Materials and Methods

Cell culture and virus

Vero cells obtained from ATCC (Manassas, VA, USA) were grown in MEM media supplemented with 10% (v/v) FBS, 2 mM L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). All cell types were incubated in 5% CO_2 at 37 °C incubator. DENV2 serotype 2 (DENV-2) strain 16681 was propagated in the *Aedes albopictus* C6/36 cell line. The cells cultured in Leibowitz-15 culture medium supplemented with 1% FBS and 10% tryptose phosphate broth (TPB) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The virus was harvested after 5 days of infection and keep frozen until used.

Cell viability assay

To determine the toxicity of KERRA, the cell viability assay was performed in Vero after KERRA treatment. Briefly, the Vero cells were plated approximately 15,000 cells per well in 96-well format a day before experiment. The various concentrations of KERRA ranging from 0-1 mg/mL were treated to the cells. The cell viability was measured at 24, 48, 72 hours after treatment using PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The cell viability was analyzed as the percentage of cell viability (% cell viability) relative to that of non-treatment control as following equation.

% Cell viability = ([OD570-OD595] Test/[OD570-OD595] Control) x 100

The percentage of cell viability was used to calculate the half maximal cytotoxicity concentration (CC50) by using nonlinear-regression analysis of GraphPad version 10 (GraphPad Software, Inc., La Jolla, CA, USA).

Time of addition assay

The anti-viral activity and step of inhibition of KERRA to inhibit dengue virus infection (serotype 2 stain 16681) was determined using time of addition assay. Briefly, Vero cells were plated approximately 15,000 cells per well in 96-well format a day before experiment.

Dengue virus was prepared in MEM media (2% FBS) for 400 FFU/reaction. KERRA was prepared at the concentration of 31.25, 62.5, and 125 μg/mL. Three conditions were set up including (1) pre-treatment condition: KERRA was applied to the cells prior to infection, (2) co-treatment condition: KERRA was introduced simultaneously with viral inoculation and (3) post-treatment condition: KERRA was added after viral entry into the cells. The cells were harvested at 72 hours after virus infection and determined for the expression level of intracellular envelope (E) protein following the cell-based ELISA method.

Cell-based ELISA

The anti-viral activity of KERRA to inhibit dengue virus infection (serotype 2 stain 16681) was determined using cell-based ELISA assay. Vero cells were plated approximately 15,000 cells per well in 96-well format a day before experiment. The day of experiment, dengue virus was prepared in MEM media (2% FBS) and added to the wells (400 FFU/well). The plate was incubated for 2 hours to allow the virus to enter the cells. Then after, the sub-lethal doses of KERRA ranging from 0-250 μg/mL were treated to the cells. The amount of intracellular viral protein was measured at 72 hours post of infection. Briefly, the infected cells were fixed with 4% formaldehyde followed by permeabilized with 0.2% Triton X. The monoclonal antibody specific to dengue envelope protein (E protein), namely clone 4G2, were added to the cells. The cells were incubated at 4 °C overnight before adding the secondary antibody, HRP-conjugated rabbit anti-mouse IgG. The cells were washed three times with PBS containing 0.1% Tween-20 (0.1% PBST). The 3,3,5,5-tetramethylbenzidine (TMB) substrate (Invitrogen, Carlsbad, CA, USA) was added to the wells. The reaction was stop using 2N sulfuric acid after 5 min incubation. The absorbance was measured at wavelength 450 nm and used to calculate a percentage of E antigen (% E antigen) relative to infected cells without treatment (set as 100%) as following equation.

$$\% \text{ E antigen} = (OD450^{Test}/OD450^{Control}) \times 100$$

The % E antigen was used to calculate the half maximal efficiency concentration (EC50) by using nonlinear-regression analysis of GraphPad version 10 (GraphPad Software, Inc., La Jolla, CA, USA).

Immunofluorescence assay (IFA)

Vero cells were plated approximately 15,000 cells per well in 96-well format a day before experiment. The day of experiment, dengue virus was prepared in MEM media (2% FBS) and added to the wells (400 FFU/well). The plate was incubated for 2 hours to allow the virus to enter the cells. Then after, the sub-lethal doses of KERRA ranging from 0-250 μg/mL were treated to the cells. The number of infected cells was measured at 72 hours post of infection. The cells were fixed with 4% formaldehyde followed by permeabilized with 0.2% Triton X. The monoclonal antibody specific to dengue envelope protein (E protein), namely clone 4G2, were added to the cells. The cells were incubated at 4 °C overnight before adding the secondary antibody, Alexa488-conjugated rabbit anti-mouse IgG and Hoechst 333442 (Thermo Fisher Scientific, Waltham, MA, USA). The plate was added for 30 minutes in dark conditions at room temperature and washed three times. The stained cells were observed under a fluorescence microscope (Nikon Instruments, Inc., Melville, NY, USA).

Results

Toxicity of KERRA in Vero cells.

Cell viability assay was performed to determine the toxicity of KERRA in Vero cells. The cells were treated with 31.25, 62.5, 125, 250, 500, and 1,000 μg/mL of KERRA for 24, 48, and 72 hours. The reducing capacity of the living cells was measured representing the cell viability upon the treatment relative to non-treatment control (Figure 1A-C). The toxicity of KERRA was observed in dose dependent manner. Low dose treatment (31.25-250 μg/mL) showed non-toxic to the Vero cells (cell viability more that 85%) whereas the concentration

of 500 μg/mL and higher dose caused significant toxicity to the cells. KERRA obtained CC50 of 485.0 μg/mL (R squared = 0.9087) at 24 hours of incubation, 483.7 μg/mL (R squared = 0.8510) at 48 hours of incubation, 348.3 μg/mL (R squared = 0.9131) at 72 hours of incubation, judged by non-linear regression analysis (Figure 1D).

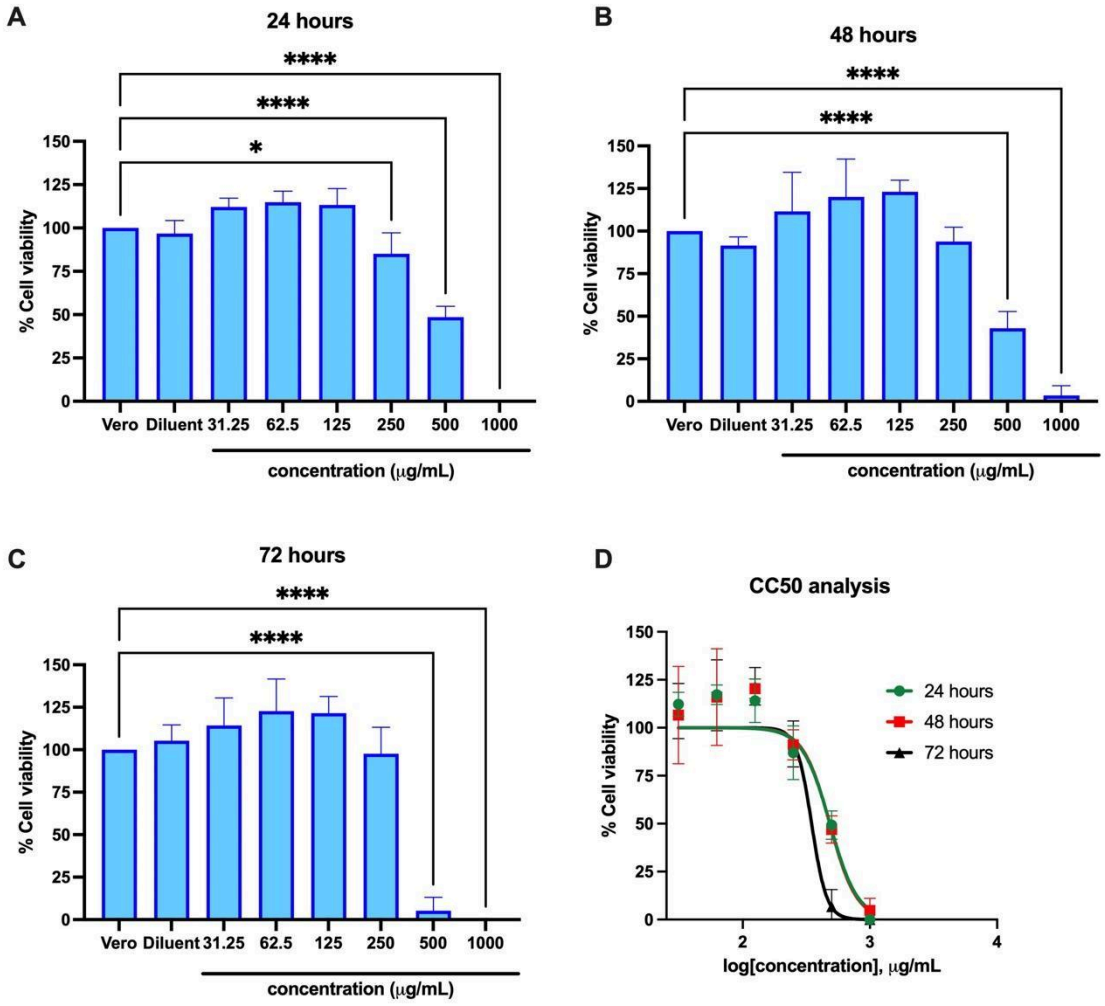

Figure 1 Cytotoxicity of KERRA in Vero cells. Vero cells were treated with KERRA at the concentration of 0, 31.25, 62.5, 125, 250, 500, 1,000 μg/mL. The cell viability was measured

at 24 hours (A), 48 hours (B), 72 hours (B) of treatment and represented as the percentage of cell viability relative to non-treatment control. The CC50 was calculated using non-linear regression of GraphPad version 10. Results are presented as mean ± SEM (error bars) from three independent experiments. Statistical significance was determined using One-Way ANOVA, with *$p < .05$, and ****$p < 0.00001$.

KERRA inhibited dengue virus infection at post-entry step.

Sub-lethal concentrations of KERRA were evaluated for their antiviral activity against dengue virus (DENV) serotype 2 strain 16681 infection. To elucidate its mechanism of action, a time-of-addition assay was performed. KERRA at concentrations of 31.25, 62.5, and 125 µg/mL was administered at different stages of infection including (1) *pre-treatment condition*: KERRA was applied to the cells prior to infection, (2) *co-treatment condition*: KERRA was introduced simultaneously with viral inoculation and (3) *post-treatment condition*: KERRA was added after viral entry into the cells. The antiviral effect of KERRA was assessed by quantifying the intracellular expression levels of the viral envelope (E) antigen under each condition (Figure 2). A significant reduction in E antigen expression was observed exclusively in the post-treatment condition, whereas no significant inhibitory effect was detected in the pre-treatment or co-treatment conditions. These findings suggest that KERRA exerts its antiviral activity post-entry due to it did not prevent viral attachment or internalization in pre-treatment and co-treatment condition.

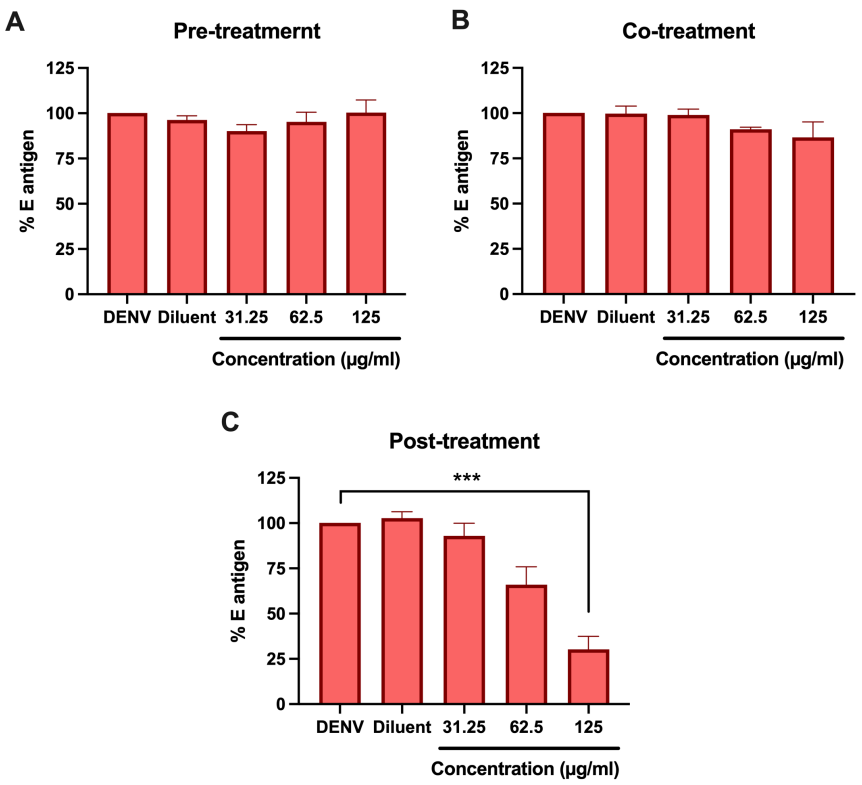

Figure 2 Anti-viral activity of KERRA to inhibit DENV infection in different steps of infection. Vero cells were infected with DENV. KERRA at the concentration of 31.25, 62.5, and 125 µg/mL was administered at different stages of infection including pre-treatment (A), co-treatment (B), and co-treatment condition (C). The infected cells were determined for % E antigen by using cell-based ELISA assay relative to that of infected cell without treatment (set as 100%). Results are presented as mean ± SEM (error bars) from three independent experiments. Statistical significance was determined using student's t-test, with ***p < 0.0001.

Efficiency of KERRA to inhibit DENV infection.

To determine the efficiency of KERRA on inhibiting DENV infection, various concentrations of KERRA was added to the Vero cells after virus infection (post-treatment condition). At 72 hours after infection, the expression level of intracellular viral envelope protein was determined using the cell-based ELISA assay. Unlike the neutralizing

monoclonal antibody which failed to inhibit dengue virus in the post treatment condition, the result showed that KERRA potentially decreased the envelope protein expression in dose-dependent manner. Treatment of the infected cells with 62.5, 125, and 250 μg/mL of KERRA reduced the % E antigen to 49.26%, 18.92%, and 0%, respectively (Figure 3A) with EC50 of 60.85 μg/mL (R squared = 0.9456) judged by non-linear regression analysis (Figure 3B).

An immunofluorescence assay was conducted to confirm the effect of KERRA on reducing the number of infected cells. The percentage of infected cells was assessed 72 hours post-infection and compared between conditions with and without KERRA treatment. Consistent with the results of the cell-based ELISA, KERRA treatment led to a dose-dependent reduction in the number of infected cells (Figure 4). Notably, significant reductions were observed at concentrations of 62.5, 125, and 250 μg/mL which correlated to the dramatical reduction of intracellular E antigen. Taken together, these data showed that KERRA effectively DENV replication.

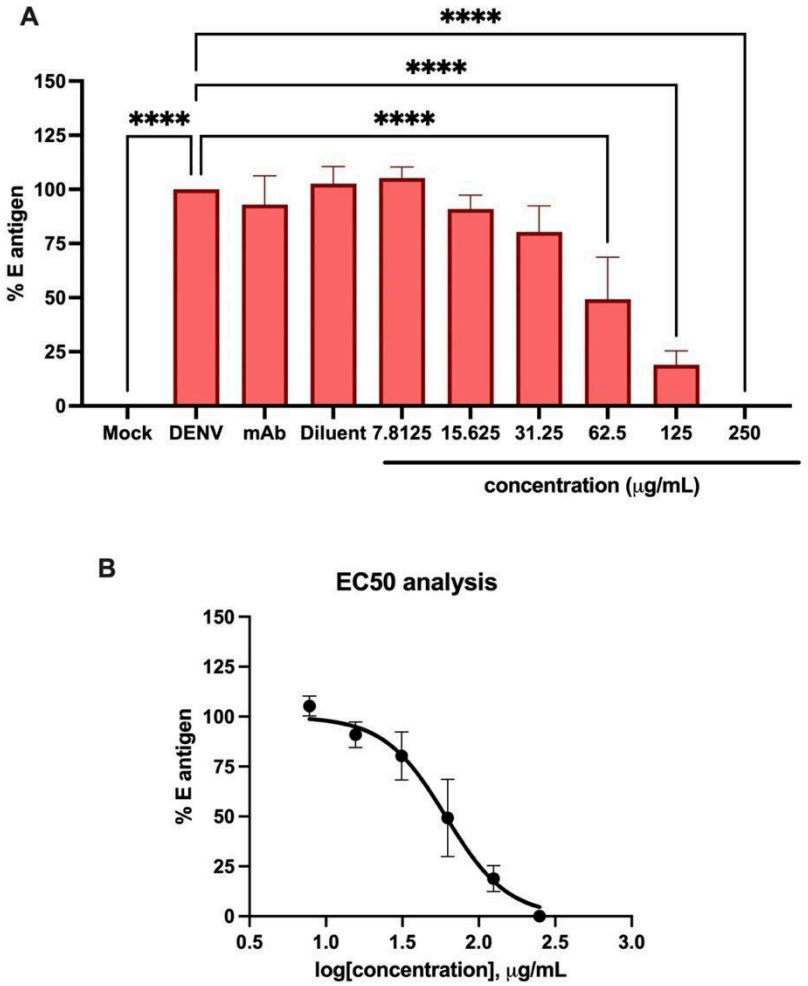

Figure 3 Anti-viral activity of KERRA to inhibit dengue virus infection. Vero cells were infected with DENV. KERRA at the concentration of 7.8125-250μg/mL were added to the cell after infection. The infected cells were harvested at 72 hours after infection and determined for %E antigen by using cell-based ELISA assay relative to that of infected cell without treatment (set as 100%) (A). The EC50 value was calculated using non-linear regression of GraphPad version 10. Results are presented as mean ± SEM (error bars) from three independent experiments. Statistical significance was determined using One-Way ANOVA, with ****$p < 0.00001$.

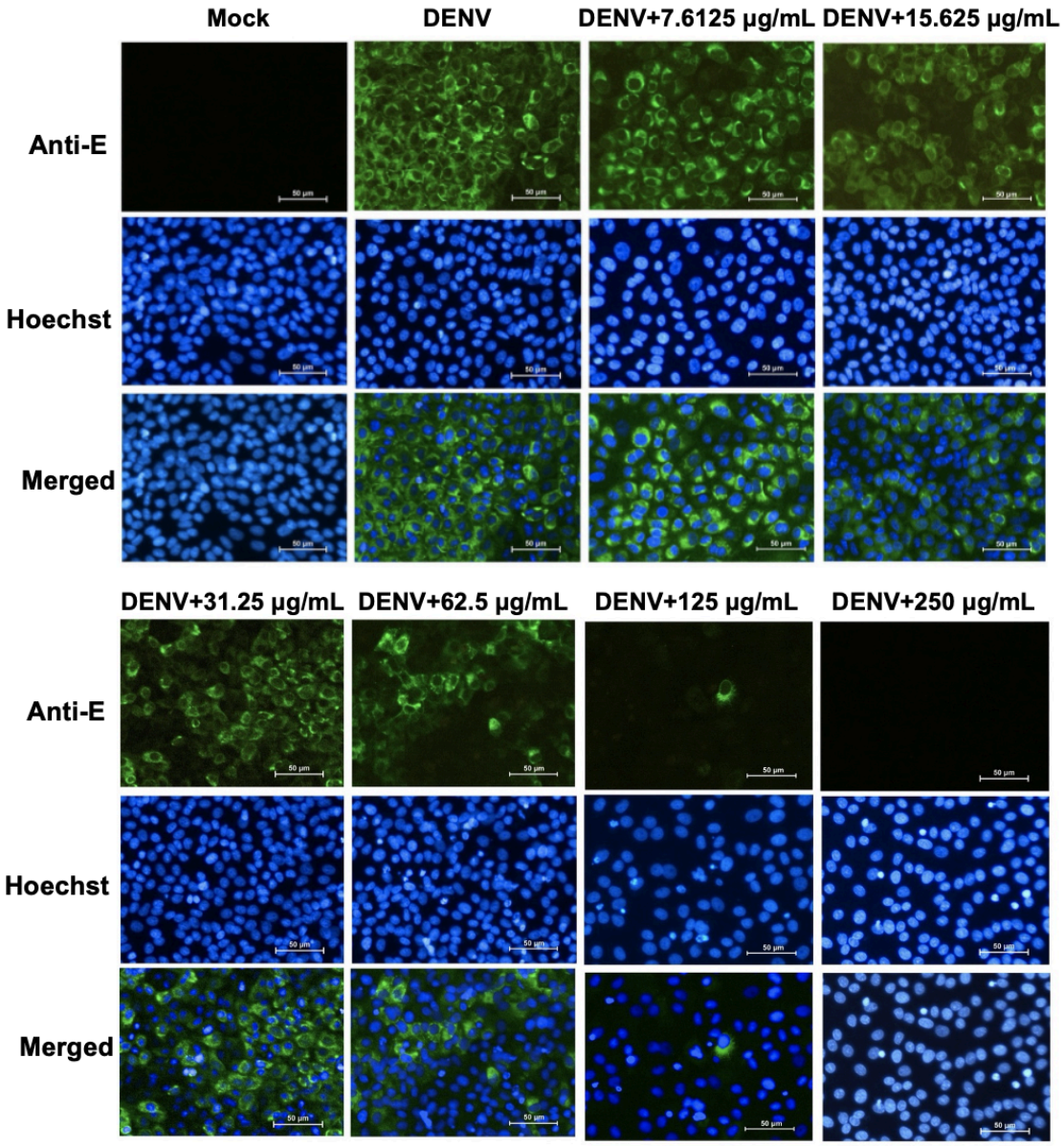

Figure 4 KERRA reduced the number of DENV infected cells. Vero cells were infected with DENV. KERRA at the concentration of 7.8125-250 µg/mL were added to the cell after infection. The number of infected cells was determined 72 hours post-infection using an E antigen-specific antibody (green). The total number of cells was visualized using Hoechst staining (blue). Scale bar was 50 micrometers.