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

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

Abstract

Flaviviruses have increasingly emerged and re-emerged in recent decades, infecting millions of people annually. Zika virus (ZIKV) 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. Here, we sought to investigate the antiviral potential of rosemary extract (RE) against ZIKV in human dermal fibroblasts (HFF-1), one of the earliest cellular targets of infection. ZIKV was treated with various concentrations of RE or its major polyphenols, including rosmarinic acid (RA), carnosic acid (CA), and carnosol (CO), and the 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 virucidal 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 infectivity when ZIKV was pre-treated with each compound individually. 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 viral replication cycle. This study highlights the antiviral potential of RE and its individual components, wFigurearranting further

77 investigation into the mechanisms underlying their activity and their effects on other medically

78 important 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, pre-membrane, and capsid proteins, along with seven non-structural (NS) proteins, including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5[3,4]. ZIKV is primarily transmitted by female *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, including keratinocytes, fibroblasts, Langerhans cells, dendritic cells, and mast cells, representing the first line of defense against the virus[9,10]. Fibroblasts are the

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

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

118 An attractive approach to developing therapeutics is the use of medicinal plants, as they
119 possess a diverse array of bioactive secondary metabolites[14]. These compounds have been
120 shaped by millions of years of evolution to provide plants selective advantages, including
121 protection against oxidative stress, ultraviolet radiation, predators, and infection[16,17]. Historically,
122 humans have used plants as a source of medicines for treating various diseases, a practice that
123 continues today[18]. Plants have played a significant role in modern drug discovery, making up
124 approximately 6% of new molecular entities approved by the U.S. Food and Drug

125 Administration between the years 2000 and 2016[18,19]. In fact, many drugs currently available on
126 the market were isolated from plants, including the anti-inflammatory acetylsalicylic acid
127 (aspirin), the narcotic morphine, and the anti-glaucoma drug pilocarpine[14,20]. In the context of
128 mosquito-borne infections, quinine, derived from the bark of the cinchona tree, stands out as one
129 of the most important anti-malarial drugs in history and is listed on the WHO's Model List of
130 Essential Medicines for severe malaria[21]. Beyond their well-documented antioxidant and anti-
131 inflammatory properties, several plant-derived secondary metabolites have demonstrated
132 antiviral activity *in vitro* and *in vivo*, including quercetin and fisetin against DENV2, baicalein
133 against JEV and influenza virus, and epigallocatechin-3-gallate against herpes simplex virus
134 (HSV), Epstein Barr virus, and DENV, to name a few[22,23]. However, relatively few studies have
135 explored the antiviral potential of medicinal plants and their individual components against
136 ZIKV, highlighting a gap in the field.

137 *Rosmarinus officinalis* L. (rosemary) is a medicinal herb belonging to the *Lamiaceae*
138 family and is native to the Mediterranean region. Rosemary extract (RE) is widely used as a
139 natural preservative in food processing and has been extensively studied for its antioxidant, anti-
140 inflammatory, antibacterial, antitumor, neuroprotective, and other therapeutic properties[24–26].
141 These can be attributed to its rich polyphenolic composition, including phenolic acids like
142 rosmarinic (RA), ursolic, and caffeic acids, as well as phenolic diterpenoids like carnosic acid
143 (CA), carnosol (CO), and rosmanol[27]. Although the antibacterial activity of RE has been well
144 characterized[28], little is known about its antiviral activity. To the best of our knowledge, no
145 studies have explored the antiviral activity of RE in the context of flavivirus infection, although
146 it has been shown to be effective against human respiratory syncytial virus (hRSV)[29] and HSV[30–
147 33]. Additionally, RA, a major polyphenol in RE, was identified as a potential antiviral agent

148 against ZIKV and DENV *in silico*[34–36]. RA has also been shown to inhibit all four serotypes of
149 DENV *in vitro*[36] and significantly reduce mortality, viral loads, and pro-inflammatory cytokine
150 levels in mice infected with a lethal dose of JEV[37]. In the current study, we demonstrate that RE
151 potently inhibits ZIKV infection by directly interacting with virus particles before infection and
152 disrupting later stages of the viral replication cycle. Furthermore, we found that CA and CO, but
153 not RA, exert virucidal activity against ZIKV. These findings highlight the antiviral potential of
154 RE and its individual components, warranting further investigation into their mechanism of
155 action and activity against other flaviviruses.

156 **Materials and Methods**

157 *Cells and virus*

158 Human foreskin fibroblast cells (HFF-1; ATTC, SCRC-1041) and Vero E6 cells (African
159 green monkey kidney cells; ATTC, CRL-1586) were maintained in Dulbecco's modified Eagle's
160 medium (DMEM; Sigma, D6546) supplemented with 10% Serum Plus II (Sigma, 14009C) and
161 1% penicillin/streptomycin (pen/strep; Gibco, 15140-122) at 37°C with 5% CO_2. The
162 PRAVBC59 strain of ZIKV (ATCC, VR1843) was propagated in Vero cells. Briefly, Vero
163 monolayers were infected with ZIKV at a multiplicity of infection (MOI) of 0.01 and incubated
164 at 37°C and 5% CO_2 for 1 h. The viral inoculum was removed, and cells were incubated for 5
165 days in culture medium containing 2% Serum Plus II and 1% pen/strep. Viral stocks were
166 quantified by plaque assay, aliquoted, and stored at -80°C.

167 *Reagent preparation*

168 Rosemary extract was prepared by grinding and passing whole dried *Rosmarinus officinalis* L.
169 leaves (purchased from Sobeys, Mississauga, ON, Canada; product of Turkey) through a mesh
170 sieve. Extraction was performed following protocols established by the US National Cancer
171 Institute. Ground leaves (5 g) were steeped overnight in 30 mL of dichloromethane-methanol
172 (1:1). A slight vacuum was used to collect the filtrate, and a methanol extraction was performed
173 for 30 min. The solvent was removed by rotary evaporation. For antiviral experiments, RE was
174 dissolved in dimethyl sulfoxide (DMSO; Sigma, D2650) at a final concentration of 100 mg/mL
175 and RA (Sigma, R4033), CA (Sigma, C0609), and CO (Sigma, C9617) were dissolved in DMSO
176 at a final concentration of 100 mM. Aliquots were stored protected from light at -20°C until
177 further use. For HPLC analysis, RE was dissolved in DMSO (Sigma, 34869) at 50 mg/mL and

178 authentic standards of RA (Cayman Chemicals, 70900), CA (Cayman Chemicals, 89820), and
179 CO (Cayman Chemicals, 89800) were dissolved in DMSO at 5 mg/mL immediately before use.

180 *Cell viability assay*

181 To determine the impact of RE treatment on cell viability, HFF-1 and Vero cells were
182 seeded in 96-well tissue culture plates and incubated at 37°C with 5% CO_2 until ~90% confluent.
183 Cells were treated in triplicate with DMEM containing 20 to 200 µg/mL of RE for 1 h at 37°C
184 with 5% CO_2, using 1% DMSO as a vehicle control. Subsequently, the medium was replaced
185 with fresh culture medium containing 10% Serum Plus II and 1% pen/strep, and cells were
186 incubated for either 96 h (HFF-1) or 120 h (Vero) at 37°C with 5% CO_2. These conditions
187 correspond to cells treated before or during infection or infected with pre-treated ZIKV.
188 Additionally, HFF-1 cells were incubated with 20 to 200 µg/mL of RE in DMEM with 10%
189 Serum Plus II and 1% pen/strep for 96 h, corresponding to cells treated after infection in the
190 time-of-addition analysis. Following incubation, culture medium was replaced with 100 µL of
191 fresh medium and 10 µL of WST-1 cell proliferation reagent (Roche, 5015944001). After 2 h,
192 absorbance was measured at 460 nm using a spectrophotometer (Bio-Tek, Synergy HT-1,
193 191356) and triplicates were averaged. The cytotoxic concentration of RE that reduced viability
194 by 50% (CC_{50}) was calculated by non-linear regression analysis (GraphPad Prism).

195 *Plaque reduction assay*

196 Plaque reduction assay was used to screen the antiviral activity of RE and three major
197 polyphenol constituents commonly reported in the literature[38–41], including RA, CA, and CO.
198 100 µL of ZIKV (290,000 PFU) was treated with 25 to 200 µg/mL of RE or 25 to 100 µM of
199 each compound individually for 1 h at 37°C with 5% CO_2, using 1% DMSO as a vehicle control.

200 The infectivity of each sample was then quantified by plaque assay. The inhibitory concentration
201 that reduced plaque numbers by 50% (IC_{50}) was calculated by non-linear regression analysis
202 (GraphPad Prism).

203 *Time-of-addition analysis in HFF-1 cells*

204 To determine which stages of infection are targeted by RE, time-of-addition analysis was
205 performed. Briefly, HFF-1 cells were seeded on 24-well plates and incubated at 37°C with 5%
206 CO_2 until ~90% confluent. Cells were infected with ZIKV at an MOI of 1 under four different
207 treatment conditions, with 1% DMSO included as a vehicle control. 1) Cells were pre-treated
208 with 20 to 100 µg/mL of RE for 1 h at 37°C. The supernatant was removed, and wells were
209 washed once with 1X phosphate-buffered saline (PBS; Gibco, 10010023) before viral
210 inoculation. 2) Cells were infected with ZIKV and simultaneously treated with 20 to 100 µg/mL
211 of RE for 1 h at 37°C. 3) Cells were infected with ZIKV for 1 h at 37°C, the inoculum was
212 removed, and culture medium containing 20 to 100 µg/mL of RE was added to each well for the
213 entire post-infection period. 4) ZIKV was incubated with 20 to 100 µg/mL of RE for 1 h at 37°C
214 before being added to the cells. In all treatment conditions, cells were washed once with PBS
215 after the 1 h adsorption period to remove unbound ZIKV. Next, 500 µL of culture medium with
216 10% Serum Plus II and 1% pen/strep was added to each well and cells were incubated at 37°C
217 and 5% CO_2. Supernatant was collected, aliquoted, and stored at -80°C after 0 and 96 h post-
218 infection. Viral titers were quantified by plaque assay and genome copies were quantified by
219 two-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The inhibitory
220 concentration that reduced viral titers or genome copies by 50% (IC_{50}) was calculated by non-
221 linear regression analysis (GraphPad Prism).

222 *Plaque assay*

223 Vero E6 cells were seeded in 12- and 24-well plates and incubated at 37°C with 5% CO_2
224 until 90% confluent. Serial 1:10 dilutions were prepared for pre-treated ZIKV stocks (plaque
225 reduction assay) or supernatant harvested from HFF-1 cells (time-of-addition analysis) 96 h post-
226 infection, whereas a 1:2 dilution was prepared for samples collected 0 h post-infection. Cells
227 were infected in duplicate with 200 µL (1:10 dilutions) or 100 µL (1:2 dilutions) of each dilution
228 in 12- or 24-well plates, respectively. Following a 1 h adsorption period at 37°C, viral inoculum
229 was removed, and cells were overlaid with 1 mL (12-well) or 0.5 mL (24-well) of a 1:1 mixture
230 of carboxymethylcellulose (CMC) and DMEM with 2% Serum Plus II and 1% pen/strep. Plates
231 were incubated at 37°C with 5% CO_2 for 5 days for plaque development, the overlay was
232 discarded, and wells were washed twice with PBS. Cells were simultaneously fixed and stained
233 with 1% crystal violet dissolved in 8% formaldehyde and 30% ethanol for 30 min at room
234 temperature. Excess stain was removed using tap water and plaques were counted. Plaque
235 forming units per mL (PFU/mL) was calculated for each sample using the following equation:

236 $$\frac{\text{PFU}}{\text{mL}} = \frac{(\text{Average Number of Plaques})(\text{Dilution Factor})}{\text{Volume of Inoculum}}$$

237 *RNA isolation and two-step RT-qPCR*

238 Viral RNA was isolated from cell-free supernatant collected 96 h post-infection using the
239 QIAamp Viral RNA Kit (Qiagen, 52904) according to the manufacturer's instructions. cDNA
240 was generated by reverse transcription using RNA to cDNA EcoDry Premix kits (Takara Bio,
241 639549) in a SimpliAmp Thermal Cycler (Applied Biosystems, 228001548) and diluted 1:20 in
242 molecular grade water (Sigma, 693520). The StepOnePlus Real-Time PCR System (Applied
243 Biosystems, 272004476) was used to perform qPCR with KAPA SYBR Fast Master Mix

244 (KAPA Biosystems, KM4103) and amplification efficiency optimized primers at a final reaction
245 volume of 10 µL (1 µL cDNA + 9 µL master mix). The following forward and reverse primer set
246 was used: 5'-GCAAACGCGGTCGCAAACCT-3' and 5'-TGCTAACGCGAAGCCAGGGT-3'
247 (Integrated DNA Technologies)[42]. To determine the relative number of ZIKV copies in cell-free
248 supernatant, a standard curve was generated using ZIKV gBlock gene fragments (Integrated
249 DNA Technologies).

250 *High-performance liquid chromatography (HPLC)*

251 The polyphenolic composition of RE was analyzed using a Dionex UltiMate 3000
252 UHPLC system (Thermo Scientific) equipped with a Kinetex C18 column (100 mm × 4.6 mm ×
253 2.6 µm; Phenomenex, 00D-4462-E0) maintained at 30°C. The mobile phase consisted of (A)
254 water (Sigma, 270733) containing 1% (v/v) glacial acetic acid (Sigma, AX0074-6) and (B)
255 acetonitrile (VWR, BDH83639.400) containing 1% (v/v) glacial acetic acid. The following
256 gradient elution was performed: 0–25 min, 95–5% A; 25–28 min, 5% A; 28–30 min, 5–95% A;
257 30–35 min, 95% A. RE was dissolved in DMSO at 50 mg/mL and diluted ten-fold in 50:50 (v:v)
258 A:B to a final concentration of 5 mg/mL for analysis. Authentic standards of RA, CA, and CO
259 were prepared in DMSO at 5 mg/mL and diluted ten-fold in 50:50 (v:v) A:B to 500 µg/mL,
260 followed by serial two-fold dilutions. The injection volume was 5 µL, with a flow rate of 1.0
261 mL/min and detection wavelength of 280 nm. Peaks were identified by comparing retention
262 times with those of authentic standards. Quantification was performed using the external
263 standardization method in Chromeleon (v.7.1.1.1127), with standard curves generated by
264 plotting each concentration (31.75, 62.5, 125, 250, and 500 µg/mL) against the relative peak
265 area.

266

267 *Statistical analysis*

268 One-way ANOVA analysis was used to evaluate WST-1 viability assay and RT-qPCR
269 data, and two-way ANOVA analysis was used to evaluate plaque assay data. Multiple
270 comparisons were performed using Dunnett's *post-hoc* test where appropriate. CC_{50} and IC_{50}
271 values were determined by analyzing normalized data (% control) using non-linear regression
272 (variable slope). Results from all experiments were expressed as mean ± SEM and considered
273 significant if $p<0.05$ between the vehicle control (DMSO) and RE or polyphenol treated groups.
274 Statistical analyses were conducted using GraphPad Prism (v.10.5.0).

275 **Results**

276 *Cytotoxicity of RE in cell culture*

277 WST-1 assay was used to evaluate the cytotoxic effects of RE treatment on HFF-1 and
278 Vero cells. Both cell lines were exposed to increasing concentrations of RE (20–200 µg/mL) or
279 an equivalent volume of DMSO (vehicle control). Incubation periods were selected to reflect the
280 conditions used in the different functional assays. Results are expressed as a percentage of the
281 vehicle control (set to 100% viability) for each independent experiment. No difference in
282 viability was observed in HFF-1 cells treated with 20 to 80 µg/mL of RE for 1 h followed by a
283 96 h incubation compared to DMSO treated cells. However, there was a significant decrease in
284 viability beginning at 100 µg/mL, with a CC_{50} of 120.2 µg/mL (Fig. 1a and 1c). Comparatively,
285 HFF-1 cells treated for the entire 96 h incubation period showed a slight increase in cell viability
286 at 40 µg/mL ($p<0.01$). There was a significant decrease in viability beginning at 80 µg/mL, with
287 a CC_{50} of 107.3 µg/mL (Fig. 1b and 1d). Vero cells were unaffected by treatment with 20 µg/mL
288 of RE for 1 h followed by a 120 h incubation (Fig. 2a).

289 *RE dose-dependently inhibits ZIKV replication in Vero cells*

290 To test the potential antiviral activity of RE, ZIKV was pre-treated with increasing
291 concentrations of RE (25–200 µg/mL) or an equivalent volume of DMSO for 1 h. The viral titer
292 of each sample was then quantified by plaque assay. Results demonstrated a significant dose-
293 dependent decrease in infectious virions relative to the control (Fig. 2b, $p<0.0001$ for all tested
294 concentrations). Concentrations of 25, 50, 75, 100, 125, 150, 175, and 200 µg/mL caused a 52.77
295 ± 5.66%, 69.06 ± 2.88%, 85.63 ± 1.49%, 92.83 ± 0.51%, 93.28 ± 0.63%, 96.74 ± 0.29%, 98.86 ±
296 0.06%, and 99.55 ± 0.10% reduction in ZIKV titers, respectively (Fig. 2c, IC_{50} = 25.06 µg/mL).
297 Due to the nature of this assay, the selectivity index (SI) was not calculated. The highest

298 concentration of RE that ZIKV was pre-treated with was 200 µg/mL, but this was serially diluted
299 ten-fold before being added to Vero cells. As a result, Vero cells were exposed to a maximum
300 concentration of 20 µg/mL of RE, which was not cytotoxic (Fig. 2a).

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

302 To determine the antiviral potential of RE against ZIKV infection and its effect on
303 different stages of the viral replication cycle, HFF-1 cells were treated with RE before,
304 concurrent with, or after viral inoculation, or ZIKV was pre-treated with RE prior to infection.
305 Increasing concentrations of RE ranging from 20 to 100 µg/mL or an equivalent volume of
306 DMSO were used in all experiments. Based on what is frequently used in the literature[10,43–46],
307 cells were infected at an MOI of 1 and cell-free supernatant was collected 0 and 96 h post-
308 infection, which is when ZIKV titers peak in HFF-1 cells (Supplemental Fig. 1). The collected
309 supernatants were aliquoted and used for both plaque assay and RT-qPCR analysis. Percent
310 reductions were calculated relative to the DMSO control group within each respective treatment
311 condition. There were no statistically significant differences in viral titers between samples
312 collected 0 h post-infection for all treatment conditions (data not shown). No antiviral effects
313 were observed in HFF-1 cells treated with RE for 1 h prior to infection (Fig. 3a and 3b).
314 Treatment of HFF-1 cells during the adsorption period significantly enhanced ZIKV replication,
315 with viral titers increasing by 195.47 ± 53.63% ($p<0.0001$), 104.50 ± 18.36% ($p<0.01$), 98.68 ±
316 27.32% ($p<0.01$), 195.89 ± 65.67% ($p<0.0001$), and 88.65 ± 41.33% ($p<0.05$) and genome copy
317 numbers increasing by 320.97 ± 36.65% ($p<0.001$), 274.66 ± 11.34% ($p<0.01$), 247.84 ± 61.02%
318 ($p<0.01$), 303.73 ± 79.16% ($p<0.01$), and 264.92 ± 68.04% ($p<0.01$) at concentrations of 20, 40,
319 60, 80, and 100 µg/mL, respectively, relative to the control. However, these changes did not
320 follow a consistent trend across increasing concentrations of RE, as there was no clear dose-

321 response relationship (Fig. 4a and 4b). RE demonstrated the most potent antiviral activity in cells
322 treated after the 1 h infection period, as infectious virion production was significantly inhibited
323 by every concentration tested (Fig. 5a, $p<0.0001$). There was a 70.90 ± 9.68% and 99.99 ±
324 0.01% reduction in viral titers in cells treated with 20 and 40 µg/mL of RE and a 100 ± 0.00%
325 reduction in viral titers below the detectable limit in cells treated with 60, 80, and 100 µg/mL of
326 RE (Fig. 5c, IC_{50} = 18.56 µg/mL, SI = 5.78). Genome copy numbers were also reduced by 95.87
327 ± 2.17% ($p<0.0001$), 99.60 ± 0.10% ($p<0.0001$), 99.61 ± 0.13% ($p<0.0001$), and 99.95 ± 0.03%
328 ($p<0.0001$) at concentrations of 40, 60, 80, and 100 µg/mL, respectively (Fig. 5b and 5d, IC_{50} =
329 23.12 µg/mL, SI = 4.64). However, concentrations of 80 µg/mL and above were cytotoxic in
330 HFF-1 cells treated with RE for 96 h (Fig. 1b). Lastly, pre-treatment of ZIKV with RE for 1 h
331 prior to infection significantly increased viral titers by 65.99 ± 44.52% ($p<0.05$) at 20 µg/mL and
332 reduced viral titers by 99.70 ± 0.30% ($p<0.0001$) at 60 µg/mL and 100 ± 0.00% ($p<0.0001$) at 80
333 and 100 µg/mL (Fig. 6a and 6c, IC_{50} = 56.25 µg/mL, SI = 2.14 µg/mL). Similarly, genome copy
334 numbers decreased by 55.14 ± 22.42% ($p<0.01$), 99.84 ± 0.12% ($p<0.0001$), 99.90 ± 0.05%
335 ($p<0.0001$), and 99.91 ± 0.06% ($p<0.0001$) at concentrations of 40, 60, 80, and 100 µg/mL,
336 respectively (Fig. 6b and 6d, IC_{50} = 39.63 µg/mL, SI = 3.03 µg/mL). However, 100 µg/mL of RE
337 was cytotoxic when HFF-1 cells were treated for 1 h followed by a 96 h incubation (Fig. 1a).

338 *CA and CO, but not RA, exhibit virucidal activity against ZIKV in Vero cells*

339 To assess whether three main polyphenols in RE exert similar antiviral effects, ZIKV was
340 pre-treated with 25 to 100 µM of each compound individually or an equivalent volume of DMSO
341 for 1 h. The remaining infectivity of each sample was quantified by plaque assay. No effect on
342 viral replication was observed when ZIKV was pre-treated with RA (Fig. 7a). However, there
343 was a significant dose-dependent decrease in infectivity with CA (Fig. 8a) and CO (Fig. 9a).

344 Treatment with CA resulted in a 30.69 ± 6.52% ($p<0.01$), 69.50 ± 5.23% ($p<0.0001$), and 99.27
345 ± 0.12% ($p<0.0001$) reduction in viral titers at concentrations of 25, 50, and 100 μM,
346 respectively, corresponding to an IC_{50} of 34.83 μM (Fig. 8b). Similarly, CO caused a 67.04 ±
347 1.97% ($p<0.001$), 93.81 ± 1.64% ($p<0.0001$), and 98.96 ± 0.16% ($p<0.0001$) reduction in viral
348 titers at concentrations of 25, 50, and 100 μM, respectively, with an IC_{50} of 19.55 μM (Fig. 9b).

349 *HPLC analysis of RE*

350 HPLC analysis was performed to identify and quantify CA, CO, and RA in the RE used
351 in this study. A representative chromatogram for the identified compounds is shown in Fig. 10,
352 and the corresponding results are presented in Table 1. The most abundant polyphenol per 1 mg
353 of RE was CA (78.41 ± 4.64 μg/mg), followed by RA (27.69 ± 0.23 μg/mg) and CO (11.96 ±
354 0.32 μg/mg), with retention times of 17.62, 7.57, and 15.81 min, respectively. These amounts
355 correspond to concentrations of 235.86 ± 13.96 μM CA, 76.85 ± 0.64 μM RA, and 36.20 ± 0.97
356 μM CO when 1 mg of RE is dissolved in 1 mL. Accordingly, at the lowest concentration of RE
357 tested in the plaque reduction assay (25 μg/mL), ZIKV was exposed to approximately 5.90 ±
358 0.35 μM CA, 1.92 ± 0.02 μM RA, and 0.91 ± 0.02 μM CO.

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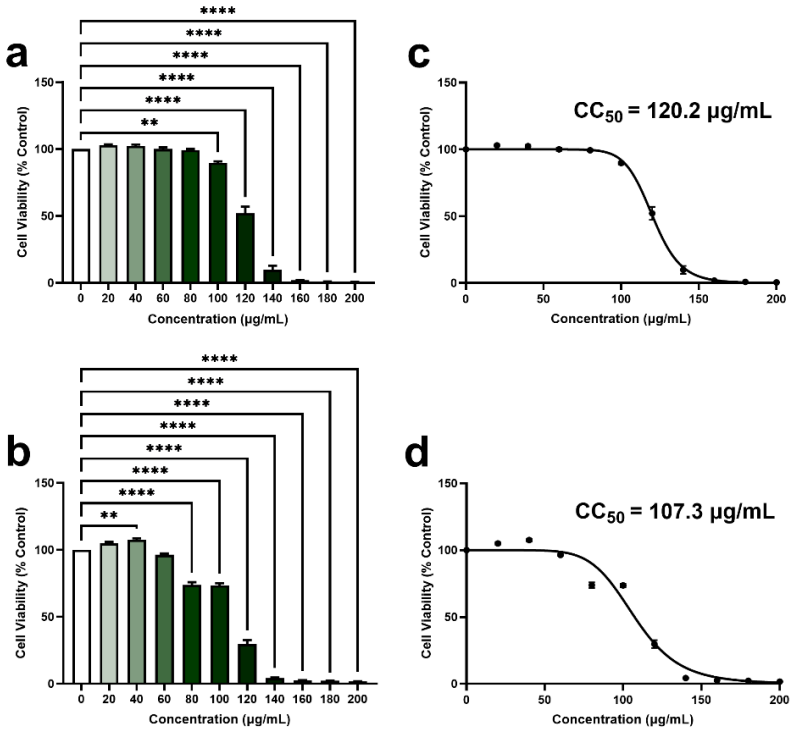

Figure 1: Cell viability of HFF-1 cells treated with increasing concentrations of RE. (a) HFF-1 cells were incubated with increasing concentrations of RE or a vehicle control (DMSO) for 1 h followed by 96 h with fresh medium. (b) HFF-1 cells were incubated with increasing concentrations of RE or a vehicle 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 test 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 (0 µg/mL). 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).

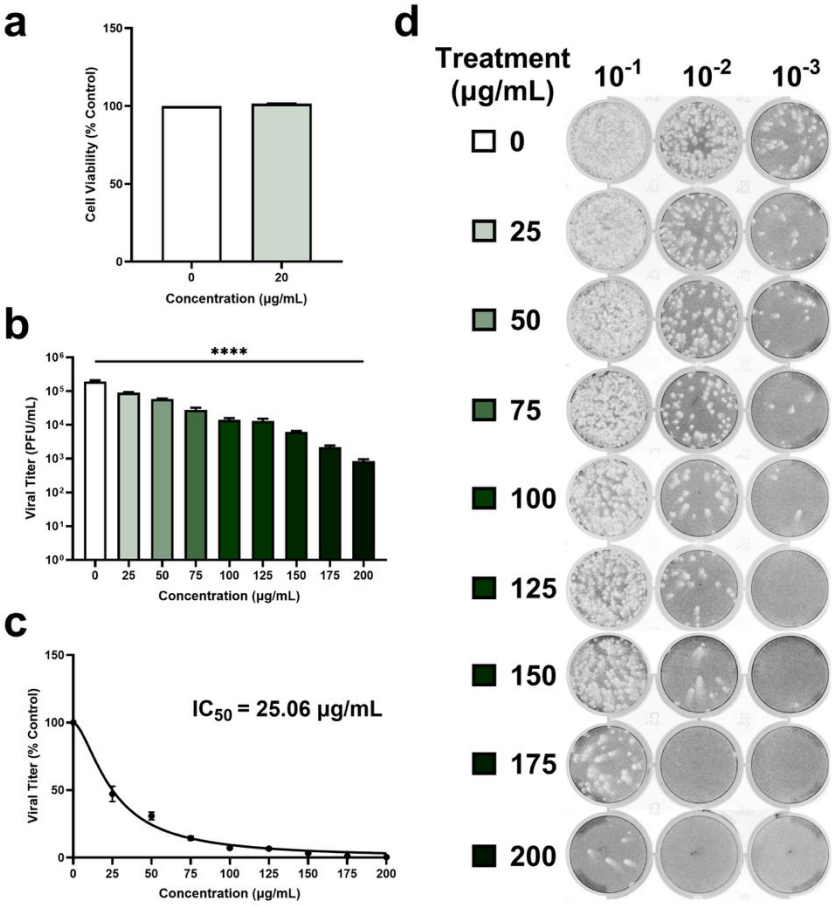

Figure 2: RE inhibits ZIKV replication in Vero cells in a dose-dependent manner. (a) Vero cells were incubated with increasing concentrations of RE or a vehicle control (DMSO) for 1 h followed by 120 h with fresh medium. Cell viability was determined by a WST-1 assay. Data are expressed as % of control ± SEM for n = 3 independent experiments. Vero cells 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 test 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. The viral titer of each sample was then determined by plaque assay. Data are expressed as mean PFU/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A one-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in viral titers following RE treatment. ****$p<0.0001$ relative to ZIKV incubated with DMSO (0 µg/mL) for all concentrations tested. (c) The IC_{50} value was calculated by non-linear regression analysis (inhibitor vs normalized response – variable slope). (d) Plaque assay images were taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

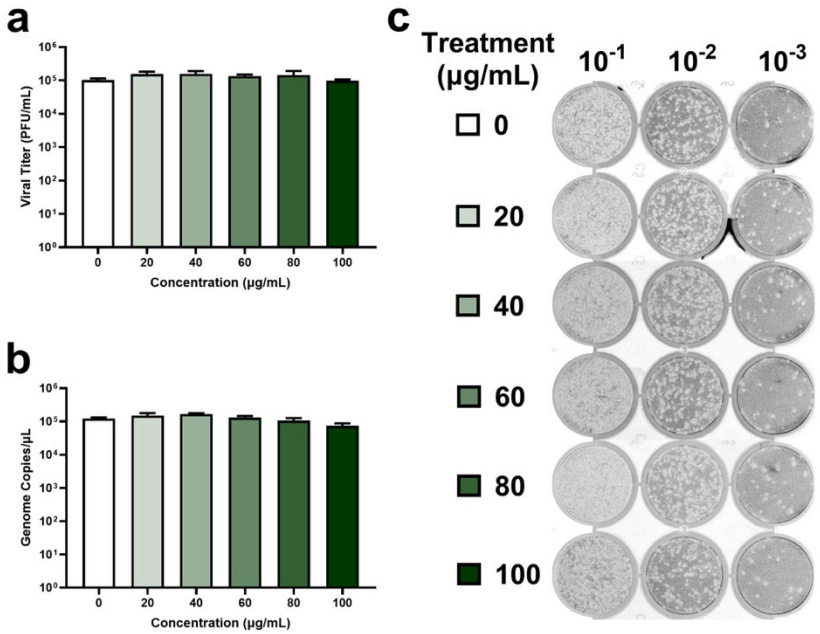

Figure 3: Treatment of HFF-1 cells with RE before infection does not impact ZIKV replication. (a) HFF-1 cells were treated with increasing concentrations of RE or a vehicle control (DMSO) for 1 h and subsequently infected with ZIKV (MOI = 1). Cell-free supernatants were collected 0 and 96 h post-infection, and the viral titer were quantified by plaque assay. Data are expressed as mean PFU/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A two-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in viral titers following RE treatment relative to HFF-1 cells treated with DMSO (0 µg/mL). (b) Viral RNA was extracted from cell-free supernatants collected 96 h post-infection and quantified by RT-qPCR. Data are expressed as mean genome copies/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A one-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in genome copy numbers following RE treatment relative to HFF-1 cells treated with DMSO (0 µg/mL). (c) Plaque assay images were taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

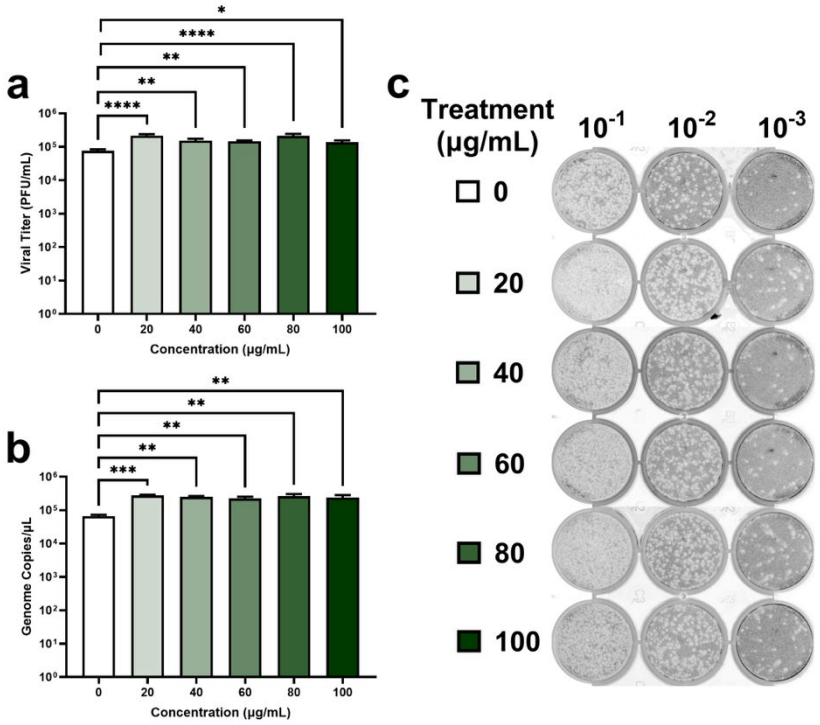

Figure 4: Treatment of HFF-1 cells with RE during infection enhances ZIKV replication. (a) HFF-1 cells were infected with ZIKV (MOI = 1) and simultaneously treated with increasing concentrations of RE or a vehicle control (DMSO) for 1 h. Cell-free supernatants were collected 0 and 96 h post-infection, 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 performed in duplicate. A two-way ANOVA and Dunnett's multiple comparisons test 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 (0 µg/mL). (b) Viral RNA was extracted from cell-free supernatants collected 96 h post-infection and quantified by RT-qPCR. Data are expressed as mean genome copies/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A one-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in genome copy numbers following RE treatment. **$p<0.01$, ***$p<0.001$ relative to HFF-1 cells treated with DMSO (0 µg/mL). (c) Plaque assay images were taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

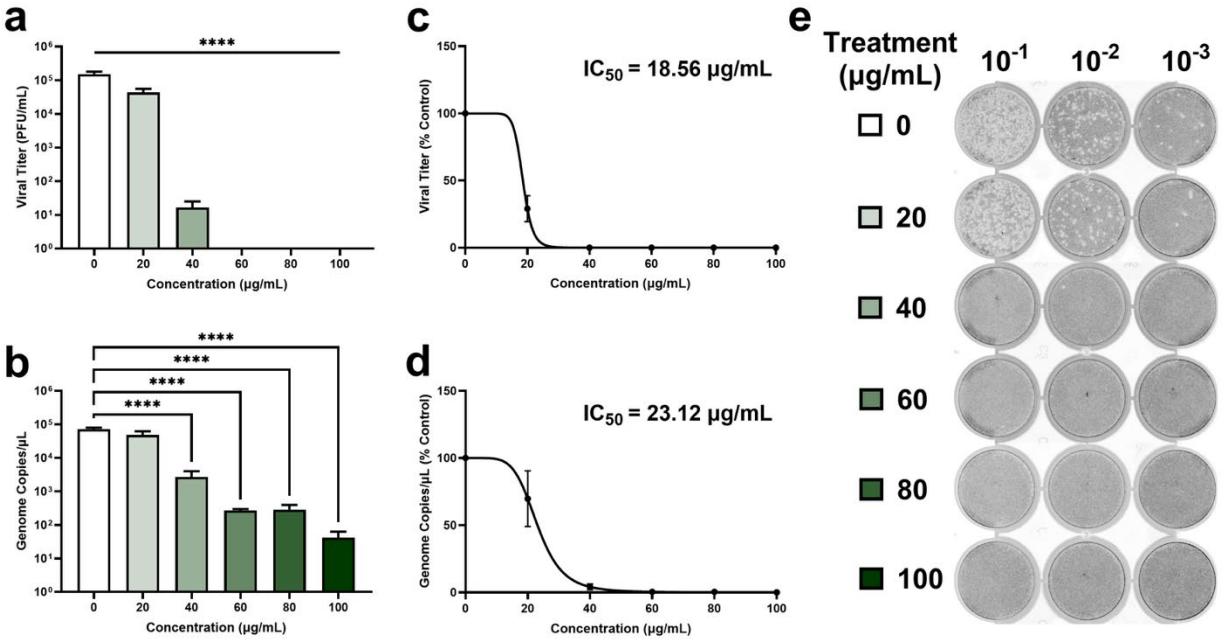

Figure 5: Treatment of HFF-1 cells with RE after infection inhibits ZIKV replication. (a) HFF-1 cells were infected with ZIKV (MOI = 1) for 1 h and subsequently treated with increasing concentrations of RE or a vehicle control (DMSO) for the entire post-infection period. Cell-free supernatants were collected 0 and 96 h post-infection and viral titers were quantified by plaque assay. Data are expressed as mean PFU/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A two-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in viral titers following RE treatment. ****$p<0.0001$ relative to HFF-1 cells treated with DMSO (0 µg/mL). (b) Viral RNA was extracted from cell-free supernatants collected 96 h post-infection and quantified by RT-qPCR. Data are expressed as mean genome copies/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A one-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in genome copy numbers following RE treatment. ****$p<0.0001$ relative to HFF-1 cells treated with DMSO (0 µg/mL). (c,d) IC_{50} values were calculated by non-linear regression analysis (inhibitor vs normalized response – variable slope). (e) Plaque assay images were taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

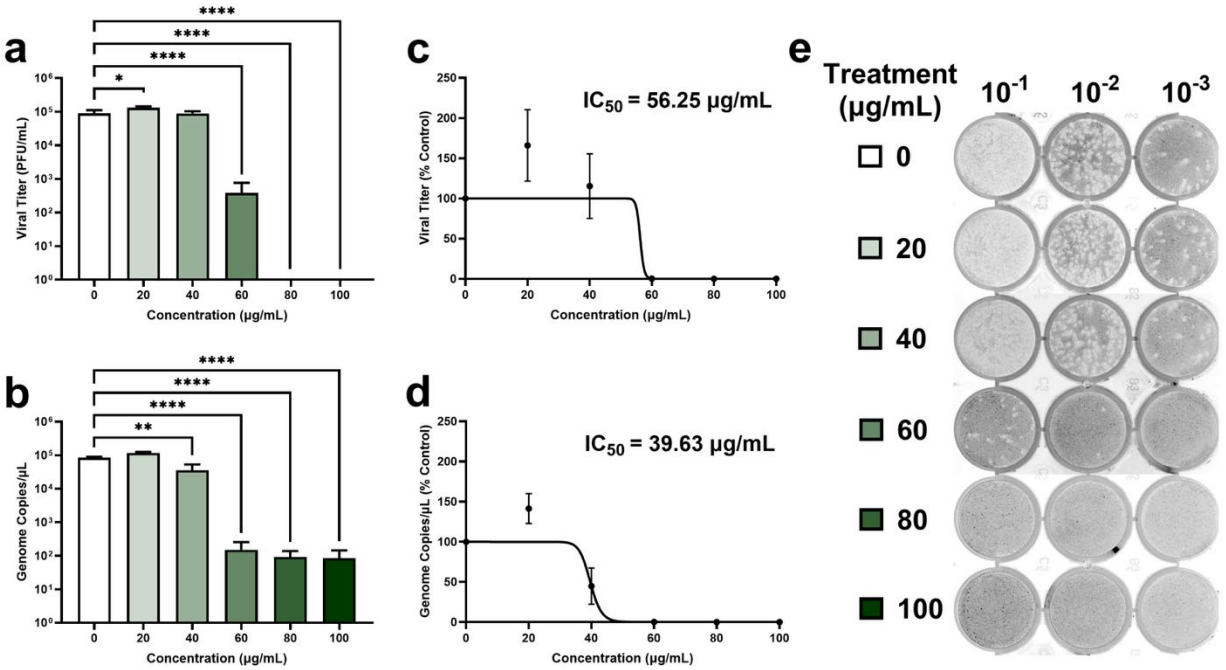

Figure 6: RE exhibits virucidal activity against ZIKV in HFF-1 cells. (a) ZIKV was incubated with increasing concentrations of RE or an equal volume of a vehicle control (DMSO) for 1 h. Subsequently, HFF-1 cells were infected with each sample (MOI = 1) and cell-free supernatants were collected 0 and 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 performed in duplicate. A two-way ANOVA and Dunnett's multiple comparisons test 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 (0 µg/mL). (b) Viral RNA was extracted from cell-free supernatants collected 96 h post-infection and quantified by RT-qPCR. Data are expressed as mean genome copies/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A one-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in genome copy numbers following RE treatment. **$p<0.01$, ****$p<0.0001$ relative to HFF-1 cells treated with DMSO (0 µg/mL). (c,d) IC_{50} values were calculated by non-linear regression analysis (inhibitor vs normalized response – variable slope). (e) Plaque assay images were taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

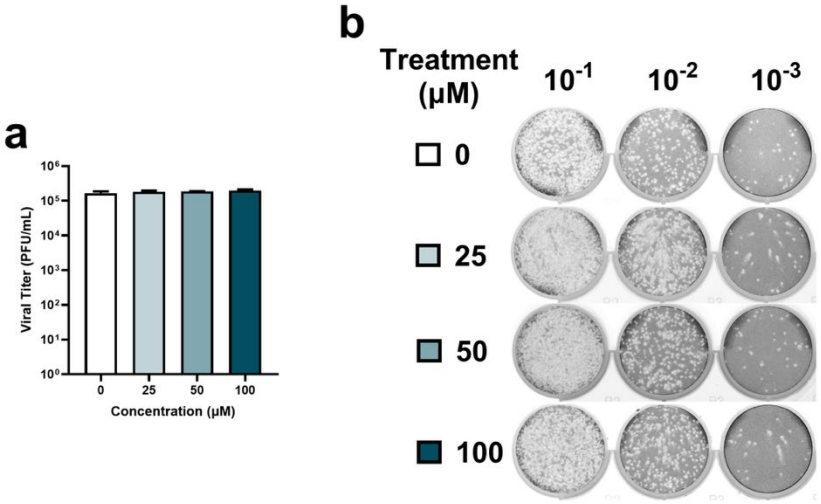

Figure 7: RA has no effect on ZIKV infection. (a) The virucidal activity of RA was evaluated by plaque reduction assay. ZIKV (290,000 PFU) was incubated with increasing concentrations of RA or an equal volume of a vehicle control (DMSO) for 1 h. Viral titers in each sample were then determined by plaque assay. Data are expressed as mean PFU/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A one-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in viral titers following RA treatment relative to DMSO (0 µg/mL). (b) Plaque assay images were taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

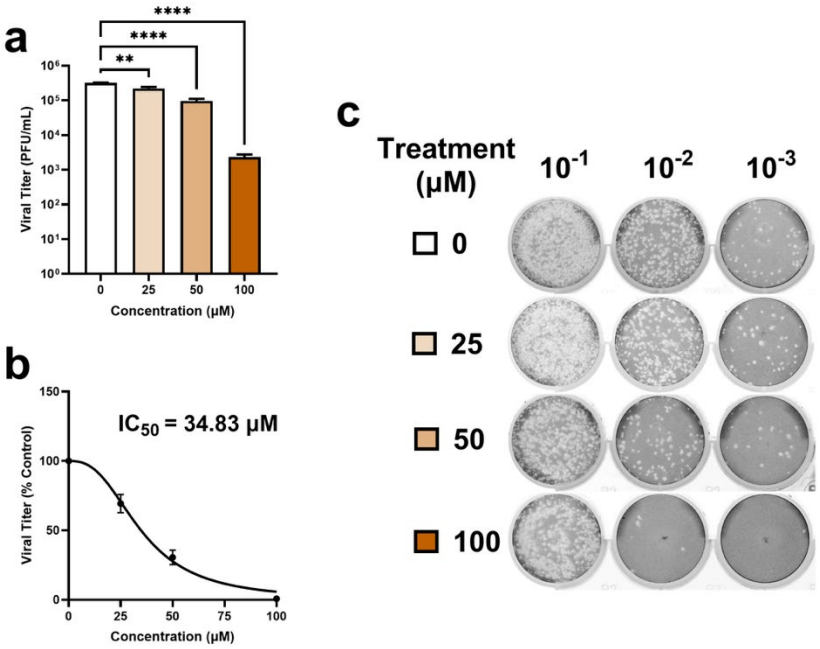

Figure 8: CA inhibits ZIKV infection in a dose-dependent manner. (a) The virucidal activity of CA was evaluated by plaque reduction assay. ZIKV (290,000 PFU) was incubated with increasing concentrations of CA or an equal volume of a vehicle control (DMSO) for 1 h. Viral titers in each sample were then determined by plaque assay. Data are expressed as mean PFU/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A one-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in viral titers following CA treatment. **$p<0.01$, ****$p<0.0001$ relative to ZIKV incubated with DMSO (0 µg/mL). (b) The IC_{50} value was calculated by non-linear regression analysis (inhibitor vs normalized response – variable slope). (c) Plaque assay images were taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

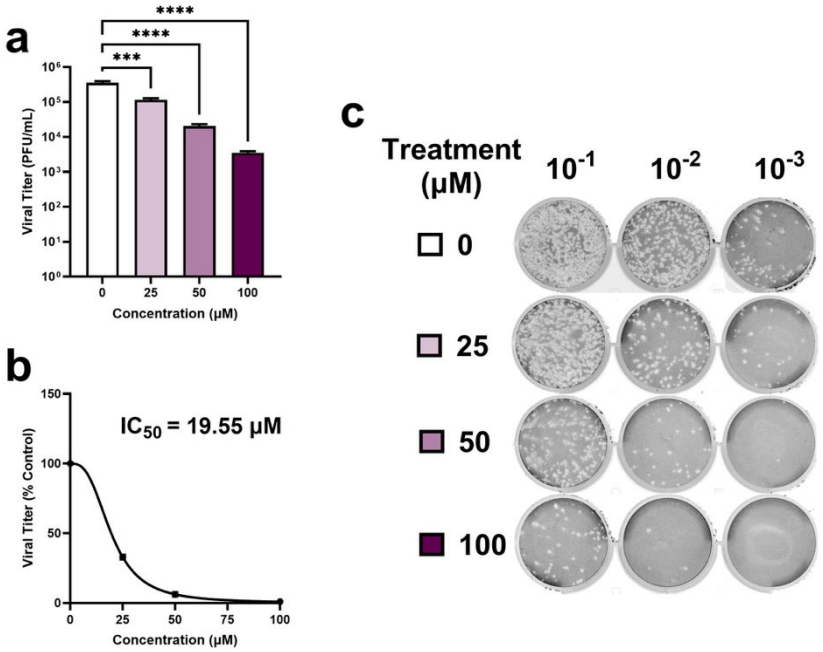

Figure 9: CO inhibits ZIKV infection in a dose-dependent manner. (a) The virucidal activity of CO was evaluated by plaque reduction assay. ZIKV (290,000 PFU) was incubated with increasing concentrations of CO or an equal volume of a vehicle control (DMSO) for 1 h. Viral titers in each sample were then determined by plaque assay. Data are expressed as mean PFU/mL ± SEM for n = 3 independent experiments, each performed in duplicate. A one-way ANOVA and Dunnett's multiple comparisons test were used to determine differences in viral titers following CO treatment. ***$p<0.001$, ****$p<0.0001$ relative to ZIKV incubated with DMSO (0 μg/mL). (b) The IC_{50} value was calculated by non-linear regression analysis (inhibitor vs normalized response – variable slope). (c) Plaque assay images were taken using an Amersham Typhoon Biomolecular Imager and represent wells from one independent experiment in its entirety.

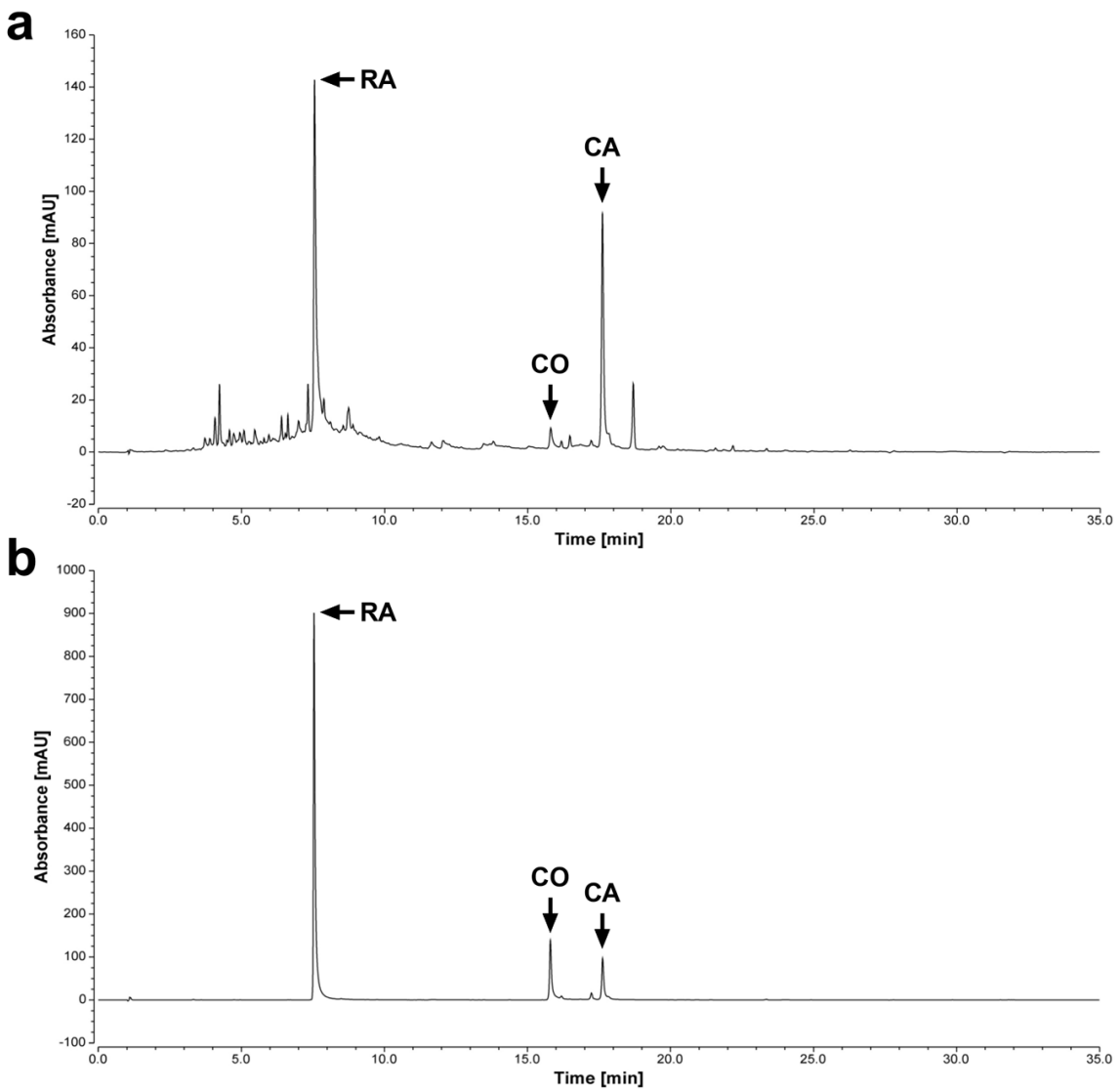

Figure 10: Separation and identification of major polyphenols in RE. Representative HPLC chromatograms of (a) RE and (b) a standard mixture of CA, CO, and RA. HPLC analysis was performed using a Kinetex C18 column and chromatograms were recorded at 284 nm. Peaks were identified by comparing retention times with authentic standards.

Table 1: Quantification of major polyphenols in RE. HPLC analysis was performed using a Kinetex C18 column and detection at 284 nm. Concentrations of CA, CO, and RA were determined using the external standardization method with authentic standards. Data are expressed as mean ± SEM for n = 3 independent experiments.

Compound	Molecular weight (g/mol)	Retention time (min)	Concentration (µg/mg of RE)	Concentration (µM) in 1 mg/mL of RE	Concentration (µM) in 25 µg/mL of RE
CA	332.42	17.62 ± 0.01	78.41 ± 4.64	235.86 ± 13.96	5.90 ± 0.35
CO	330.42	15.82 ± 0.01	11.96 ± 0.32	36.20 ± 0.97	0.91 ± 0.02
RA	360.32	7.57 ± 0.01	27.69 ± 0.23	76.85 ± 0.64	1.92 ± 0.02

506 **Discussion**

507 Although cases of ZIKV have significantly declined since reaching their peak in 2016,
508 the virus continues to circulate at low levels in tropical and subtropical regions, posing an
509 ongoing threat to human health[15]. Predictive models estimate that by 2050, 1.3 billion people
510 may be at risk of ZIKV infection, as rising global temperatures continue to expand the
511 distribution of competent vectors and create conditions suitable for ZIKV transmission[47]. Despite
512 extensive efforts by the global scientific community, there are no vaccines or antiviral treatments
513 against ZIKV currently available, leaving us unprepared for potential future outbreaks[3]. This
514 study aimed to evaluate the antiviral potential of RE against ZIKV in human dermal fibroblasts,
515 one of the earliest cellular targets of infection. Here, we provide novel evidence demonstrating
516 RE exerts potent *in vitro* antiviral effects against ZIKV. RE was found to significantly impair
517 ZIKV infection in Vero cells when the virus was treated before infection (Fig. 2). To assess the
518 effects of RE on the ZIKV replication cycle, HFF-1 cells were treated before, during, and after
519 infection or ZIKV was treated before infection. No antiviral activity was observed when HFF-1
520 cells were treated before (Fig. 3) or during infection (Fig. 4), however, ZIKV replication
521 unexpectedly increased in the latter condition. There was a significant decrease in viral titers and
522 genome copies when ZIKV was pre-treated with RE, indicating RE may target the initial stages
523 of the viral replication cycle in HFF-1 cells (Fig. 6). RE was most effective when HFF-1 cells
524 were treated post-infection, indicating RE may also target the later stages of the viral replication
525 cycle (Fig. 5). Of the major compounds tested, CA (Fig. 8) and CO (Fig. 9), but not RA (Fig. 7),
526 possess antiviral activity against ZIKV. To the best of our knowledge, this is the first study
527 describing the antiviral activity of RE in the context of flavivirus infection, specifically ZIKV.

528 To determine the antiviral potential of RE against ZIKV, we employed a plaque reduction
529 assay. This approach is commonly used in the literature[36,48–50] to screen different compounds for

530 antiviral activity and involves directly treating the virus before infecting a susceptible cell line,
531 such as Vero. Here, we observed a significant dose-dependent decrease in viral replication with
532 all concentrations of RE, with the lowest concentration causing a 52.77% reduction in viral titers
533 (Fig. 2, IC_{50} = 25.06 µg/mL). Although very few studies have investigated the antiviral effects of
534 RE, comparable results have been shown against HSV-1[30,32] and HSV-2[30] in Vero cells.
535 However, Nolkemper et al. reported IC_{50} values of 0.646 µg/mL against HSV-1 and 1.055
536 µg/mL against HSV-2, which are much lower than those observed in the current study[30]. This
537 discrepancy can be potentially attributed to differences between the viruses and procedures used.
538 For example, Nolkemper et al. pre-treated 200 PFU of HSV before adding the entire inoculum to
539 RC-37 cells (African green monkey kidney cells), whereas we pre-treated 290,000 PFU of ZIKV
540 and infected Vero cells with serially diluted inoculum (10^{-1} to 10^{-3}). Furthermore, variations in
541 extraction solvents (water vs. methanol in our study) can substantially influence the chemical
542 composition and corresponding bioactivity of RE[51]. Overall, our results are in general agreement
543 with the literature, as several classes of natural products with anti-ZIKV activity exert a virucidal
544 effect by directly interacting with virus particles or exhibit biological effects during viral
545 adsorption and internalization[22].
546 To identify which stage(s) of the ZIKV replication cycle are affected by RE, we
547 performed four experiments using HFF-1 cells: 1) treatment of cells before infection, 2)
548 simultaneous treatment and infection of cells, 3) treatment of cells after infection, and 4)
549 treatment of ZIKV before infection. Fibroblasts are among the first cells that encounter ZIKV at
550 the site of inoculation, therefore, HFF-1 cells were selected as a relevant infection model. Results
551 show that treating cells before or during the adsorption phase does not impair ZIKV replication
552 (Fig. 3 and 4). Nolkemper et al. found similar results, with no effect on HSV-2 and a minimal

553 24% reduction in HSV-1 replication when RC-37 cells were treated with a non-cytotoxic
554 concentration of RE before infection. However, treatment of RC-37 cells during infection caused
555 a 36% and 67% reduction in HSV-1 and HSV-2 replication, respectively[30]. In another study, 20
556 µg/mL RE caused a 66-fold reduction in hRSV production in A549 cells treated before infection,
557 conflicting with our results, though a different virus and cell type[29]. Moreover, Lin *et al.*
558 demonstrated a significant 21.9% reduction in viral VP1 mRNA and an 88.6% reduction in
559 EV71 replication in human rhabdomyosarcoma (RD) cells treated with 10 µM of RA, one of the
560 main constituents found in RE, during infection. Similar to our findings, RA minimally inhibited
561 EV71 by 5.54% in RD cells treated before infection[52]. Surprisingly, we observed a statistically
562 significant increase in ZIKV replication in cells treated during infection with every concentration
563 of RE (Fig. 4), as well as in cells infected with ZIKV pre-treated with 20 µg/mL (Fig. 5). As far
564 as we know, this effect has not been reported in the literature. Considering that pH can have a
565 significant impact on ZIKV infectivity[53], we measured the pH of various concentrations of RE
566 prepared in DMEM. No major changes in pH were observed in comparison to DMSO, with all
567 values falling within a range of approximately 8.2 to 8.5 (data not shown). Therefore, the
568 increase in virus production may have occurred due to an experimental artefact, but further
569 investigation is required to determine if underlying mechanisms are at play.
570 Unlike the results above, RE was shown to exert potent antiviral activity when cells were
571 treated post-infection. There was a considerable 70.90% decrease in infectious virion production
572 (IC_{50} = 18.56 µg/mL, SI = 5.78) at the lowest concentration tested (20 µg/mL) and a 95.87%
573 decrease in genome copy numbers (IC_{50} = 23.12 µg/mL, SI = 4.64) at 40 µg/mL, with viral titers
574 falling below the detectable limit at 60 µg/mL and beyond (Fig. 5). This is in agreement with
575 Horvath et al., who found a 90.46% reduction in HSV-2 genome concentrations in Vero cells

576 treated with 0.47 mg/mL of RE after infection[33]. Similarly, Al-Megrin *et al.* reported that 30 and
577 40 µg/mL of RE reduces plaque formation of HSV-1 by 55% and HSV-2 by 65%, respectively,
578 when incorporated into the overlay[32]. In contrast to these findings, RE had no effect on HSV-1 or
579 HSV-2 plaque formation in RC-37 cells[30]. Of the main polyphenols found in RE, CA has been
580 shown to reduce HSV-2[33] and hRSV[29] genome concentrations in cells treated post-infection,
581 whereas EV71[52] mRNA and progeny virus production were reduced in RA treated cells, further
582 supporting our results. Based on an *in-silico* study, RE may interfere with the NS5 RNA-
583 dependent RNA polymerase (RdRp), which is essential for replicating the ZIKV genome. This is
584 due to inhibitory potential of CA (-6.8 kcal/mol), CO (-7.0 kcal/mol), RA (6.9 kcal/mol), and
585 several other compounds, which were predicted to have high binding energy against the RdRp,
586 although *in vitro* studies are required to validate these data[26]. Collectively, these data suggest that
587 RE may act on the later stages of the viral replication cycle, however, further research is
588 necessary to determine the underlying mechanisms, such as effects on gene expression or virion
589 assembly and release.

590 As seen with the plaque reduction assay, pre-treatment of ZIKV with RE significantly
591 inhibited infection in HFF-1 cells, resulting in a 99.70% reduction in progeny virions at a
592 concentration of 60 µg/mL and complete inhibition at 80 µg/mL (Fig. 6, IC_{50} = 56.25 µg/mL, SI
593 = 2.14). Viral RNA was also significantly reduced by 55.14%, 99.84%, 99.90%, and 99.91% at
594 concentrations of 40, 60, 80, and 100 µg/mL, respectively (IC_{50} = 39.63 µg/mL, SI = 3.03
595 µg/mL). Similar results were reported for HSV pre-treated with the maximum non-cytotoxic
596 concentration of RE (exact concentration not specified) in RC-37 cells, resulting in 97% and
597 99% reductions in HSV-1 and HSV-2, respectively[30]. Unlike our plaque reduction assay results,
598 no decrease in viral titers was observed when ZIKV was pre-treated with concentrations below

599 60 μg/mL, which may be due to the distinct species and tissue origin of each cell line, as well as
600 fundamental differences between the assays. For example, ZIKV was treated with RE for 1 h and
601 the infectivity of each sample was quantified by plaque reduction assay, whereas in the time-of-
602 addition analysis, plaque assay was used to quantify the release of infectious virions by HFF-1
603 cells after infection with pre-treated ZIKV. Since no antiviral effects were observed in HFF-1
604 cells treated during infection, these findings indicate that RE directly interacts with extracellular
605 ZIKV particles by potentially binding to viral proteins involved in mediating viral entry or
606 damaging virions, thereby impairing their ability to infect host cells.

607 To determine whether three major polyphenols in RE exert anti-ZIKV activity, each
608 compound was individually screened using a plaque reduction assay. Pre-treatment of ZIKV
609 with CA and CO significantly reduced ZIKV plaque formation in a dose-dependent manner, with
610 IC_{50} values of 34.83 and 19.55 μM, respectively (Fig. 8 and 9). Although both compounds are
611 phenolic diterpenoids bearing a catechol moiety, CA carries a carboxyl moiety, whereas CO
612 carries a lactone moiety[54]. These structural differences suggest that the lactone moiety may
613 contribute to the lower IC_{50} of CO. In comparison, RA had no effect on ZIKV infection at any
614 concentration tested (Fig. 7). This was somewhat unexpected, as RA is the only compound with
615 existing evidence of antiviral activity against flaviviruses. Pre-treatment of DENV with RA has
616 been shown to inhibit all four serotypes *in vitro*[36], as well as significantly reduce mortality, viral
617 mRNA, and proinflammatory cytokine levels in mice peritoneally treated with RA and infected
618 with a lethal dose of JEV[37]. There is also *in vitro* evidence demonstrating the efficacy of RA
619 against the influenza A virus H1N1[48], EV71[52,55], and hepatitis B virus[56], as well as *in vivo*
620 evidence against a lethal dose of EV71 in mice[57]. Based on the structural similarities among
621 flaviviruses, we anticipated that RA would exert antiviral activity against ZIKV. Its failure to do

622 so indicates that its effects may be specific to DENV and JEV. The antiviral activity of CA has
623 also been well characterized, as it was shown to be effective against HSV-2[33], hRSV[29], and
624 influenza A virus H1N1[48]. However, another study reported no effect against influenza A virus
625 H1N1[29]. Interestingly, we did not anticipate CO to have such a profound effect, as only two
626 groups have analyzed its antiviral properties with conflicting results[29,58]. HPLC analysis revealed
627 that the lowest concentration of RE tested by plaque reduction assay (25 µg/mL), which caused a
628 52.77% reduction in viral titers (Fig. 2), contained approximately 5.90 µM CA and 0.91 µM CO
629 (Table 1). These concentrations are substantially lower than the IC_{50} values determined for each
630 compound individually, suggesting that additional compounds within RE, or synergistic
631 interactions between compounds, underlie its antiviral activity and warrant further investigation.
632 There are several limitations worth considering. Many antiviral studies include a positive
633 control to validate assay performance and ensure the proper interpretation of results. Since no
634 clinically approved antiviral exists for ZIKV, we did not include a positive control, although a
635 repurposed drug with *in vitro* anti-ZIKV activity could theoretically serve this purpose.
636 Secondly, depending on soil characteristics, climate, light exposure, and other growing
637 conditions, the chemical composition of plant extracts may vary, limiting their reproducibility.
638 To address this, studies should report the composition of each extract, where possible, to
639 facilitate comparisons across the literature. Lastly, while *in vitro* studies provide valuable
640 insights and are necessary during the early stages of investigation, the observed effects may not
641 directly translate to humans. The human body consists of many diverse cell types interacting
642 within complex tissues and organ systems, and antiviral efficacy can be influenced by
643 metabolism, immune responses, bioavailability, and other factors. Therefore, it is important to
644 evaluate antivirals in small animal models, as these are more physiologically relevant. However,

645 a lack of immunocompetent animal models for ZIKV and other flaviviruses remains a challenge
646 that must be addressed moving forward.

647 As mosquitoes and flaviviruses continue to expand into new regions and increasingly
648 threaten human health, new antiviral agents are urgently needed. In this study, we demonstrate
649 that RE exhibits potent antiviral activity against ZIKV by directly interacting with virus particles
650 before infection in Vero and HFF-1 cells and interfering with post-entry processes of the ZIKV
651 replication cycle in HFF-1 cells. Screening three major polyphenols found in RE revealed that
652 CA and CO significantly reduce ZIKV infectivity and are promising candidates for follow-up
653 studies. Whether RE can also inhibit other medically important flaviviruses, such as DENV and
654 WNV, remains to be determined. Collectively, these findings provide novel evidence supporting
655 the antiviral potential of RE and its individual components, warranting further investigation into
656 their mechanisms of action and effects in additional cell and viral models.

Authorship Contributions

JTM, RWEC, JMC, EIP, and AJM conceived and designed the study. RWEC, JMC, and DD assisted with experimental planning. JTM performed the experiments with assistance from RWEC, NJH, and CAL. JTM and AJM analyzed the data and wrote the manuscript. All authors contributed to discussion and editing of the manuscript.

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Conflicts of interests and disclosures

The authors declare no conflicts of interest.

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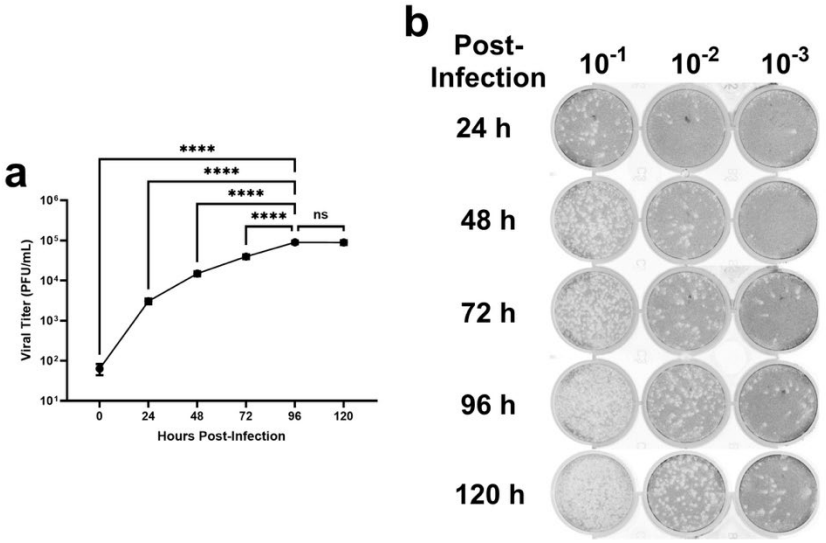
815 **Supplemental Material**



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