



## Antiviral activity of selected flavonoids against Chikungunya virus



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### ABSTRACT

This study focuses on the antiviral activity of selected flavonoids against the Chikungunya virus (CHIKV), a mosquito-transmitted virus that can cause incapacitating arthritis in infected individuals. Based on the results of screening on Vero cells, the tested compounds were evaluated further with various assays, including cytotoxicity assay, virus yield assay by quantitative reverse transcription polymerase chain reaction (qRT-PCR), virus RNA replication assay with a CHIKV replicon cell line, Western blotting, and quantitative immunofluorescence assay. Baicalein, fisetin, and quercetagenin displayed potent inhibition of CHIKV infection, with 50% inhibitory concentrations [IC<sub>50</sub>] of 1.891 μg/ml (6.997 μM), 8.444 μg/ml (29.5 μM), and 13.85 μg/ml (43.52 μM), respectively, and with minimal cytotoxicity. The time-of-addition studies and various antiviral assays demonstrated that baicalein and quercetagenin mainly inhibited CHIKV binding to the Vero cells and displayed potent activity against extracellular CHIKV particles. The qRT-PCR, immunofluorescence assay, and Western blot analyses indicated that each of these flavonoids affects CHIKV RNA production and viral protein expression. These data provide the first evidence of the intracellular anti-CHIKV activity of baicalein, fisetin, and quercetagenin.

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### 1. Introduction

The Chikungunya virus (CHIKV; genus *Alphavirus*, family *Togaviridae*) is an enveloped virus with a positive-sense single-stranded RNA genome (Johnston and Peters, 1996). CHIKV is transmitted through the bites of its vectors, *Aedes aegypti* and *Ae. albopictus* (de Lamballerie et al., 2008). In addition to its known endemic areas, which include parts of Africa, islands in the Indian and Pacific Oceans, southern Europe, and Southeast Asia (CDC, 2015), the virus has emerged in other areas. The outbreaks that occurred in Réunion and its neighboring islands in 2005 were among the worst cases ever reported. They involved about one-third of the population and led to more than 250 deaths among the 785,000 inhabitants (Bonn, 2006).

Since then, sporadic epidemics have occurred. In October 2013, CHIKV cases were reported in Caribbean islands, from which the virus spread to South and Central America. Although the travel-related cases seem more applicable in explanations of the existence of CHIKV in United States, the risk of autochthonous cases cannot be dismissed because the activity of the vectors are greater with changes in climate and temperature. As a result of the rapid dispersion of CHIKV, it was projected that more than 1 million people would be infected by the end of 2014 (Higgs and Vanlandingham, 2015). Between the year of 2014 and 2016, more 2 million cases of chikungunya were reported in America and another states in the region (Alfaro-Tolozza et al., 2015; Rodriguez-Morales et al., 2015).

CHIKV has three different genotypes, which were named after their region of origin: West African, Asian, and East/Central/South African (Powers et al., 2000). The CHIKV genome with ~11.8 kb contains two open reading frames, one that encodes for nonstructural proteins, including nsP1, nsP2, nsP3, and nsP4, and

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one for structural proteins such as capsid and the envelope proteins (E1, E2, E3, and 6 k) (Tsetsarkin et al., 2011). Although CHIKV belongs to the Old World alphaviruses, which generally cause polyarthritides and rashes, its new and unique pathogenicity has crossed into the encephalitic group (Smith et al., 2009; Gerardin et al., 2008).

There is currently no specific approved antiviral agent or licensed vaccine to prevent CHIKV infection; the only available treatments are supportive, such as nonsteroidal anti-inflammatory drugs taken to alleviate arthralgia and myalgia (Gerardin et al., 2008). Ribavirin, as one of the approved broad-spectrum antiviral drugs, has been tested on patients with CHIKV infection and has led to improvements in joint pain, but further research should be conducted to confirm its effectiveness against CHIKV infection (Queyriaux et al., 2008; Ravichandran and Manian, 2008; Briolant et al., 2004). Many compounds such as arbidol, mycophenolic acid, harringtonine, and chloroquine have also shown antiviral activity against CHIKV *in vitro* and, more recently, *in vivo* (Delogu et al., 2011; Khan et al., 2011; Kaur et al., 2013; Shimizu et al., 1972). However, clinical trials have shown that chloroquine is ineffective against CHIKV infection (de Lamballerie et al., 2009).

Flavonoids are polyphenolic compounds that exist ubiquitously in plants as low-molecular-weight secondary metabolites in the form of aglycones, glycosides, and methylated derivatives (Tapas et al., 2008). Flavonoids are used in traditional Eastern medicine and have antioxidant, antitumor, antiproliferative, anti-inflammatory, and pro-apoptotic activities (Williams et al., 2004; Singh et al., 2008; Sung et al., 2007; Garcia-Mediavilla et al., 2007). Baicalein ( $C_{15}H_{10}O_5$ ; Fig. 1) is a flavone, a type of flavonoid, that is extracted from Huangchin plants (*Scutellaria baicalensis* and *Scutellaria lateriflora*). In 2012, Cotin and colleagues reported the potential of baicalein as an antiviral candidate to treat human

cytomegalovirus (HCMV) infection. Baicalein can reduce the expression of the HCMV immediate early gene (IE-1 protein) and cause total ablation of IE-2 expression (Cotin et al., 2012). We previously demonstrated the dynamic role of baicalein in the inhibition of different stages of dengue virus type-2 replication *in vitro* (Zandi et al., 2012).

Flavonols, fisetin ( $C_{15}H_{10}O_6$ ), and quercetagenin ( $C_{15}H_{10}O_8$ ) are variants based on the position of the substituents that are attached to the basic structure of the flavane nucleus, 2-phenyl-benzo[ $\alpha$ ]pyrane (Fig. 1). Their potential role as antiviral agents was described in previous reports. Fisetin has shown significant *in vitro* antiviral activity against the replication of dengue virus type-2 and enterovirus A71 (Zandi et al., 2011; Lin et al., 2012). Quercetagenin has potential antiviral activity against HCMV and the hepatitis C virus (Cotin et al., 2012; Ahmed-Belkacem et al., 2014). This study was performed to increase the chances of identifying a potent antiviral agent against CHIKV, preferably with low cytotoxicity *in vitro*. According to the results of various assays, baicalein, quercetagenin, and fisetin show potent intracellular inhibitory effects against the early stages of CHIKV replication.

## 2. Materials and methods

### 2.1. Virus and cells

The East/Central/South African genotype of CHIKV (accession number: MY/065/08/FN295485) was used in this study. The virus was isolated from the Johor's outbreak in 2008 and was propagated in BHK-21 cells (baby hamster kidney; ATCC CCL-10) maintained in Eagle's minimum essential medium (EMEM; Gibco, NY, USA) supplemented with 2% inactivated fetal bovine serum (FBS). The virus titration was based on the Reed-Muench endpoint calculation

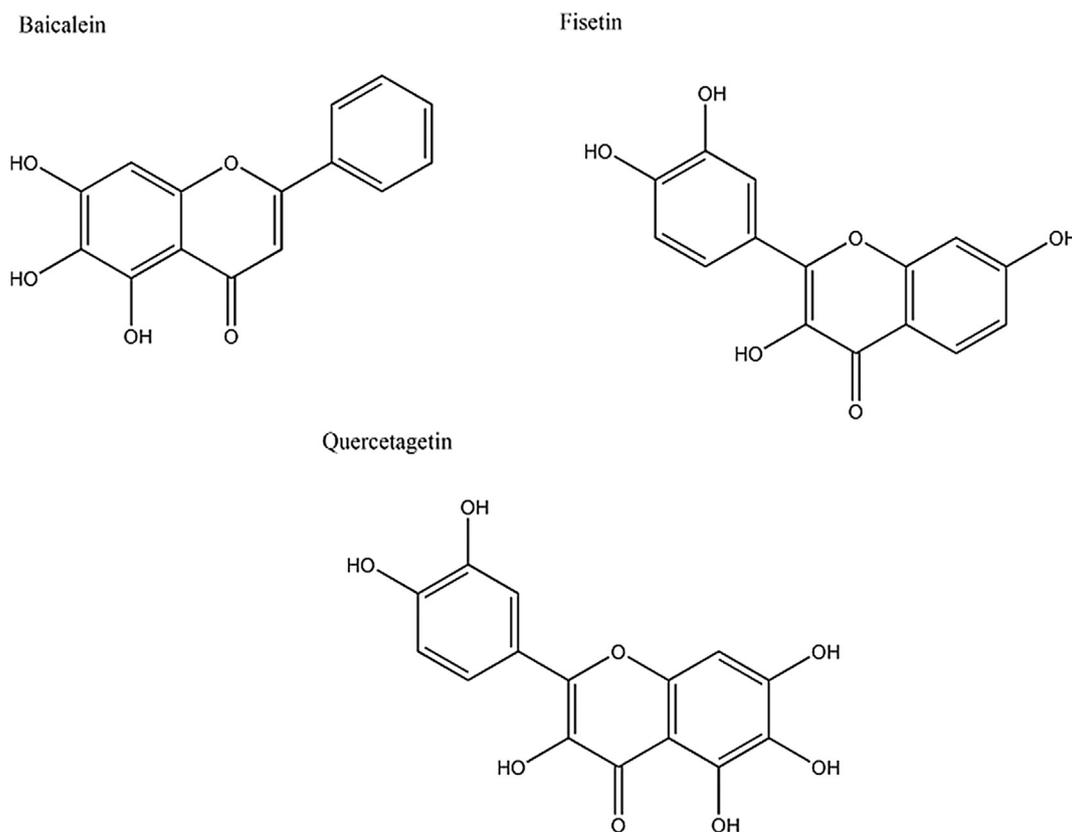


Fig. 1. The chemical structure of flavonoids.

method to determine the 50% infectious dose (TCID<sub>50</sub>) (Reed and Muench, 1938). Virus titration and all antiviral assays were performed with Vero cells (African Green monkey kidney; ATCC CCL-81) that were maintained under the same conditions as the BHK-21 cells.

The CHIKV replicon BHK-21 cell line, which contains RNA encoding CHIKV replicase complex proteins, puromycin acetyltransferase, enhanced green fluorescent protein, and *Renilla* luciferase (Rluc) markers as described previously (Pohjala et al., 2011), was used to screen the compounds of interest. The CHIKV replicon cell line was maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 8% FBS and 2% tryptose-broth phosphate. All cells were grown at 37 °C with 5% CO<sub>2</sub>.

## 2.2. Flavonoids

Baicalein and fisetin were purchased from Sigma-Aldrich (St. Louis, MO, USA), and quercetagenin was purchased from Indofine Chemical Co. (Hillsborough, NJ). The stock solutions for all compounds were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and filtered through a syringe filter with a 0.2- $\mu$ m pore size (Millipore, Billerica, MA) before they were diluted to the particular concentrations at the time of usage. The stocks were stored at -20 °C.

## 2.3. Nucleoside analogue

Ribavirin (Sigma-Aldrich) was used as a control compound with known anti-CHIKV activity (Briolant et al., 2004; Ravichandran and Manian, 2008).

## 2.4. Cytotoxicity study

The cytotoxicity of compounds was evaluated against Vero and BHK-21 cells with an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) assay kit (Promega, Madison, WI) according to the manufacturers' instructions. Briefly, a monolayer of cells was prepared in a 96-well cell culture plate. The cells were then treated with increasing concentrations of the compounds in triplicate, followed by 48-h incubation at 37 °C with 5% CO<sub>2</sub>. The MTS solution was then added, and the cells were incubated for 4 h at 37 °C with 5% CO<sub>2</sub>. The absorbance of each well was measured at a 495-nm wavelength with an Infinite 200 Pro multiplate reader (Tecan, Männedorf, Switzerland). The half maximal cytotoxic concentration (CC<sub>50</sub>) and the maximum non-toxic dose (MNTD) for each compound were determined with Graph Pad Prism 5 software (San Diego, CA).

## 2.5. Primary antiviral screening

As an antiviral screening assay using infectious CHIKV, a continuous treatment assay was performed to determine any plausible antiviral activity of a compound against any stage of the virus life cycle. This assay was modified and performed according to the method proposed by Moghaddam et al. (2014). In this assay, three compounds were screened for its antiviral activity. A monolayer of Vero cells was prepared in a 96-well cell tissue culture plate. The cells were then treated with increasing concentrations of each compound toward the MNTD concentration of each compound. The compounds were diluted in the working media, i.e., EMEM supplemented with 2% FBS. The vehicle control wells were treated with 0.1% DMSO, which was also diluted in the working media. The cells were concurrently infected with CHIKV with a multiplicity of infection [MOI] of 1, and the plate was then

incubated for 2 h at 37 °C with 5% CO<sub>2</sub>. After 2 h of incubation, the supernatant was removed from each well, and the newly prepared treatment with the same concentration as the previous stage was added into the respective wells.

The plate was then incubated for 48 h under the same conditions as previously described until the cytopathic effect (CPE) appeared in the virus control wells (i.e., the cells treated with CHIKV). The plate was examined daily for the presentation of the CPE, and after 2 d the score for CPE inhibition was determined by comparison with the observed CPE in the virus control wells. MTS assay was also performed to confirm the results. The tested compounds with significant inhibition against the in vitro replication of CHIKV in this phase were selected for further experimentation.

## 2.6. Anti-CHIKV assay using CHIKV replicon cell line

A CHIKV BHK-21 replicon cell line was used to evaluate the compounds for their activity against the CHIKV replicase complex. This assay was modified according to Pohjala et al. A monolayer of CHIKV replicon cells was prepared in a 96-well white plate (Corning Inc., Corning, NY) and treated with increasing concentrations of each compound of interest. The vehicle control wells were treated with 0.1% DMSO that was also diluted in the working media. After 48 h incubation at 37 °C with 5% CO<sub>2</sub>, the activity of the Rluc reporter, expressed by the CHIKV replicon, was detected with a *Renilla* luciferase assay kit (Promega) according to the manufacturer's protocols. The luminescence signal was then measured with GloMAX 20/20 Luminometer (Promega) and plotted against the log transformation of the concentrations of the compounds, and a sigmoidal curve fit with variable slope was created to obtain the half maximal inhibitory concentration (IC<sub>50</sub>) value for each compound.

## 2.7. Virus yield reduction assay using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

A qRT-PCR assay was used to evaluate the CHIKV yield during the antiviral studies. Briefly, the viral RNA was extracted from the supernatant of the infected cells, and the amplification of the 136 base region of the positive-strand nsP3 encoding sequence was performed as described by Chiam and colleagues (Chiam et al., 2013). The primers were nsP3-F (5'-GCG CGT AAG TCC AAG GGA AT-3') and nsP3-R (5'-AGC ATC CAG GTC TGA CGG G-3'). The cDNA was first generated from the RNA extracted from the supernatants (Qiagen, Hilden, Germany) of the previous assay plates, using nsP3-R primer and Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The unincorporated primers were then digested with 20U of Exonuclease I (New England Biolabs, Ipswich, MA). qRT-PCR was performed with a Step-OnePlus Real-Time PCR System with 2 $\times$  Power SYBR Green PCR Master Mix (Life Technologies) following the manufacturer's protocol and using serially diluted standards. The standards were prepared as referred to by Chiam et al. (Chiam et al., 2013). The cycling parameters were 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The amplified product was verified by melting curve analysis.

## 2.8. Time-of-addition assay

The time-of-addition study was performed to determine the optimal time of drug treatment to achieve inhibition of virus infection (Ravichandran and Manian, 2008; Daelemans et al., 2011). This assay was performed using the highest concentration of each compound but a lower concentration than the MNTD on CHIKV-infected Vero cells in 96-well plates. For the pre-infection assays,

the cell monolayers were treated with compounds for 2 h and 1 h before infection with CHIKV (MOI, 1). For the cotreatment assay (0 h), Vero cells were treated with each compound and at the same time of CHIKV inoculation to the cells. For pretreatment and cotreatment assays, infected cells were incubated in the absence of compounds. For the posttreatment assay, Vero cells were infected with CHIKV, and the compounds were added at 2, 3, 4, or 5 h after infection. For the virus control, CHIKV was added to the respective wells at 0 h. The negative controls contained only 0.1% DMSO diluted with the vehicle/working media. The plate was then incubated for 48 h at 37 °C with 5% CO<sub>2</sub> followed by virus yield reduction assay.

### 2.9. Binding assay

Binding assay was performed abased on appropriate modification of method used in Farshadpour et al., 2014. To analyze the effects of selected compounds on the attachment of CHIKV to the Vero cells, the confluent Vero monolayers in 24-well cell culture microplates were incubated with CHIKV (MOI, 1) in the presence or absence of increasing concentrations of the compounds for 1 h at 4 °C. The cells were then washed twice with sterile phosphate-buffered saline solution (PBS) and overlaid with EMEM containing 2% FBS in the absence of drugs. After 2 d of incubation at 37 °C, the anti-binding activity of the compounds was determined with virus yield reduction assay as described above.

### 2.10. Anti-entry assay

A monolayer of Vero cells was prepared in a 24-well plate and incubated with CHIKV (MOI, 1) at 4 °C for 1 h. After washing with PBS to remove the non-adsorbed virus, different concentrations of compounds were added to the respective wells before incubation for 1 h at 37 °C. The cells were washed with PBS and treated with citrate buffer (pH 3) to inactivate the absorbed but noninternalized viruses. The vehicle control wells were treated with 0.1% DMSO that was also diluted in the working media. EMEM supplemented with 2% inactivated FBS was then added to the wells, and the plate was incubated for 48 h at 37 °C with 5% CO<sub>2</sub>. The activity of the compounds against CHIKV internalization has been evaluated by virus yield reduction assay as described above.

### 2.11. Post-entry antiviral assay

This assay was modified according to Zandi et al. (2012). Monolayers of Vero cells were grown in 24-well cell culture plates. The Vero cells were then inoculated with CHIKV (MOI, 1) and incubated for 2 h at 37 °C with 5% CO<sub>2</sub> before treatment with increasing concentrations of compounds prepared in EMEM containing 2% FBS. The plate was incubated for 48 h at 37 °C with 5% CO<sub>2</sub>. After 2 d, the antiviral effect of each compound was determined with a virus yield reduction assay, as mentioned above. Since ribavirin antiviral activity was known to act intracellularly or particularly, in RNA replication, it was only used as a control for this antiviral assay.

### 2.12. Virus inactivation activity

Different concentrations of baicalein, fisetin, and quercetagenin were mixed with a CHIKV suspension with an MOI of 1 and incubated for 2 h at 37 °C. Vero cells were then infected with the diluted treated viral suspension to eliminate the potential effects of the compounds against virus adsorption. After 1 h adsorption at 37 °C, the cells were washed twice with PBS and overlaid by EMEM

containing 2% FBS, followed by incubation at 37 °C for 2 d with 5% CO<sub>2</sub>. The activity of each compound was then determined using a virus yield reduction assay, as mentioned above.

### 2.13. Detection of RNA replication of intermediate species

Monolayers of Vero cells were grown in 24-well cell culture plates. The Vero cells were then inoculated with CHIKV (MOI, 1) and incubated for 2 h at 37 °C with 5% CO<sub>2</sub> before treatment with increasing concentrations of compounds prepared in EMEM containing 2% FBS. The plate was incubated for 8 h at 37 °C with 5% CO<sub>2</sub>. After 8 h, the antiviral effect of each compound was determined with a virus yield reduction assay, as mentioned above. The intracellular RNA was extracted by using RNeasy Mini Kit (Qiagen, Hilden, Germany). Specific primers were used for negative-strand nsP3 assay as well as E1 positive-strand assay. The cDNA of negative-strand nsP3 was generated with Tag nsP3-F primer (CCTCCGCGGCCGTCATGGTGGCGAGCGCGTAAGTCCAAGGGAAT). The qRT-PCR for negative-strand nsP3 assay was performed by using Tag primer (CCTCCGCGGCCGTCATGGTGGCGA) and nsP3-R as reverse primer (AGCATCCAGGTCTGACGGG). The cDNA of E1 positive-strand assay was generated with CHIKV E1-R (ATC-GAATGCACCCGACACT). The qRT-PCR for E1 positive-strand assay was performed by using the same reverse primer with CHIKV E1-F as forward primer (TCGACGCGCCCTCTTAA).

### 2.14. Immunofluorescence assay

Prepared monolayers of Vero cells in 96-well plates were infected with CHIKV (MOI, 1) and incubated for 2 h at 37 °C before treatment with different concentrations of tested compounds. The vehicle control wells were treated with 0.1% DMSO that was also diluted in the working media. The CHIKV-infected cells treated with the compounds were then incubated for 24 h at 37 °C with 5% CO<sub>2</sub> and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were then washed three times with PBS before the addition of monoclonal rabbit anti-CHIKV E2 antibody (1:300) and incubated for 1 h at 37 °C. After 1 h, the plate was washed three times with PBS before the addition of anti-rabbit immunoglobulin G Fab2 (1:1000) conjugated with Alexa Fluor (RT) 488 (Cell Signaling Technology, Danvers, MA) as a secondary antibody and incubated for 1 h at 37 °C. The nuclei were stained with DAPI (Thermo Fisher Scientific, Waltham, MA) for 15 min at room temperature and then washed three times with PBS. The images were captured by a high-content screening system (Operetta; PerkinElmer, Waltham, MA), and the immunofluorescence signal was measured and analyzed with Harmony Software Version 3.5.1 (PerkinElmer).

### 2.15. Western blotting

A monolayer of Vero cells was prepared in a 75-cm<sup>2</sup> tissue culture flask and infected with CHIKV at an MOI of 1. After 2 h, the infected cells were treated with different concentrations of the selected compounds. The flasks were then incubated at 37 °C with 5% CO<sub>2</sub> for 2 d. The cells were then scraped, washed with PBS, and lysed using 1% Triton X100 (Sigma-Aldrich) containing complete protease inhibitor cocktail (Sigma-Aldrich) at 4 °C for 45 min. Cellular debris was pelleted out by centrifugation at 16,000 g for 5 min. A Micro BCA Protein Assay Kit (Thermo Fisher Scientific) was used to quantify the protein concentration for each sample. Lysate containing 100 µg of protein was denatured using sodium dodecyl sulfate-loading buffer, and the proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The gel was then equilibrated in Towbin buffer (0.025 M Tris, 0.192 M glycine, 20% methanol) for 10 min, and the proteins were transferred to a polyvinylidene fluoride membrane using the Bio-Rad wet transfer system (Hercules, CA). For detection of nsP1 and nsP3, the membranes were blocked with PBS 1% casein blocker (Bio Rad) for 1 h at room temperature on a shaker. The blots were rinsed three times with PBS Tween 20 before being incubated with primary anti-CHIKV nsP1, anti-CHIKV nsP3, or anti-CHIKV E2 rabbit polyclonal antibodies in 1% casein solution. The blots were then washed three times for 15 min each with PBS containing Tween 20, followed by incubation with the secondary goat anti-rabbit immunoglobulin G (Abcam, Cambridge, U.K.) antibodies conjugated with horseradish peroxidase for 1 h at room temperature on an orbital shaker. The membranes were washed three times for 15 min each with PBS containing Tween 20. For the loading control, separate blots containing the same samples were incubated in 1% casein with primary anti- $\beta$ -actin mouse monoclonal antibody conjugated with horseradish peroxidase (Cell Signaling Technology) for 1 h at room temperature on a shaker. The blots were then washed three times for 15 min each with PBS containing Tween 20. The membranes were developed by the colorimetric method using appropriate substrates (Thermo Fisher Scientific).

### 2.16. Statistical analyses

A one-way analysis of variance (ANOVA) test was conducted with Graph Pad Prism 5 software to evaluate the significance of the data from the antiviral assays. For the time-of-addition studies, the additional Dunnett's multiple comparison posttest was performed to determine the most effective time for the compound inhibitory activity against CHIKV.

## 3. Results

### 3.1. Cytotoxicity of compounds

The CC<sub>50</sub> and the MNTD of compounds on Vero cells and/or BHK-21 cells were determined by MTS assay. The CC<sub>50</sub> and the MNTD values of each compound are presented in Table 1. There was no observed cytotoxicity against the cells treated with 0.1% DMSO.

### 3.2. Primary screening

Non-cytotoxic concentrations of compounds were tested on CHIKV-infected Vero cells to identify effective compounds. A CPE inhibition assay (Fig. 2(a)) and MTS assay (Fig. 2(b)) were used at 48 hpi. It was determined that baicalein ( $P = 0.0052$ ), fisetin ( $P = 0.0052$ ), and quercetagenin ( $P = 0.0052$ ) were the only compounds that exerted significant inhibition against in vitro replication of CHIKV in a dose-dependent manner (Fig. 2). Since the compounds showed potential inhibitory activity against in vitro replication of CHIKV, it was decided to perform further evaluation using the specific antiviral assays.

### 3.3. CHIKV replicon cell line-based assay

The results for the antiviral activity of the tested compounds on the CHIKV replicon cell line showed that baicalein inhibited the *Rluc* activity with an IC<sub>50</sub> of 3.243  $\mu\text{g/ml}$  (12  $\mu\text{M}$ ). However, fisetin's IC<sub>50</sub> of 44.27  $\mu\text{g/ml}$  (154.66  $\mu\text{M}$ ) showed a moderate inhibitory effect against the CHIKV replicon because the IC<sub>50</sub> cannot be achieved due to cytotoxicity on BHK-21 cells. Treatment by quercetagenin showed inhibitory activity against CHIKV replicon, with an IC<sub>50</sub> of 13.53  $\mu\text{g/ml}$  (42.52  $\mu\text{M}$ ) (Fig. 3). Based on these data, baicalein is the most potent inhibitor of CHIKV RNA.

### 3.4. Time-of-addition assay

A time-of-addition assay was conducted to identify the optimum time for the compounds (all at 100  $\mu\text{g/ml}$ ) to exert their antiviral effects against CHIKV infection. The data shown in Fig. 4 suggest that the tested flavonoids probably act in the early stages of in vitro CHIKV replication. As shown in Fig. 4, fisetin exhibited potent anti-CHIKV activity even after 4 hpi. Based on the virus yield, it could be seen that baicalein and quercetagenin inhibited CHIKV efficiently only when added before infection or together with the virus; when they were added after infection, the activity was reduced, and neither compound exhibited significant anti-CHIKV activity when they were added to the infected cells 4 h after infection.

### 3.5. Virus inactivation

As shown in Fig. 5, baicalein ( $P = 0.0052$ ) and quercetagenin ( $P = 0.0064$ ) exerted potent dose-dependent activity against extracellular CHIKV particles as compared to fisetin. The IC<sub>50</sub> values were 52.71  $\mu\text{g/ml}$  (184.15  $\mu\text{M}$ ), 9.934  $\mu\text{g/ml}$  (31.21  $\mu\text{M}$ ), and 11.64  $\mu\text{g/ml}$  (for 43.07  $\mu\text{M}$ ) fisetin, quercetagenin, and baicalein, respectively, which shows that fisetin does not exert a significant effect against CHIKV extracellular particles.

### 3.6. Virus binding assay

As shown in Fig. 6, baicalein ( $P = 0.0051$ ) and quercetagenin ( $P = 0.0063$ ) exerted significant dose-dependent inhibition against the attachment of CHIKV to Vero cells. The IC<sub>50</sub> values were 28.04  $\mu\text{g/ml}$  (103.76  $\mu\text{M}$ ) and 8.050  $\mu\text{g/ml}$  (25.3  $\mu\text{M}$ ), respectively, for baicalein and quercetagenin. Fisetin did not exhibit a significant effect against CHIKV attachment and adsorption to the Vero cells.

### 3.7. Activity of compounds against CHIKV internalization

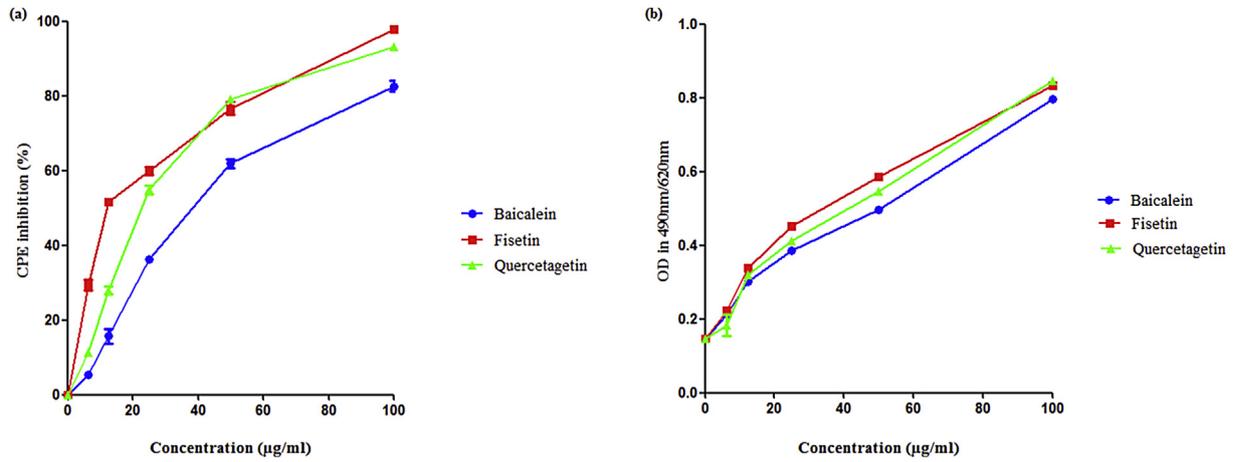
Data from the anti-entry activity assay showed that the tested compounds did not strongly interfere with CHIKV internalization to the Vero cells. However, moderate dose-dependent inhibition against CHIKV internalization could be seen when the compounds were added during virus internalization (Fig. 7).

**Table 1**

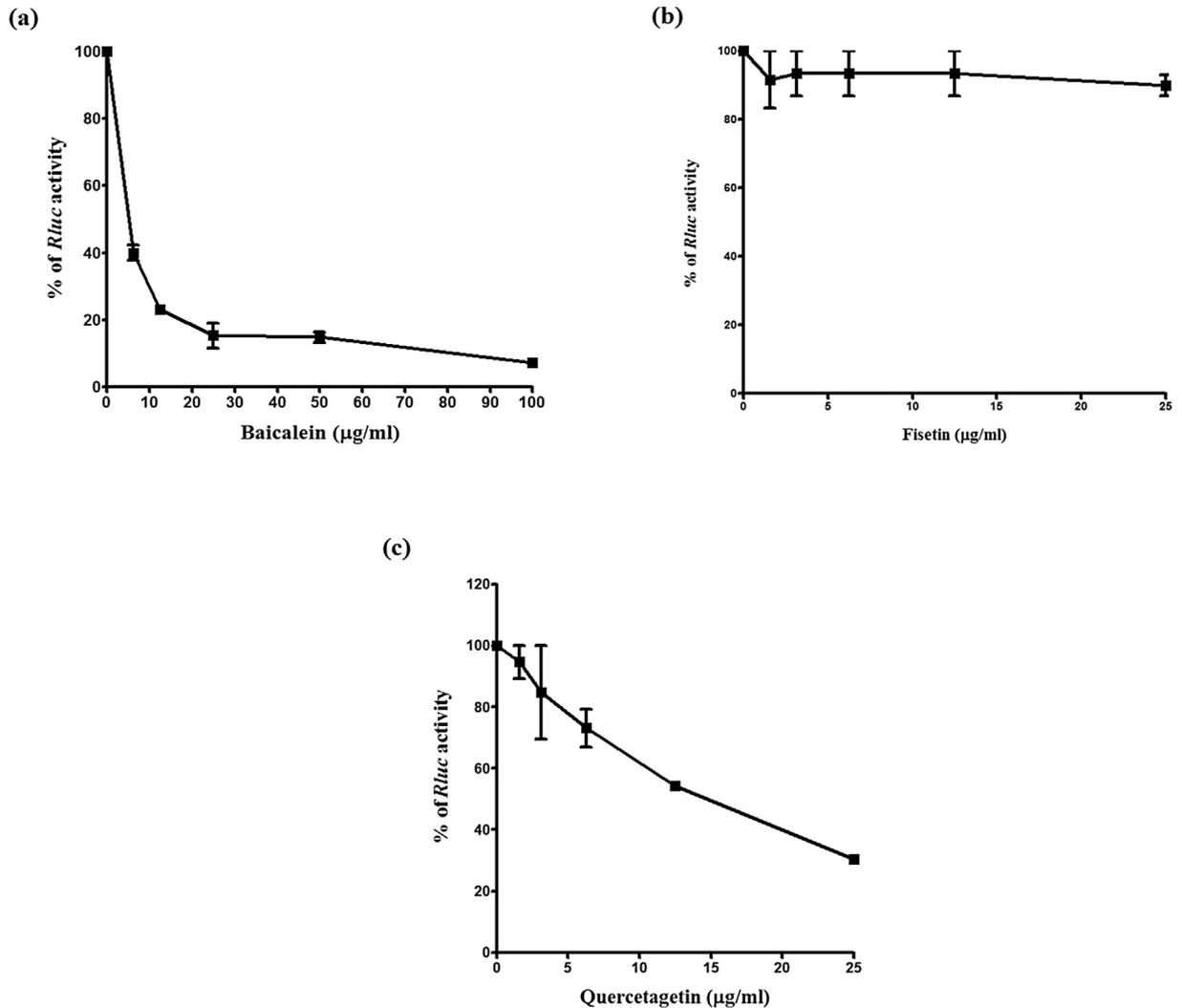
The CC<sub>50</sub> and MNTD values of the flavonoid compounds and nucleoside analogue on Vero cells and BHK-21 cells.

Flavonoids & nucleoside analogue	Vero cells		BHK-21 cells	
	CC <sub>50</sub> ( $\mu\text{g/ml}$ )	MNTD ( $\mu\text{g/ml}$ )	CC <sub>50</sub> ( $\mu\text{g/ml}$ )	MNTD ( $\mu\text{g/ml}$ )
Baicalein	356.3	183.2	474.5	197.2
Fisetin	194.4	>100	212.3	72.68
Quercetagenin	226.7	173.5	52.42	32.1
Ribavirin	>600	799.2	NA <sup>a</sup>	NA <sup>a</sup>

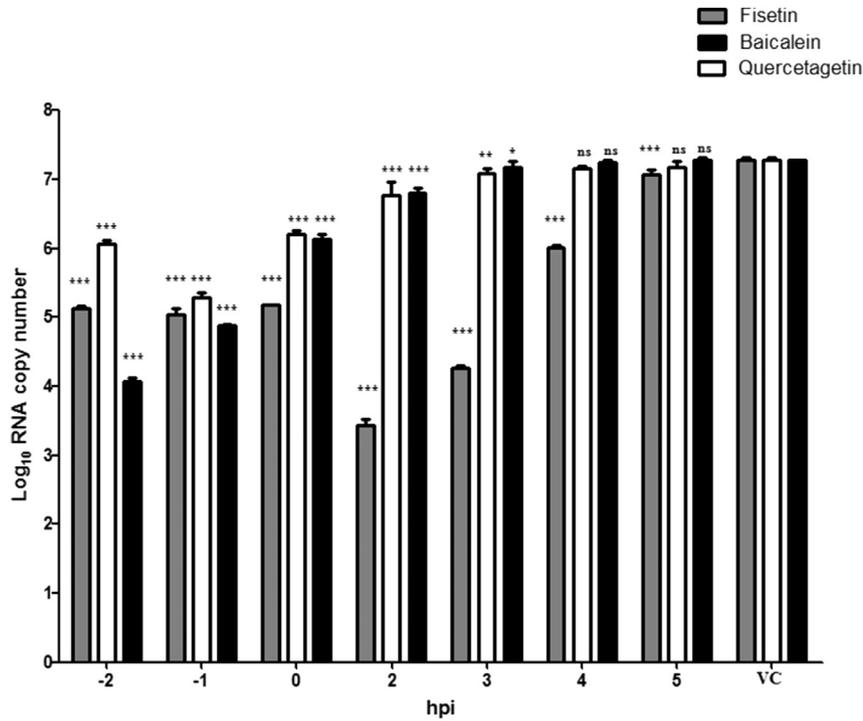
<sup>a</sup> NA = not applicable because it was not used in the CHIKV replicon cell line assay.



**Fig. 2.** (a) The percentage of inhibition assay result obtained from primary antiviral screening. The results are normalized to the respective values obtained from the non-treated CHIKV-infected control cells ("0" on the X-axis is referring to the non-treated positive CHIKV-infected controls). Statistical significance is analyzed from a one-way ANOVA (Kruskal-Wallis test),  $P < 0.05$ . Error bars represent standard errors of triplicate means. (b) Flavonoids increased survival of CHIKV-infected cells obtained through the MTS assay for primary antiviral screening. The results are normalized to the respective values obtained from the non-treated CHIKV-infected control cells ("0" on the X-axis is referring to the non-treated positive CHIKV-infected controls). Statistical significance is analyzed from a one-way ANOVA (Kruskal-Wallis test),  $P < 0.05$ . Error bars represent standard errors of triplicate means.



**Fig. 3.** Evaluation of the anti-CHIKV activity of flavonoid compounds by using the CHIKV replicon cell line. All of the three flavonoid compounds; baicalein in (a), fisetin in (b) and quercetagenin in (c), are able to reduce the percentage of *Rluc* activity produced by the CHIKV replicon in a dose-dependent manner. The *Rluc* activity was measured at 48 h post treatment. Vehicle-treated (0.1% DMSO) cells were used as control ("0" concentration). Data from triplicate assays were plotted and analyzed using one-way ANOVA (Kruskal-Wallis test), Graph Pad Prism Version 5, Graph Pad Software Inc., San Diego, CA),  $P < 0.05$ . Error bars represent standard errors of triplicate means.

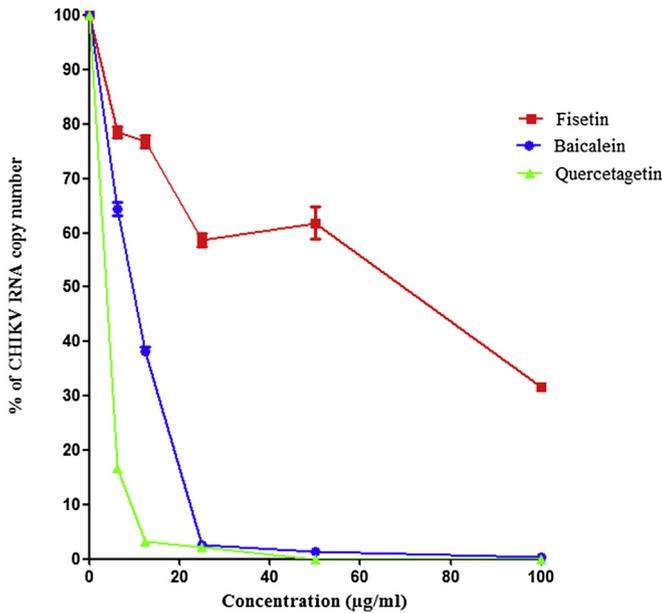


**Fig. 4.** Three flavonoids efficiently reduced the CHIKV RNA production at the early hour of the treatment. The RNA copy numbers of CHIKV reduced significantly at the early hour of baicalein (–2, –1, 0, 2 and 3 hpi), fisetin (all time of treatment) and quercetagetin (–2, –1, 0, 2 and 3 hpi) treatment in the time-of-addition assay. “VC” is referring to the non-treated CHIKV-infected controls. Statistical significance was analyzed from a one-way ANOVA and Dunnett’s multiple comparison posttest. \*\*\*,  $P < 0.0001$ . In and Dunnett’s multiple comparison posttest, each hour of treatment was compared to the CHIKV-positive control wells. “ns” is referring to not significant. Error bars represent standard errors of triplicate means.

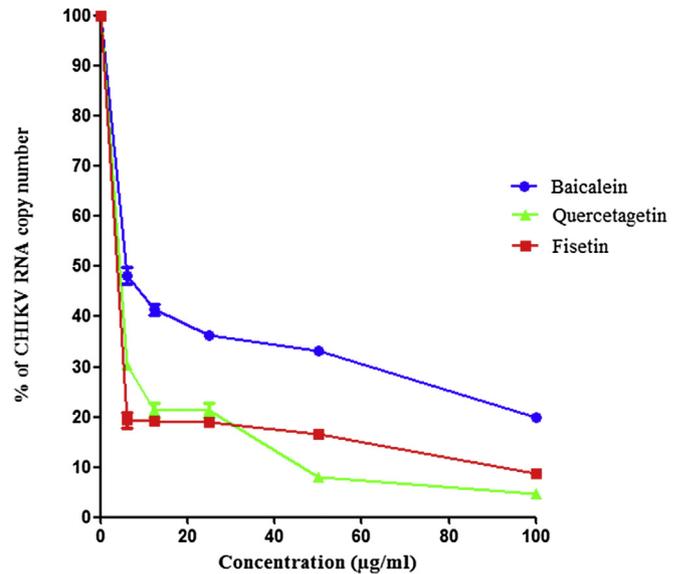
**3.8. Antiviral activity against post-entry stages of CHIKV replication**

As shown in Fig. 8, baicalein ( $P = 0.0058$ ), with an  $IC_{50}$  of 1.891  $\mu\text{g/ml}$  (6.997  $\mu\text{M}$ ) and an SI of 188.4 showed more potent intracellular anti-CHIKV activity than quercetagetin ( $P = 0.0081$ ;

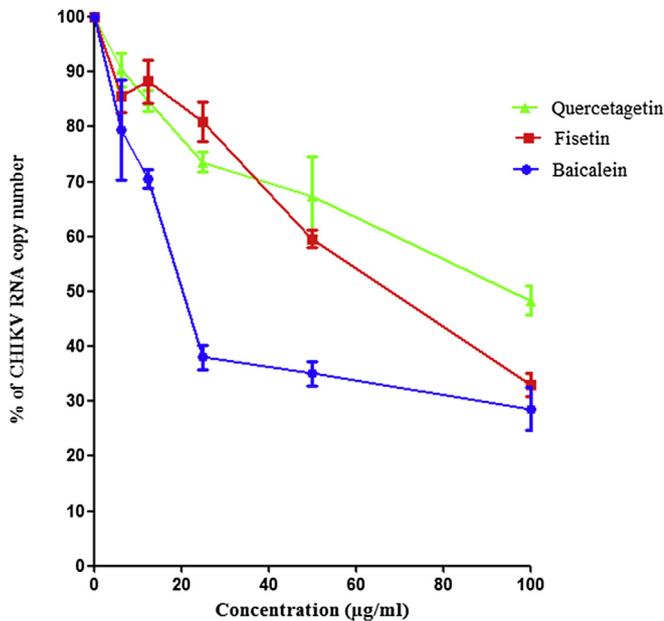
$IC_{50} = 13.85 \mu\text{g/ml}$  (43.52  $\mu\text{M}$ );  $SI = 16.3$ ) and fisetin ( $P = 0.0054$ ;  $IC_{50} = 8.444 \mu\text{g/ml}$  (29.5  $\mu\text{M}$ );  $SI = 23.02$ ). However, compared to the  $IC_{50}$  value of ribavirin as a positive control with defined anti-CHIKV activity (11.07  $\mu\text{g/ml}$ ; related  $SI = 54.2$ ), it could be concluded that the tested compounds exert stronger intracellular



**Fig. 5.** Baicalein and quercetagetin exhibit potent virucidal activity against CHIKV. Statistical analysis was performed by using one-way ANOVA (Kruskal-Wallis test) where  $P < 0.05$  is significant. (“0” on the X-axis is referring to the non-treated CHIKV-infected controls). Error bars represent standard errors of triplicate means.



**Fig. 6.** Baicalein, fisetin and quercetagetin possessed the anti-adsorption activity against CHIKV. Statistical analysis was performed by using one-way ANOVA (Kruskal-Wallis test) where  $P < 0.05$  is significant. (“0” on the X-axis is referring to the non-treated CHIKV-infected controls). Error bars represent standard errors of triplicate means.

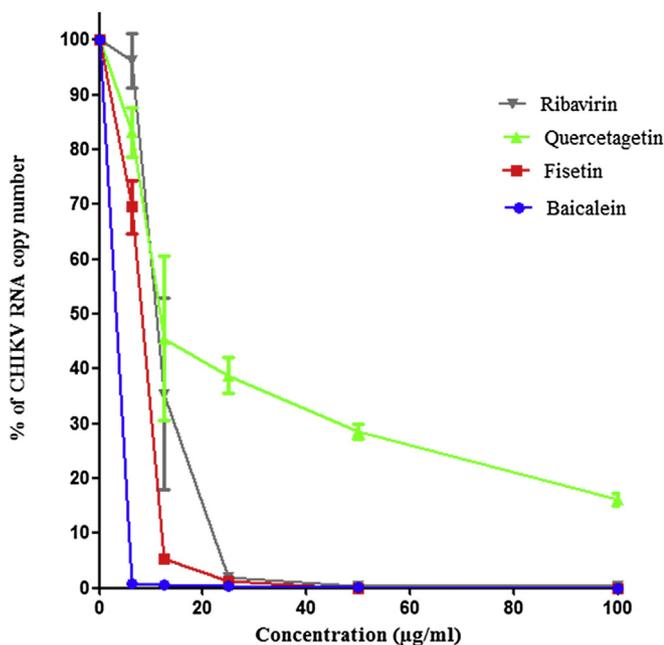


**Fig. 7.** Baicalein, fisetin and quercetagenin exhibit the antiviral activity at the entry stage of CHIKV infection. Statistical analysis was performed by using one-way ANOVA (Kruskal-Wallis test) where  $P < 0.05$  is significant. ("0" on the X-axis is referring to the non-treated CHIKV-infected controls). Error bars represent standard errors of triplicate means.

anti-CHIKV activity.

### 3.9. Detection of RNA replication intermediate species

Since the kinetics of active CHIKV RNA replication can be deduced from the result of negative-strand qRT-PCR, Fig. 9(b)



**Fig. 8.** Baicalein, fisetin and quercetagenin exhibit the antiviral activity at the post-entry stage of CHIKV infection. Statistical analysis was performed by using one-way ANOVA (Kruskal-Wallis test) where  $P < 0.05$  is significant. ("0" on the X-axis is referring to the non-treated CHIKV-infected controls). Error bars represent standard errors of triplicate means.

shows the yield of RNA through the assay regardless of the treatment with the compounds. However, we hypothesized the interference between converting the negative-strand RNA to positive-strand RNA as well as production of the envelope, since there is reduction in the RNA yield of positive-strand qRT-PCR (baicalein,  $P = 0.0060$ ; fisetin,  $P = 0.0047$ ; quercetagenin,  $P = 0.0061$ ) Fig. 9(a) and E1 qRT-PCR (baicalein,  $P = 0.0051$ ; fisetin,  $P = 0.0049$ ; quercetagenin,  $P = 0.0051$ ) Fig. 9 (c).

### 3.10. Immunoblot assay

To investigate the effects of the tested compounds on CHIKV protein synthesis, Western blot analyses were performed on CHIKV E2, nsP1, and nsP3 proteins. In addition, the anti-CHIKV E2 antibody used also detected the precursor of E2 (pE2), which contains E2 and E3 (Fig. 10(a) and (b)). A dose-dependent reduction of the CHIKV E2, nsP1, and nsP3 proteins was observed upon treatment with baicalein and fisetin. As shown in Fig. 10, baicalein was effective at 100 µg/ml, and fisetin was effective at concentrations as low as 12.5 µg/ml. Quercetagenin, however, did not show any reduction in CHIKV E2, nsP1, and nsP3 production; this finding might be a hint about the effects of quercetagenin during the later stages of the CHIKV replication cycle, which might be before the assembly of the virus particles and/or virus release. For the detection of CHIKV nsP1 and nsP3 proteins, a baicalein concentration of 100 µg/ml and a fisetin concentration as low as 12.5 µg/ml were comparable to the negative control. This finding would suggest that baicalein and fisetin might inhibit the production of CHIKV protein, especially the proteins that are involved in the negative-strand synthesis and part of the replicase unit.  $\beta$ -actin was used as a loading control in this experiment to ensure that the integrity of the cells are still intact and that the concentration of the flavonoids used in this experiment did not affect the synthesis and expression of the host cellular proteins.

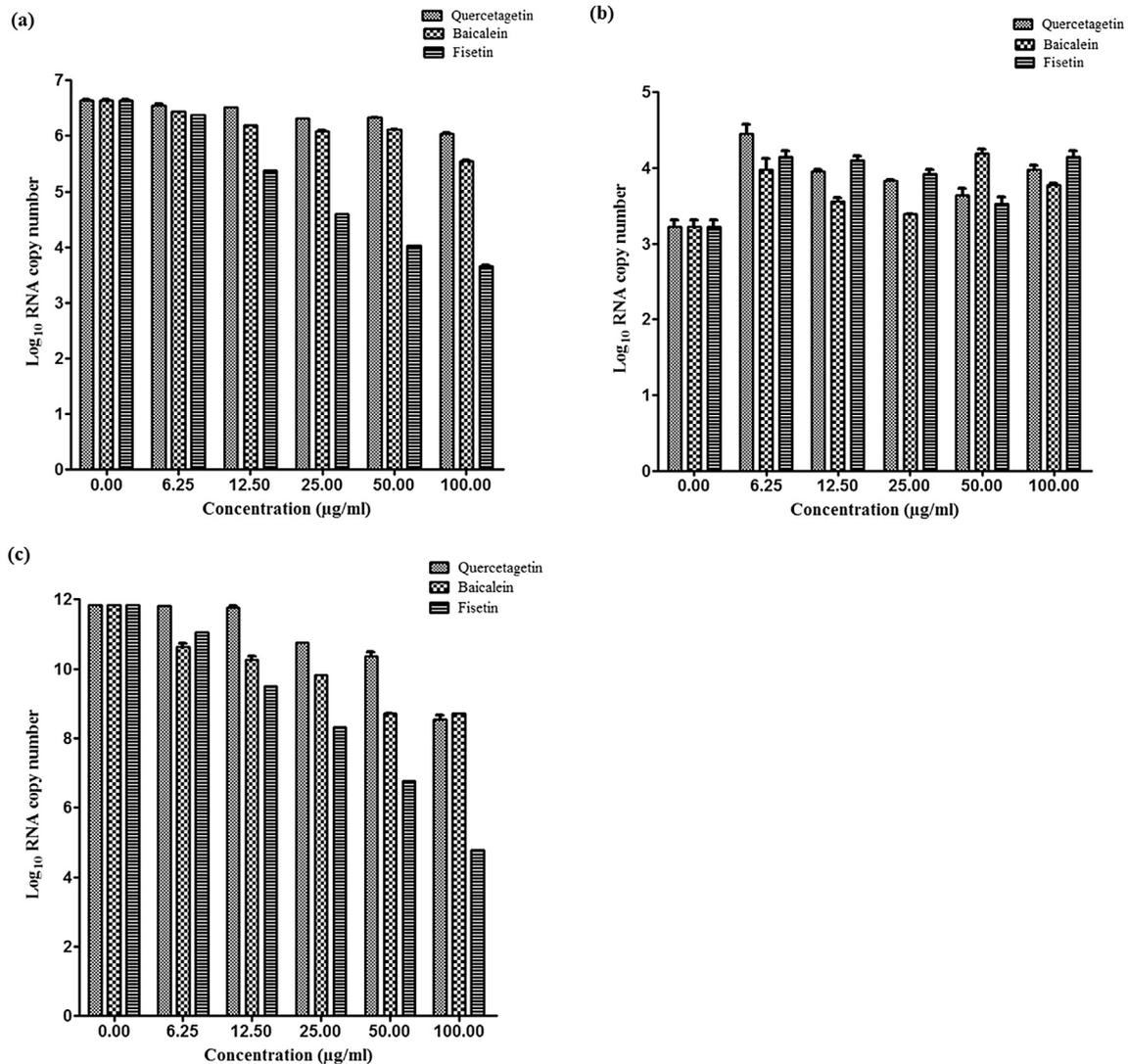
### 3.11. Immunofluorescence assay

An immunofluorescence assay was performed to detect the CHIKV antigen in the tested cells as the indicator for the successful infection and replication of CHIKV and to determine the extent of inhibition that can be provided by the tested compounds. As shown in Fig. 11, all of the analyzed compounds and ribavirin displayed dose-dependent inhibition of CHIKV infection compared to the vehicle control. Based on the automated calculation by (high-throughput screening) HTS machine, it has been revealed that a concentration of 25 µg/ml of fisetin is able to inhibit  $\geq 90\%$  CHIKV antigen, whereas baicalein, quercetagenin, and ribavirin retain about  $\geq 50\%$  CHIKV antigen with the same concentration.

## 4. Discussion

With the lack of a vaccine and antiviral treatment for CHIKV infection, various alternatives have been evaluated to increase the likelihood of finding an effective antiviral agent. In this experiment, one flavone (baicalein) and two flavonols (fisetin and quercetagenin) were selected for evaluation of their anti-CHIKV activities. During the primary antiviral screening assay, these three compounds were selected for antiviral candidates because they displayed a dose-dependent inhibition against CHIKV replication with minimal toxicity and were thus regarded as having potential for use as therapeutic agents.

Based on a rapid antiviral screening test, a CHIKV replicon cell line that eliminates the viral entry and particle release steps was also used. Although the reduction of *Rluc* activity was not significant after treatment with either fisetin or quercetagenin compared



**Fig. 9.** Fisetin, baicalein and quercetagenin reduced the efficiency of converting negative-strand RNA to positive strand (a) RNA thus, interfere with the effectiveness of envelope production (c). The kinetics of the replication is active as shown in (b) but the yield of the positive-strand RNA is reduced (a). The intracellular CHIKV RNA was extracted at 8 hpi. Statistical analysis was performed by using one-way ANOVA (Kruskal-Wallis test) where  $P < 0.05$  is significant. ("0" on the X-axis is referring to the non-treated CHIKV-infected controls). Error bars represent standard errors of triplicate means.

to baicalein, it can be suggested that the observed antiviral properties from those two compounds, especially fisetin, could be a result of their effects against important cellular factors involved in CHIKV replication.

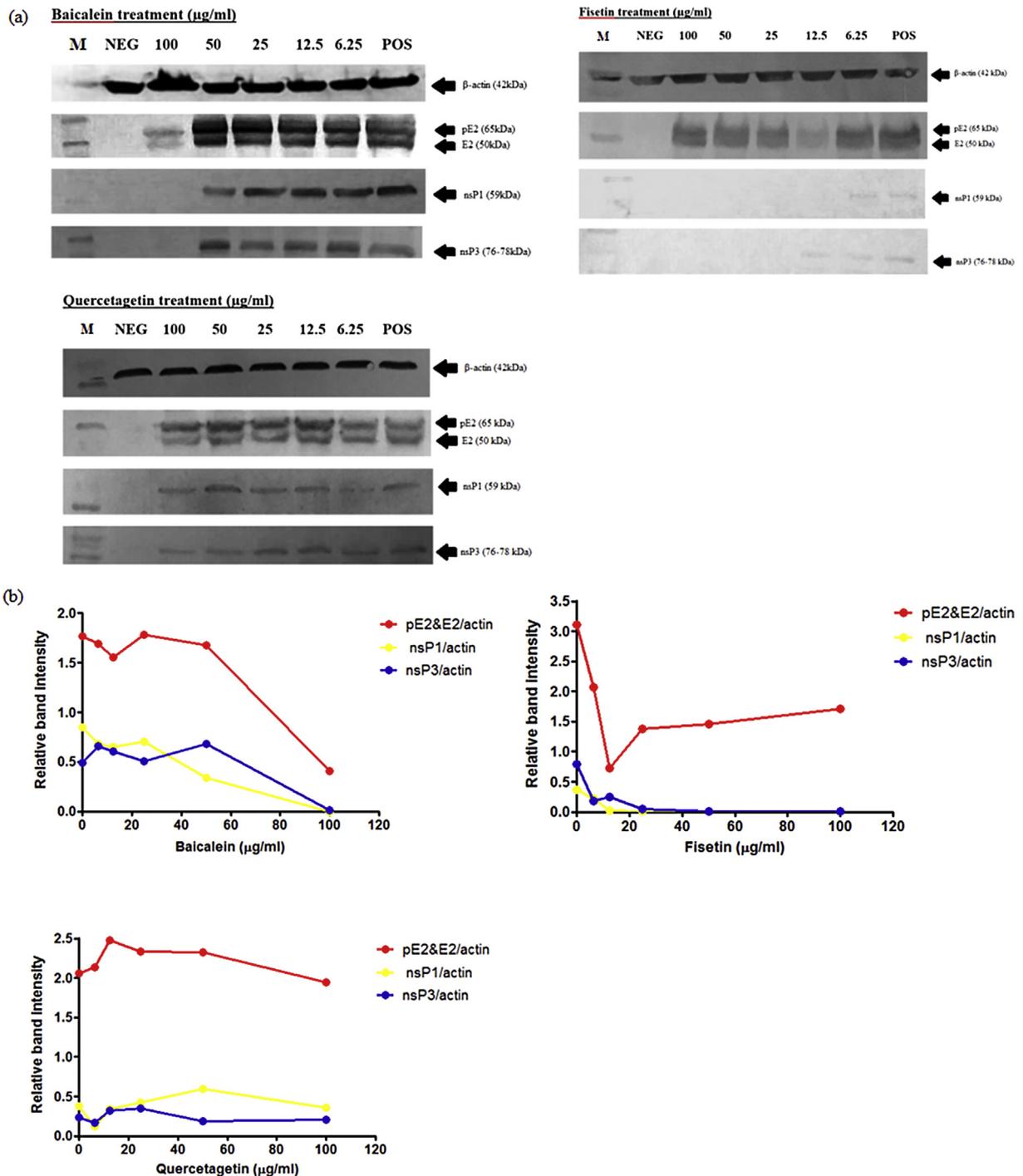
The data from the time course study suggest that all three tested compounds exhibit inhibitory effects against the early events of the CHIKV replication cycle. Almost 100% of CHIKV replication efficiency could be inhibited by baicalein at -2, -1, 0, 2 and 3 hpi, by fisetin at all time of treatment, and by quercetagenin at -2, -1, 0, 2 and 3 hpi. We believe that these bioflavonoid compounds act in the early hours and possibly the early stages of CHIKV infection. However, it has been revealed that fisetin showed more potent inhibitory activity against CHIKV replication than the other tested compounds when it was added after CHIKV internalization into the Vero cells.

Baicalein and fisetin, with  $IC_{50}$  values of 1.891 (6.997  $\mu$ M) and 8.444  $\mu$ g/ml (29.5  $\mu$ M), respectively, exhibited stronger antiviral activity against CHIKV intracellular replication than quercetagenin. The calculated SI values for baicalein (188.4) and fisetin (23.02) also confirm their potency as potential candidates for further

investigation toward anti-CHIKV drug discovery. Quercetagenin showed significant effects against CHIKV binding to Vero cells, with an  $IC_{50}$  of 8.050  $\mu$ g/ml (25.3  $\mu$ M) and an SI of 16.3.

The detection RNA replication of intermediate species by performing qPCR after 8 h pi allow us to identify roughly the mechanism of action of the compounds in CHIV intracellular replication. The reduction of the RNA yield on positive-strand and E1 qRT-PCR is contradict with the active kinetics of replication as shown by negative-strand qRT-PCR result. The only relevant explanation is that the compounds interfere at the stage of converting negative-strand RNA to positive-strand RNA.

The data from western blot analyses indicated that baicalein treatment resulted in a decrease in the production of CHIKV proteins, especially at 100  $\mu$ g/ml. Fisetin treatment resulted in a dose-dependent decrease in the production of CHIKV nonstructural proteins, which began at concentrations as low as 12.5  $\mu$ g/ml. The observed decrease in the expressions of nsP3 protein might suggest that the antiviral function of baicalein and fisetin began with the inhibition of translation of nonstructural proteins, leading to a decrease in the production levels of the replicase units. Because this

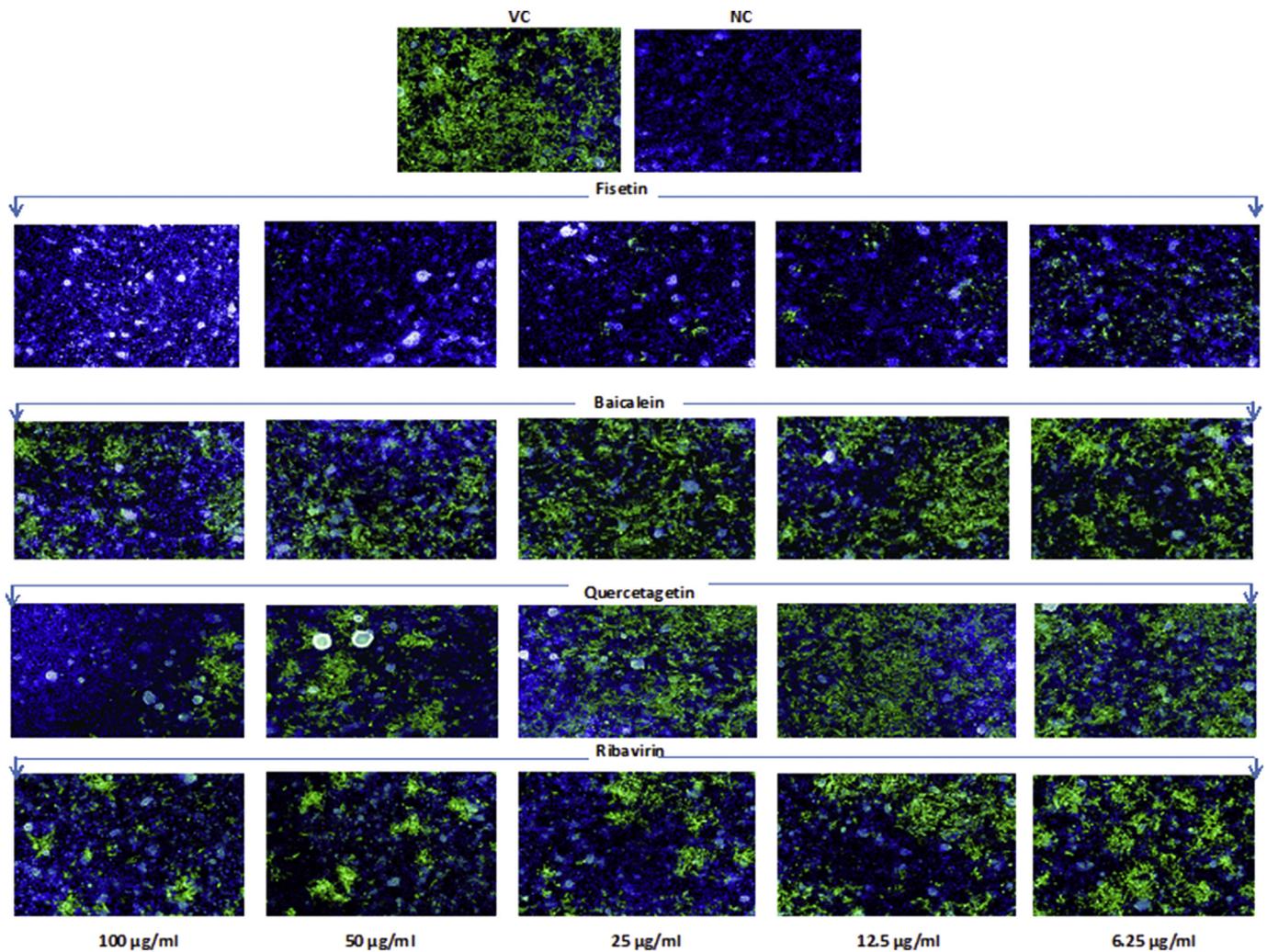


**Fig. 10.** (a) Baicalein and fisetin suppressed the accumulation of CHIKV-encoded proteins. Western blot analysis was performed to determine the effect of baicalein, fisetin and quercetagenin on production of CHIKV nsP1, nsP3 and E2 proteins. Bands of the specific proteins as observed on the blot. A dose dependent reduction of CHIKV nsP1, nsP3 and E2 proteins were observed upon baicalein and fisetin treatments for 48 h β-actin is used as a loading control for each set of samples. (b) The above mentioned observation was confirmed by calculating the relative band intensity (calculated from the band intensity of sample/band intensity of β-actin loading control) using Image J.

would likely result in a reduction of negative-sense RNA levels to function as templates for the synthesis of positive-sense RNA strands, the detection of nsP1, which is involved in negative-strand synthesis, is very appropriate, and both baicalein and fisetin showed the expected result.

Because quercetagenin showed no reduction in the level of CHIKV proteins in Western blot analyses, the observed post-treatment antiviral effects could reflect activity against the later

stages, such as assembly and release. Quercetagenin showed a potent effect against CHIKV binding to Vero cells (SI = 28.16) in addition to its neutralizing effect against extracellular CHIKV particles (SI = 22.8), which can confirm the potential of this compound as an inhibitor of the early stages of in vitro CHIKV replication. The results from immunofluorescence assay for detection of the CHIKV E2 protein on the surface of infected cells also confirms the role of quercetagenin as an inhibitor of E2 presentation in infected and



**Fig. 11.** Baicalein, fisetin and quercetagenin suppress the CHIKV antigen in Vero cells. A pattern of a dose-dependent inhibition by the three flavonoid compounds and ribavirin (fisetin > quercetagenin > ribavirin > baicalein) on the CHIKV E2 protein detection can be seen. Detection of the CHIKV E2 protein is used as the indication of the successful CHIKV infection. Cell nuclei are stained with DAPI (blue) and CHIKV infection is indicated by Alexa Fluor 488 (green) staining. VC: virus control; NC: negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treated cells, because the detection of the E2 antigen indicates the success of CHIKV replication and assembly in the cells, even though it was revealed in the Western blotting experiment that quercetagenin has no inhibitory effect against CHIKV E2 synthesis.

Fisetin potentially inhibited the CHIKV antigen even at the lowest concentration of all compared tested compounds. This finding is consistent with the observed effect of this compound in Western blotting experiments. This observation might be due to inhibition of CHIKV mRNA synthesis or genome replication and warrants further mechanistic molecular investigations. In conclusion, our findings suggest that fisetin, baicalein, and quercetagenin are deserving of further study to determine the precise details of their mode of action and potential anti-CHIKV therapeutics.

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