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Antiviral Activity of Isobutyl Gallate to Dengue Virus Serotype 2 *In Vitro*

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Abstract. Dengue is one of the most prevalent mosquito-borne diseases in Indonesia that lead to a spectrum of disease, ranging from a flu-like illness to a life-threatening dengue shock syndrome. Despite its high incidence and mortality rate, there is currently no antiviral drug available against dengue virus (DENV). Therefore, research regarding the development of antiviral to DENV is essential. The aim of this study was to evaluate the antiviral activity of isobutyl gallate, a gallic acid derivative, against DENV in vitro. Various concentration of isobutyl gallate was added to DENV-2 strain New Guinea C (NGC) and infected to Vero cell line. Inhibition of DENV replication as aligned with half inhibitory concentration (IC₅₀) value was determined by focus assay. Furthermore, the toxicity of isobutyl gallate that corresponds with cytotoxic concentration (CC₅₀) value was determined by MTT assay. Selectivity index (SI) value was defined as the ratio of CC₅₀ to IC₅₀. Isobutyl gallate showed no cytotoxic effects against Huh 7 it-1 cells with CC₅₀ values of 167.19 µg/mL and exhibited strong antiviral activity with IC₅₀ of 4.45 µg/mL, hence a significant SI value of 25.69. Isobutyl gallate exerts potent antiviral activity against DENV.

1. Introduction

Over the last decades, DENV has emerged as one of the most important mosquito-borne viral diseases in the world. Dengue fever causes a severe flu-like illness as a result of DENV infection. DENV and its vectors have been widely distributed over the last half-century, with more than 125 countries in the world are dengue endemic [1]. According to the data from World Health Organization (WHO), there are up to 100 million cases of dengue fever, and half a million cases of dengue hemorrhagic fever (DHF) require hospitalization with over 20.000 deaths each year, a major percentage of whom are



children [2]. Studies suggest that dengue is more prevalent in Indonesia, with proportions ranging between 5.7-7%. Therefore, dengue fever poses a serious risk for Indonesians [3].

Dengue infections are mostly asymptomatic. However, when they are symptomatic, the virus can cause mild dengue fever (DF), or even more severe forms of the disease, which is dengue hemorrhagic fever (DHF) that most likely progress to dengue shock syndrome (DSS) and if left untreated it can be fatal and lead to death [4]. As dengue infection is posing a serious threat, it is important to prevent both the infection and the following complication. Unfortunately, there is currently no specific antiviral available for DENV. One of the compounds that have antiviral DENV properties has been reported was gallic acid [5]. Also reported by others that one the derivative gallic acid, octyl gallic have activity combats DNA and RNA virus. Based on the previous study, our team tries to test a derivative of gallic acid as antiviral DENV [6]. One of the derivatives is isobutyl gallate, a gallic acid derivative that is commonly used in nutraceutical due to its anti-inflammatory and antioxidant properties. Gallic acid itself, a polyphenolic compound whose product of esterification has potentials in inhibiting Hepatitis C virus (HCV), possesses antibiotic, antiviral, and anticancer properties. It has been proven that gallic acid can act as a strong antiviral antioxidant and can exert its antiviral effect on several RNA viruses, including influenza virus and poliovirus [5]. However, research regarding the potential of gallic acid derivatives as DENV antiviral is still limited. Therefore, this research is conducted to evaluate the potential of isobutyl gallate as a candidate for DENV antiviral.

2. Materials and methods

This research was designed to be a laboratory-based experimental study in vitro with triple replications. It was conducted at the Department of Microbiology Faculty of Medicine Universitas Indonesia from August 2017 to May 2018.

2.1. Preparation of Isobutyl Gallate

Isobutyl gallate was synthesized from gallic acid by Dr. Ade Arsianti (Medical Faculty University of Indonesia). To make a testing solution, it was prepared through dilution, diluted in DMSO until the concentration reaches 100 mg/mL. To make isobutyl gallate at the concentration of 160 µg/mL, the diluted compound was homogenized using a vortex, and then 6.08 µl of diluted extract was added to 1900 µl of the medium. It was further diluted in serial dilution to obtain a concentration of 80, 40, 20, 10, and 5 µg/mL [6].

2.2. Preparation of Dengue Virus

Preparation of dengue virus DENV-2 strain New Guinea C (NGC) used in this study was done by the researcher of the previous study [6].

2.3. Determination of Viral Titer Using Focus Forming Unit Assay

Viral titer was measured using the focus assay method, an immunostaining procedure that uses peroxidase as a marker to count DENV viral titer. Monolayer Huh-7 was seeded in a 96-well plate. After 24 hours of incubation, the cells were treated with a mixture of DENV and various concentrations of isobutyl gallate. The cells were incubated at 37 °C for 2 hours. During the incubation period, the 96-well plate was shaken at 30 minutes intervals. After the cells had been infected with the virus, the remaining DENV was removed from the cells by washing the cells with PBS. Various concentrations of isobutyl gallate were added to the cells before they were incubated at 37 °C for 3 days.

To determine the antiviral activity, the cells were harvested before counting the viral titer using focus assay. The incubated cells that had previously been prepared were added with a serial dilution of viral supernatant and were further incubated at 37 °C with 5% CO₂ for 2 hours. After incubation, the medium was removed, and methylcellulose overlay mediums were added. Fixation was done using PBS with 10% of Formaldehyde at room temperature. The cells were washed thrice with PBS at 5 minutes intervals. Following cell washing, PBS tween 100 µl/well was added and Vero cells were washed with PBS tween after secondary antibody, IgG anti-human labeled as peroxidase had been

added to the cells. OPD substrate containing H₂O₂ was also added to Vero cell. The infected cell will form brownish foci, originated from one virus. This experiment was done in triplicate [7].

$$\text{Viral titer (FFU/mL)} = \frac{(\text{Total foci} * \text{Dilution} * \text{Volume of infection})}{1000} \quad (1)$$

2.4. Determination of IC₅₀

The results obtained from focus assay was also used to calculate IC₅₀ with the following formula:

$$\text{Infectivity (\%)} = \frac{\text{Viral titer}}{\text{Average titer DMSO}} * 100\% \quad (2)$$

2.5. Determination of CC₅₀ using MTT Assay

Cell that had been prepared was tested using MTT assay. MTT solution was mixed with DMEM and was homogenated using vortex. Three hundred microliter of MTT medium 10% was added to cell in each well plate. The cell was incubated and MTT medium 10% was drained. Two hundred microliter of DMSO 100% medium was added to cell in each well plate. The plate was put on the shaker for 30 minutes. ELISA reader was used to measuring the peak absorbance at 490nm wavelengths [7]. The results were used to calculate CC₅₀ value, represented by the percentage of cell viability, with following formula:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Average of DMSO 0.1\% absorbances}} * 100\% \quad (3)$$

3. Results and Discussions

Dengue remains one of the infectious diseases that pose a serious risk to health. It is one of the leading causes of death worldwide, especially in endemic areas. There are more than one-third of the world's population who live in endemic areas at high risk for dengue infection and more than 400 million people are infected with dengue every year. Despite the high number of cases, antiviral against DENV is currently underdeveloped. Therefore, research about DENV antiviral is needed to treat dengue infection and prevent the complication, thus can reduce the mortality rate of dengue cases worldwide.

Previous research report activity gallic acid as anti DENV [4]. In this study, the compound tested was isobutyl gallate, one of gallic acid derivatives with the molecular formula of C₁₁H₁₄O₅. Gallic acid itself is described as a colorless or yellowish-crystalline compound that can be obtained from tea leaves, tree bark, gallnuts, and berries through hydrolysis of tannins. It is commonly used in nutraceuticals due to its anti-inflammatory and antioxidant properties [6].

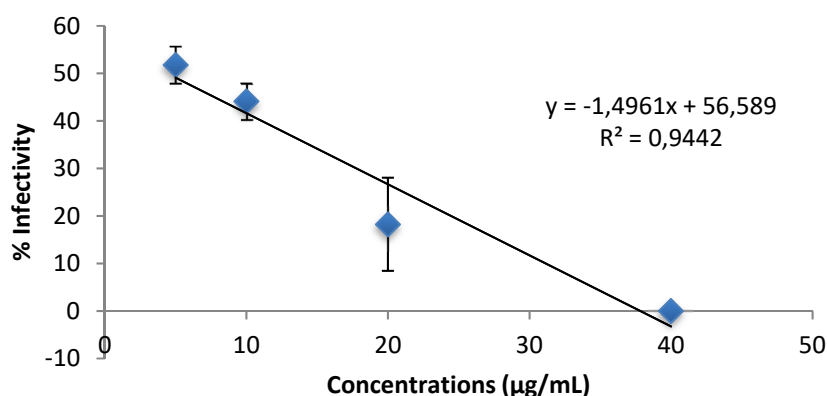
3.1. Percentage of Infectivity and IC₅₀ value

The antiviral property of Isobutyl gallate against DENV replication was determined by adding various concentrations of Isobutyl gallate to DENV and then infect to the cells. DENV after treated with isobutyl gallate at the concentration of 40 µg/mL or higher showed no DENV that still able to infect the cell (Table 1.) with 0% infectivity. When the concentration decreased to 20 µg/mL, the mean infectivity of the DENV-2 increased to 18.24 ± 9.8 (Table 1.). In addition, the results of DENV-2 infectivity showed a strong correlation between the concentration of the isobutyl gallate and the percentage of DENV-2 infectivity with R² value of 0.944 (Figure 1.)

Table 1. Average of percentage infectivity of DENV2 after treated with various concentrations of isobutyl gallate

Concentration ($\mu\text{g/mL}$)	Average of Infectivity (%) \pm SD
160	0.0
80	0.0
40	0.0
20	18.24 ± 9.8
10	44.13 ± 3.74
5	51.78 ± 3.84
DMSO	100 ± 13.21

IC_{50} of isobutyl gallate was determined from the equations obtained from the respective graph (Figure1). Based on the equation of a respected graph, we found that IC_{50} of isobutyl gallate was 4.45 $\mu\text{g/mL}$.

**Figure 1.** The linear graph with R^2 value and equation to obtain IC_{50} value isobutyl gallate

In this experiment, the virus was treated with various concentrations of isobutyl gallate and infect to the Huh 7 it-1 cell to determine antiviral activity. DMSO at a concentration of 0.1% was used as a negative control of an antiviral experiment of this study. The value of IC_{50} is described as the concentration in which the compound inhibited half of the DENV-2 infection of isobutyl gallate was 4.45 $\mu\text{g/mL}$. The results suggest that isobutyl gallate can be a good candidate for DENV antiviral because it exerted inhibitory activity against dengue virus at low concentration in vitro. This value is significantly lower compared to *Curcuma longa*, *Psidium guajava*, and *C. papaya*, whose IC_{50} value are 17.91 $\mu\text{g/mL}$, 7.2 $\mu\text{g/mL}$, and 6.57 $\mu\text{g/mL}$, respectively [9,10].

3.2. Toxicity and CC_{50} value of Isobutyl gallate

Potential cytotoxicity of isobutyl gallate towards uninfected cells was determined by measuring the viability of the cell after being treated with various concentrations of isobutyl gallate. MTT assay was used to determine the viability of the treated and control cells.

Table 2. Cell viability of Huh-7it-1 after treated with various isobutyl gallate

Concentration ($\mu\text{g/mL}$)	Average of Viability (%) \pm SD
160	58.43 ± 9.15
80	131.10 ± 15.76
40	149.20 ± 3.08
20	183.70 ± 8.40
10	195.73 ± 10.15
5	212.06 ± 22.82
DMSO	100.10 ± 6.27

The concentration of 5 to 80 $\mu\text{g/mL}$, showed a great percentage of mean viability ($>100\%$), indicating that the compound was not toxic to the cell at this range. Treated with 160 $\mu\text{g/mL}$, however, there was a decrease to 58.43% (Table 2). The CC_{50} value was defined as the concentration that resulted in 50% cytotoxicity. To obtain CC_{50} value, the data was plotted on a scattered graph, resulting in a linear regression equation (Figure 2.). By plotting, the value of R^2 was obtained and it revealed a strong correlation between the concentration of isobutyl gallate and the percentage of cell viability, higher concentration of isobutyl gallate resulted in the lower percentage of cell viability. Based on the equation of respected graph (Figure 2.), the CC_{50} value of isobutyl gallate was 167.19 $\mu\text{g/mL}$.

Moreover, isobutyl gallate has low potential cytotoxicity towards uninfected cells because, at the concentration of 5 to 80 $\mu\text{g/mL}$, the compound was not toxic to the cell. This is proven by the cell viability results that showed a great percentage of mean viability ($>100\%$) at that range. Potential toxicity was measured by the value of CC_{50} , signifying the concentration at which isobutyl gallate will become toxic to uninfected cells. Isobutyl gallate has a high value of CC_{50} ($\text{CC}_{50} = 167.19 \mu\text{g/mL}$), thus it requires a high concentration of isobutyl gallate in order to result in cytotoxicity towards uninfected cells.

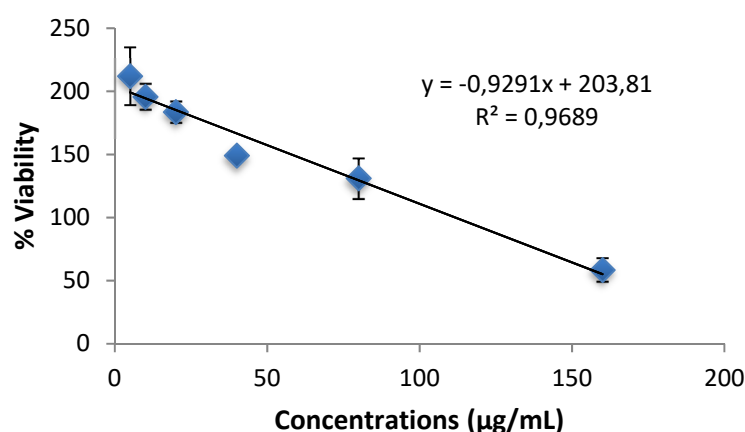


Figure 2. Regression liner of Viability of cell versus concentration of isobutyl gallate to determination correlation and CC_{50} value of isobutyl gallate

3.3. Selectivity Index (SI)

Selectivity Index (SI) is calculated by dividing the value of CC_{50} to the value of IC_{50} . Based on the aforementioned data, the selectivity index of isobutyl gallate was 25.69.

In this study showed that the selectivity index of isobutyl gallate was considered high with the value of 25.69. According to studies conducted by Valdés-García and Quispe, compounds that have SI value of above 10 are considered as selective compounds and SI value of above 10 can be considered as a significant value. It is desirable to have high SI because that means the compound can work selectively towards specific pathogen [8]. In comparison with other plant-derived polyphenolic compounds (bioflavonoids) namely daidzein and naringin, that have significantly lower SI value of 1.03 and 1.3 respectively, isobutyl gallate is more selective towards DENV. In addition, this value is about five times higher than *C. longa* SI value ($\text{SI}=4.8$) and about three times higher than that of quercetin ($\text{SI}=8.74$), which is a phenolic compound. The SI of isobutyl gallate, however, is similar to the SI of *P. guajava*, at the level of 21.28 [10,11].

4. Conclusion

Our results showed that isobutyl gallate exerts a dose-dependent inhibitory to dengue virus in vitro with CC_{50} , IC_{50} , and SI of 167.19 $\mu\text{g/mL}$, 4.45 $\mu\text{g/mL}$, and 25.69 respectively. The aforementioned data signifies that isobutyl gallate has low cytotoxicity towards uninfected cells and exhibited anti-viral property at low concentration, thus it can be a good candidate for DENV antiviral in future.

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