure 1: Infectious titers peak at 96 h in HFF-1 cells infected with ZIKV**. (a)
708 HFF-1 cells were infected with ZIKV (MOI = 1) for 1 h at 37°C. Cell-free supernatants were
709 collected 0, 24, 48, 72, 96, and 120 h post infection and viral titers in each sample were
710 quantified by plaque assay. Data are expressed as PFU/mL ±SEM for n = 3 independent
711 experiments, each replicated in duplicate. A two-way ANOVA and Dunnett's multiple
712 comparisons were used to determine differences in viral titers between each timepoint.
713 ****$p<0.0001$ relative to supernatant collected 96 h post-infection. (b) Plaque assay images
714 taken using an Amersham Typhoon Biomolecular Imager and represent wells from one
715 independent experiment in its entirety.

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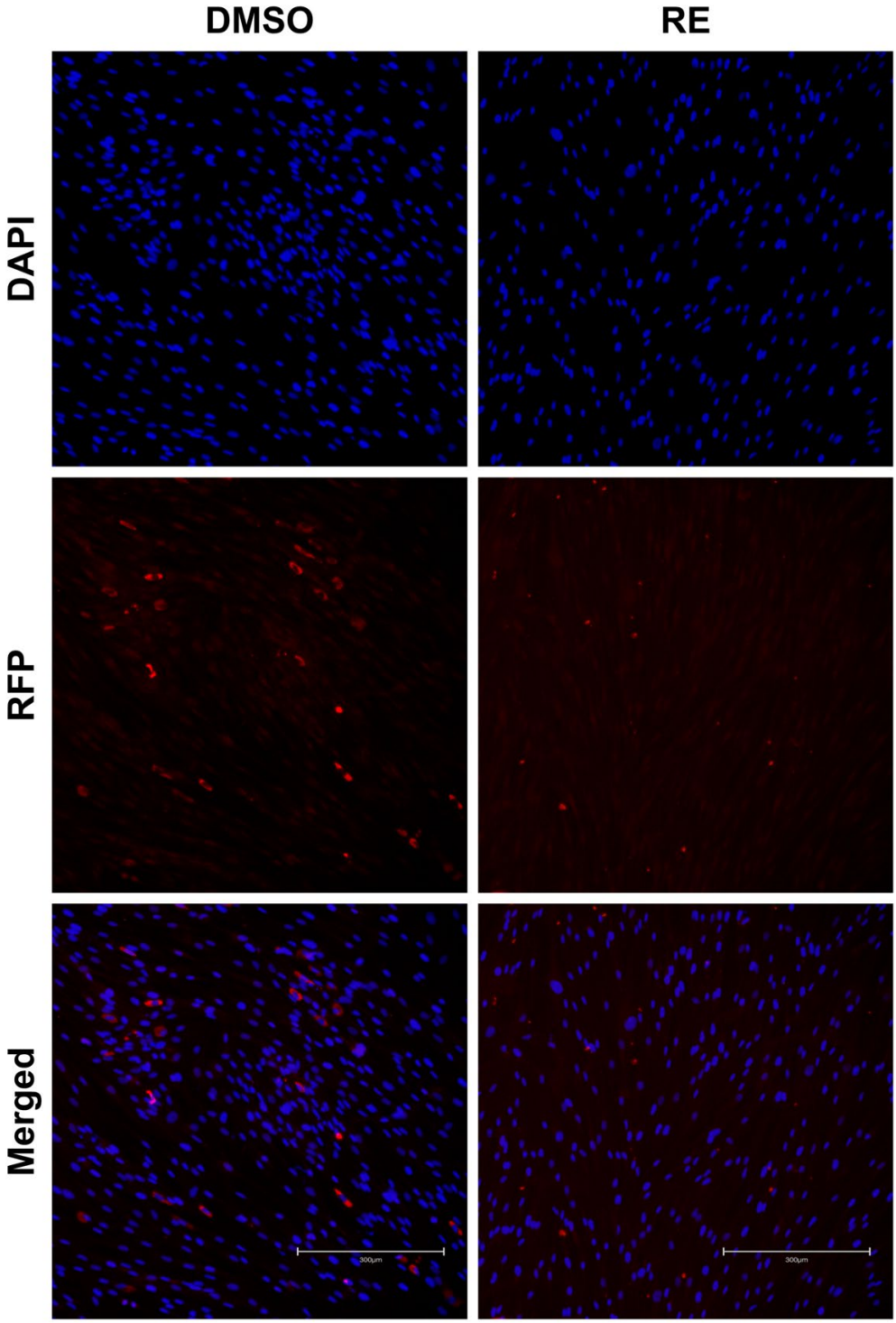

Supplemental Figure 2: Visual representation of the antiviral activity of RE against ZIKV in HFF-1 cells treated after infection. HFF-1 cells seeded in 24-well plates were infected with ZIKV (MOI = 5) for 1 h at 37°C. Cells were washed once with PBS to remove unbound virus and then treated with DMSO or 50 µg/mL of RE in DMEM with 10% FBS and 1% pen/strep at a final volume of 200 µL. After incubating for 72 h, cells were fixed with paraformaldehyde (BioShop, PAR070), permeabilized with Triton X (BioShop, TRX506), and blocked with bovine

725 serum albumin (Sigma, A7906), washing thrice with PBS between each step. Subsequently, cells
726 were stained with NucBlue (Life Technologies, R37605) and a 1:200 dilution of an RFP-
727 conjugated primary antibody specific for the ZIKV M protein (Novus Biologicals, NBP2-
728 61132AF532). Infection was visualized using an EVOS M7000 Microscope Imaging System
729 (Invitrogen) at 200x magnification.