Effectivity of quercetin as antiviral to dengue virus-2 strain New Guinea C in Huh 7-it 1 cell line

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Abstract. The prevalence of dengue virus infection (DENV) in Indonesia is still high compared to other tropical countries in the world. Unfortunately, the specific of antiviral drug to DENV is not available yet. The pure compound such as quercetin revealed a good antiviral to DENV candidat. Quercetin is a plant-derived flavonoid that can be found in wide variety of fruits and vegetables. It has been proven that quercetin able to improve body performance and reduce the risk of infection as well as inhibit DENV replication in Vero cell. However, the research on human cell line is not yet conducted. Therefore, this research aims to determine the effectivity of quercetin as antiviral drug towards DENV-2 strain New Guinea C in human cell line Huh 7 it-1. We used Focus Forming Assay and MTT assay To determine the value of IC50 and CC50, respectively. The result of CC50 value was 217.113 μg/mL and IC50 value was 18.41 μg/mL, with the SI value of 11.8. From the results imply that quercetin has low toxicity with high effectivity. Thus, quercetin suggested a good candidate of antiviral drug against DENV-2 in future.

1. Introduction
Tropical countries tend to have higher humidity rate than non-tropical countries. Humidity is one of several factors that make place suitable for mosquitos to live. One of the most concerning illness in the world is dengue fever (DF), which caused by dengue virus (DENV) transmitted from mosquitos to human, accounts for up to 390 million cases per year. But, only 500,000 infected people that need to be hospitalized and unfortunately 2.5% of them die. In 2016, there was a worldwide dengue fever outbreak, especially in Brazil where the total amount of the case reached 3 times higher than it was in 2014 [1].

In the same year, 2016, the total amount of reported case in Philippines reached 176,411 cases and 100,028 cases in Malaysia. In total in 2015, the reported cases from all over South-East Asia was 3.2 million. In Indonesia itself, the cases began to be reported from 1968 in Surabaya which accounted for 58 cases and 24 of the infected people died. In 2014, there was 100,347 cases reported case and 907
infected people died, the case fatality rate accounted for 0.904%. While, in 2015, there was increment of 0.067% of the case fatality rate, from 126,675 cases reported and 1,229 of them died [2], [3].

Unfortunately, today, there is still no commercially available antiviral drug to DENV. Beside to cure of DENV patients, antiviral drug can be used to prevent the transmission of DENV in the community due to reduction of viremia after treated with antiviral drug. The mechanism of antiviral include blocking the viral entry or virus attachment to human cells [4-7]. One of many pure compounds that can be found in nature is quercetin, even though the potency as antiviral drug to DENV is still remain unknown in the human cell line. In Vero cell, flavonoids able to inhibit the fusion of DENV-2 by blocking conformational changes in envelope protein [8].

Quercetin as one of natural compounds, flavonol group can be found mostly in plants, black tea and fruits such as apple, berries, grapes, onions, tomato [9]. It has already been used by several researches as anti-inflammation, anti-bacterial, anti-viral, and anti-fungi [10-12]. As anti-inflammation, quercetin inhibit the expression of TNF α, IL-8 and IL-1 α in glial cell to reduce apoptotic neuronal cell death [13]. In gastrointestinal tract, it will inhibit the production of COX and LOX, and down regulation the expression of IL-4 [13]. As antibacterial, quercetin able to combat Positive and negative Gram bacteria through disrupting the cell wall and cell membrane of bacteria. Quercetin also able to perform hydrogen atom transfer, single electron transfer followed by protein-transfer and sequential proton loss electron transfer as its anti-oxidant properties. As antiviral drug, quercetin also plays role in inhibiting early stage of influenza, rhinovirus and hepatitis C virus replication [14,15]. Other researchers also found that quercetin has potency as anti-fungal through inhibit biofilm formation [16]. Up to now, the potency of quercetin as antiviral drug to DENV is still unknown. In this study, we will explore the potency of quercetin in vitro using DENV serotype 2 strain New Guinea C and human cell line Huh 7it-1.

2. Materials and methods

2.1 Preparation of quercetin
Quercetin, the pure compound which used in this research, was a product of Sygma®.

2.2 Propagation of cell line
Cells used in the research was Huh 7it-1 which cultured in T-75 Flask. Propagation of the cells were done according to previous study [17]. We used cell at concentration of 5 x 10⁴ cell/mL, 200 μL/well in 48 well plate for evaluation of DENV inhibitory. Then, to determine the toxicity, in 96 well plate, we added the cell 100 μl/well at concentration of 2,3 x 10⁴ cell/mL. All of the cells were incubated in 37°C with 5% CO₂ for 48 hours.

2.3 Propagation of dengue virus
The Dengue virus serotype 2 strain new guinea C was given by Microbiology Department Faculty of Medicine Universitas Indonesia-RSCM. Propagation and titration of DENV were done according to previous study [17].

2.4 Determination of toxicity through CC₅₀ value
The toxicity level of quercetin to the cell was determined by MTT assay that evaluate the percentage of Huh 7it-1 cells viability after being treated with diverse concentration of quercetin with DMSO 0,1% as the negative control. The MTT assay was performed according to previous study. The value of absorbance reading determined the value of cell viability and toxicity. The result of toxicity percentage of each concentration was calculated to evaluate the mean value and standard deviation value, then the mean of the percentage was plotted to corresponding concentration to form concentration-mean percentage of viability curve. From the curve, the intersection of the concentration of quercetin and the regression of cell viability determined the half-toxicity concentration (CC₅₀) of quercetin [17].
2.5 Determination of antiviral activity through IC$_{50}$ value
Monolayer cell in 48 well plate as described above was used to determine antiviral activity. After the incubation, the cell was infected with mixture of DENV-2 at MOI of 0.5 FFU/cell and various concentration of quercetin ranging from 5, 10, 20, 40, 80, until 160 µg/mL for 2 hours in triplicate. Afterwards, it was added 200 µL of DMEM 10%FBS containing various concentration of quercetin was added. The plates were then be further incubated for another 3 days in 37°C with 5% CO$_2$. Supernatant was harvested and counted the titer by focus assay. The brown foci formed inside each well, including the ones in negative control well was counted manually under microscope after immunostaining. Mean value of infectivity percentage of each concentration of quercetin in triplicate was calculated through comparing the number of foci in each well to negative control well. The calculated mean value of infectivity percentage of each triplicate was plotted to corresponding concentration to from concentration-percentage of inhibition curve. The regression of non-linear equation of concentration-effect curves was utilized to obtain the half-inhibitory concentration of the IC$_{50}$ [17].

3. Results and discussions
The ideal antivirus to treat DENV infection is the one with low toxicity towards the host cell and high effectiveness to inhibit the virus. Through this, the antivirus will work effectively to minimize the virus replication in the host cell as well as keeping the cell viable. Many studies are now dedicated to find the general antivirus. Quercetin as one of flavonoid derivatives, has potential antiviral. The study conducted by Wu et al proved that quercetin able to inhibit influenza virus entry through inhibition of viral-cell infusion, due to quercetin’s interactions with envelope protein hemagglutinin (HA) expression that is required for influenza virus to attach to host cell. This study revealed the IC$_{50}$ value of quercetin was 7.756 µg/mL for strain H1N1 in MDCK cell, in vitro [14]. Other than influenza virus, quercetin also already been studied against zika virus. The study by Wong et al, using Q3G as natural derivatives of quercetin to determine its antiviral activity towards zika virus in vero cell, in vitro. Q3G proven to be able to inhibit the replication of zika virus with the IC$_{50}$ value of approximately 1.2-1.3 µmol/L or around 24.845-26.915 µg/mL [18].

3.1 Cell viability
Cell viability, or the cell ability to survive, was counted using MTT assay method that visualizes the data in the form of optical density or absorbance as the result from spectrophotometer with the wave length of 490 nm. Upon determining the absorbance, higher number of the absorbance were seen in the more concentrated-colored well that indicates more viable cells. Then, the percentage of cell viability between treated and controls were counted (table 1).

| Concentrations µg/mL | % Viability | Mean Value ± SD |
|----------------------|-------------|-----------------|
| 640                  | 2.455       | 2.070 ± 1.133   |
| 320                  | 6.219       | 6.732 ± 3.037   |
| 160                  | 63.485      | 63.532 ± 1.384  |
| 80                   | 66.183      | 65.998 ± 0.777  |
| 40                   | 76.003      | 76.049 ± 0.693  |
| 20                   | 83.472      | 83.356 ± 0.262  |
| 10                   | 86.307      | 88.197 ± 1.660  |
Through figure 1 above, CC$_{50}$ value was able to be determined through substitution of “y” in the equation with 50, resulting in X as the CC$_{50}$ value. CC$_{50}$ means the 50% cytotoxic concentration of the pure compound, as for quercetin in this research, to cause 50% death of the infected cells. Here, the CC$_{50}$ value was 217.113 μg/mL. The R$^2$ value reflects the correlation between the X and Y axis, the quercetin concentration and percentage of viability respectively. High R$^2$ means the value is above 0.5, in this case the value was 0.8547. This depicts that there was strong correlation between the concentration of quercetin and percentage of viability. As high concentration of quercetin was given, the percentage of the cell viability declined.

### 3.2 Effectivity of DENV inhibition

From the focus assay, we found that treated with quercetin inhibit dengue repliction in dose dependent manner. Treated with quercetin with concentratin more than 40 mg/ml decreased the persentage of infectivity to 0% (table 2). Treated with concentration of 20 mg/ml showed infectivity of 24.540 ± 12.270.

### Table 2. Percentage of Infectivity DENV after treated with various concentration of quercetin

| Concentrations (μg/mL) | % Infectivity 1 | % Infectivity 2 | % Infectivity 3 | Mean Value ± SD |
|------------------------|-----------------|-----------------|-----------------|-----------------|
| 160                    | 0.000           | 0.000           | 0.000           | 0.000 ± 0.000   |
| 80                     | 0.000           | 0.000           | 0.000           | 0.000 ± 0.000   |
| 40                     | 0.000           | 0.000           | 0.000           | 0.000 ± 0.000   |
| 20                     | 12.270          | 36.810          | 24.540          | 24.540 ± 12.270 |
| 10                     | 110.429         | 85.890          | 49.080          | 81.800 ± 30.879 |
| 5                      | 122.699         | 73.620          | 73.620          | 89.980 ± 28.336 |
From figure 2, IC$_{50}$ value was able to be determined, by substituting the “y” with 50 in $y = -2.6745x + 99.226$. IC$_{50}$ itself defines as the minimum concentration of quercetin, to inhibit the virus and the value here was 18.406 μg/mL. The R$^2$, which as aforementioned, functions to visualize the correlation level between the X and Y axis, concentration of quercetin and infectivity percentage respectively. The R$^2$ here was also high, which was 0.8934. This reflects the high correlation between the concentration of quercetin and the infectivity percentage. According to the chart, the correlation between quercetin concentration and infectivity percentage is inversely proportional. Higher concentration of quercetin given, will result in lower infectivity.

Other than quercetin, there are natural compound also thought to be potential antivirus against dengue virus. Catechin in the form of epigallocatechin (EGCG) in green tea has the potency to inhibit the entry of dengue virus by acting on the virion that eventually impaired the attachment of virus to host cell. The study was done by Raekiansyah et al using vero cell and 4 serotypes of dengue virus including DENV-1, DENV-2, DENV-3, DENV-4. The IC$_{50}$ for all serotypes are as follows 14.8, 18.0, 11.2 and 13.6 μM, respectively. Those value equal to 6.784 μg/mL, 8.251 μg/mL, 5.134 μg/mL and 6.234 μg/mL [19]. Curcumin, as active compound from Curcuma Longa, has also been identified as antiviral against dengue virus. This is conducted by Ichsyani et al using Huh 7 it-1 cells for in vitro method and liver and kidney of ddY mice for in vivo method. The obtained value for IC$_{50}$ and CC$_{50}$ were 17.91 μg/mL and 85.4 μg/mL, respectively. From that, the SI value determined was 4.8. In addition, evaluating from the liver and kidney of ddY mice, no histopathological abnormalities were shown [20].

In this study we found that quercetin has no toxic effect to Huh 7it-1 cell with the half-toxicity value of 217.113 μg/mL. The difference between CC$_{50}$ value in this research and research that was conducted by Zandi et al may happen due to difference in the cell that were used, in which this research used human cell line Huh 7it-1 cell and Zandi et al used Vero cell [4]. The result also different from the study by Saptawati et al, this may be due to the usage of Carica papaya instead of pure quercetin compound as this result used [21]. If compared to other compound used such as curcuma, quercetin has higher CC$_{50}$ value, which indicates that it is safer to use quercetin rather than curcuma in DENV treatment.

The IC$_{50}$ value in this study was 18.406 μg/mL. In comparison with other study such as EGCG, the IC$_{50}$ value of quercetin is higher. While for the curcuma, there is not much different regarding the IC$_{50}$ value on both studies.

![Figure 2. Linear regression curve of antiviral effect of quercetin in Huh 7 it-1 cell](image-url)
3.3 Selectivity Index (SI)
The selectivity index from this research were acquired through dividing the CC50 value with the IC50 value. SI value of this research was 11.797, it indicates that quercetin concentration to kill 50% of the dengue virus is able to be multiplied up to 11 times. In that concentration, quercetin still able to inhibit the infectivity of DENV-2 in the cells while keeping the cells viable. In comparison to curcumin which utilized same cells and virus, the SI value was only 4.8. This reflects that quercetin has more potency as antiviral against DENV-2 since it is more effective with lower cytotoxic effect.

4. Conclusion
The result of this research revealed that the half-toxicity level towards the Huh 7 it-1 cell and half-inhibition level towards DENV-2 of quercetin were 217.113 μg/mL and 18.406 μg/mL, respectively, with the SI value of 11.797. This reflects that quercetin has a potency as antiviral against DENV-2 in future.

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