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Antiviral Activity of Propyl Gallate against Replication of Dengue Virus Serotype 2: *In Vitro* and *In Silico* Study

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Abstract

BACKGROUND: Dengue fever and dengue hemorrhagic fever are infectious diseases caused by dengue virus (DENV) with high mortality rates. The mainstay of treatment for DENV infection is supportive, since there was no commercial specific antiviral drug for DENV. Propyl gallate has potential to be an antivirus for DENV. However, the mechanism is still unknown.

AIM: This study aims to identify the activity of propyl gallate in DENV-2 replication in vitro and analyze the binding energy of propyl gallate toward NS3 and NS5 protein in silico.

MATERIALS AND METHODS: We used DENV serotype 2 New Guinea C and Vero cells for *in vitro* study. Focus and MTT assay was conducted to measure inhibition percentage and to measure viability percentage. Furthermore, *in silico* was conducted to identify the binding energy and inhibition constant of propyl gallate toward NS3 dan NS5 protein.

RESULTS: The percentage inhibition of pre- and post-infection was 4.34 ± 7.53% and 30.7 ± 4.88% with viability of 94.64 ± 0.4% and 95.31 ± 3.38%, respectively. The binding energy of propyl gallate with NS5, NS3 protease, and NS3 helicase was −3.49 kcal/mol, −2.47 kcal/mol, and −3.72 kcal/mol.

CONCLUSION: Propyl gallate has high inhibition activity toward DENV-2 adhesion-replication *in vitro* with low-binding energy to NS5 and NS3 *in silico*.

Introduction

In the midst of the COVID-19 pandemic, dengue infection is still a serious public health problem, especially in tropical and sub-tropical countries. Dengue causes a wide spectrum of disease, started from subclinical disease to dengue fever, dengue hemorrhagic fever (DHF), and dengue shock syndrome which can be life-threatening. Severe dengue has a higher risk of death when not managed appropriately. The number DHF cases worldwide has increased six-fold, from 505,430 in 2000 to 3,312,040 in 2015 [1]. The endemic areas of dengue virus (DENV) include tropical and subtropical regions where Aedes mosquitoes are abundant, including Indonesia [2]. On 2017, there were 68,407 DHF cases in Indonesia, in which 493 people died [3].

DENV consist of four serotypes, DENV-1, DENV-2, DENV-3, and DENV-4. DENV has a unique genome that encodes two types of proteins, which are structural proteins and non-structural proteins. Structural proteins such as protein C, prM, and E generally facilitate viral entry to the host cell. There are many types of non-structural proteins, hence especially NS3 and NS5 functions as essential enzymes for viral replication and translation. Norazharuddin and Lai stated that DENV replication capabilities will be lost if the activity of NS3 and NS5 is minimalized [4].

Until today, the treatment for DENV infection worldwide is mainly supportive. The number of mortality cases found in DENV infection shows that supportive treatment is inadequate. A specific antiviral agent for DENV has not been found, whether natural or synthetic. A certain compound that has been proposed to be a potent antiviral drug is propyl gallate. Propyl gallate is a derivate of gallic acid, which is a natural compound from plants, most commonly used as food additives. From a study by Mahmudah [5] about the potency of propyl gallate in eliminating DENV, the half minimum cytotoxic concentration value (CC_{50}) is 223.99 μg/mL and the half minimum inhibitory concentration value (IC_{50}) is 15.57 μg/mL. However, the mechanism of DENV replication inhibition by propyl gallate is still unclear. The current literature regarding the importance of NS3 and NS5 proteins in DENV replication shows promise for researchers to find a compound that can potentially target those specific proteins [5]. Thus, this

study aims to identify the mechanism of propyl gallate as an antiviral drug for DENV, especially its' activity in inhibiting viral replication. This study will be conducted *in vitro* and *in silico*.

Materials and Methods

Virus propagation

DENV propagation was done in Vero cells using DMEM medium with 2% FBS at 37°C with 5% CO_2 for 5–7 days. The propagation process was done at the Department of Microbiology, Faculty of Medicine, University of Indonesia. The titer of DENV was calculated using focus assay method [5].

Preparation of propyl gallate

Synthesis of propyl gallate was done by esterification of gallic acid in the Department of Clinical Chemistry, Faculty of Medicine, University of Indonesia. Gallic acid was obtained from Sigma-Aldrich Chemical Company. Esterification reaction of gallic acid was carried out in oven-dried glassware and required silica gel 60 and flash column chromatography. The purified compound is later analyzed using thin-layer chromatography, high resonance mass spectrometer, and nuclear magnetic resonance spectrometer, producing propyl gallate which is an ester derivative of gallic acid.

Time addition of propyl gallate

Inhibition of pre-post and post-infection was conducted to determine inhibition during adhesion-replication and replication proses alone of DENV, respectively. Inhibition pre-post infection was done by adding propyl gallate to DENV prior infection to Vero for 2 h at 4°C. Then, monolayer cells were infected with treated DENV and incubated at 37°C and 5% CO_2 for 2 h. Negative control for experimental group was made by giving DMSO with concentration of 0.1%. After 2 h, the cells were then given medium DMEM with 10% FBS containing propyl gallate with concentration of 15.57 μg/mL (twice the IC_{50} value) and incubated at 37°C with 5% CO_2 for 48 h. Inhibition of post-infection, first of all, the monolayers of Vero cells prepared on the previous day (3) was infected with 0.5 FFU/cells of DENV, 50 μL per well. Then, the wells were incubated for 2 h at 37°C with 5% CO_2 and added with 100 μL of medium DMEM with 10% FBS containing propyl gallate with concentration of 15.57 μg/mL (twice the IC_{50} value). On the control group, the cells were added with medium contained DMSO 0,1%. After incubation for 48 h, supernatant was harvested and focus assay was conducted to determine inhibition of DENV replication [5].

Determine of percentage inhibition

Percentage of inhibition was calculated by focus assay as pervious study without modification [5]. Percentage of inhibition can be calculated using the following formula [5]:

$$\frac{\textit{Mean of control focuses} - \textit{test focuses}}{\textit{Mean of control focuses}} \times 100\%$$

Percentage of viability testing

Percentage of viability was calculated using MTT assay as previous study. Percentage of viability can be calculated with the following formula:

$$\frac{\textit{Optical density of test group}}{\textit{Optical density of control group}} \times 100\%$$

Analysis of binding energy

In silico analysis using molecular docking was conducted to determine the binding energy of propyl gallate with DENV proteins involved in viral replication. The DENV proteins used in this analysis were non-structural protein 3 (NS3) and non-structural protein 5 (NS5). This analysis was carried out using AutoDock Tools – 1.5.6 software. The ligand used was propyl gallate. The three-dimensional propyl gallate ligand with mol2 format was downloaded from http://zinc.*docking*.org website. This website provides various compound structures for macromolecule-ligand docking purposes. The non-structural proteins of DENV were downloaded from https://www.rdcb.org with the pdb format. Coordinates for docking were determined using coordinates of innate ligand. Then, docking process was done using AutoDock program. The best macromolecule-ligand conformation was the conformation requiring the lowest inhibition constant and expressed the lowest binding energy.

Statistical analysis

Data were expressed in tables and tested using JASP software. First, normality test was conducted using Shapiro–Wilk test. Then, independent t-test was used if normal distribution and Mann–Whitney test if the normal distribution.

Research ethics

This study uses Vero cells without intervention toward living creatures so this study did not require any permission from the Ethics Committee, Faculty of Medicine, University of Indonesia. This study has

Table 1: Percentage of inhibition of propyl gallate in both treatments

Treatment	Percentage of inhibition (%)			Results (mean ± SD %)	p-value
	1st repetition	2nd repetition	3rd repetition		
Post-infection (Viral replication)	1.192	−1.106	12.94	4.34 ± 7.53	0.007
Pre-post infection (Viral adhesion)	33.89	25.04	33.03	30.7 ± 4.88	

received approval from Research Module, Faculty of Medicine, University of Indonesia with letter number ND-144/UN2.F1.D1.2/PDP.01/Riset-2/2020, and permit to conduct research from Department of Microbiology, Faculty of Medicine, University of Indonesia.

Results

Inhibition activity of DENV

The activity of propyl gallate in inhibiting DENV-2 replication was expressed by percentage of inhibition (%). The average of percentage inhibition is shown in Table 1. From the results, the percentage of inhibition in the pre-post-infection group was higher that the post-infection (replication) group with significantly difference (p = 0.007).

Toxicity of propyl gallate

Other than studying the effectivity of propyl gallate in inhibiting DENV-2, toxicity effects of propyl gallate toward cells were also studied by determining the Vero cells viability. Viability of healthy cells was estimated by percentage of optical density of the treatment group and control group. The percentage of viability data is shown in Table 2. There was not significantly difference viability between pre-post-infection and post-infection group with p value of 0.750.

Bioinformatics analysis of binding energy

To support the study results, *in silico* analysis using docking with AutoDock Tools – 1.5.6 software was conducted to identify the binding energy between propyl gallate and NS3 and NS5 proteins of DENV-2. NS5 has the code 5ZQK. Throughout this process, we found that NS3 has two domains, which are helicase (code 2BHR) and protease (code 2FOM). Analysis of binding energy was done separately for the two domains of NS3. We also found that NS5 protein has an innate ligand which is s-adenosylmethionine (SAM). In other hand, NS3 protease has an innate ligand which is glycerol. NS3 helicase protein does not have a nonpolar innate ligand. The binding energy of NS3 protease and NS5 with each innate ligand was also analyzed, then compared with the binding energy of NS3 protease and NS5 with the test ligand which is propyl gallate. The conformation of each docking process is expressed in binding energy, inhibition constant, and the sum of hydrogen bonds formed, expressed in Table 3.

Discussion

Activity of propyl gallate dalam inhibiting DENV-2 replication

Propyl gallate has antiviral activity to DENV. In this study, we found that percentage of inhibition by propyl gallate was 4.34 ± 7.53% and 30.7 ± 4.88% in the post- and pre-post-infection, respectively, and significantly difference (p = 0.007). This finding showed that propyl gallate has better inhibition activity toward viral adhesion than replication inside the infected cells. We suggest that propyl gallate was better utilized as a prophylactic agent toward DENV infection than therapeutic agent. It is postulated that the weak inhibition effect of viral replication is caused by the weak affinity of propyl gallate toward DENV-2 proteins involved in viral replication, or the failure of propyl gallate to enter the cells effectively. Other study demonstrated that propyl gallate has better inhibition activity toward viral adhesion, with percentage of inhibition reaching 53 ± 9.85%, compared to percentage of inhibition toward host cell receptor of 9 ± 2.65% [6]. This finding support that propyl gallate has more effective inhibition toward viral adhesion than viral replication.

In comparison with other gallic acid derivate such as butyl gallate and methyl gallate, prophyl gallate has lower capability to inhibit DENV-2 replication [7]. Methyl gallate at concentration of 0.3 mg/mL has capability to inhibit DENV by 98% with interfering NS2B-NS3 protease activities [8].

The inhibition percentage of propyl gallate toward DENV replication in this study was different when we compared with other viruses. In other viruses, a study by Kratz *et al*. showed that propyl gallate yielded inhibition activity of 90% toward HIV-1 virus [9]. However, another study by Uozaki *et al*. shows that the reduction effect of HSV-1 viral titer in HEp-2 cells by

Table 2: Percentage of viability of propyl gallate in both treatments

Treatment	Percentage of viability (%)			Results (mean ± SD %)	p-value
	1st repetition	2nd repetition	3rd repetition		
Post-infection (Viral replication)	95.16	98.76	92.01	94.64 ± 0.4	0.750
Pre-post infection (Viral adhesion)	94.53	94.31	95.09	95.31 ± 3.38	

Table 3: Analysis of binding energy between macromolecule and ligand

Macromolecule-ligand	Binding energy (kcal/mol)	Inhibition constant (mM)	Hydrogen bonds
NS5 – propyl gallate	−3.49	2.78	0
NS5 – SAM	−6.35	22.21	1
NS3 protease – propyl gallate	−2.47	15.6	1
NS3 protease – glycerol	−1.23	124.75	2
NS3 helicase – propyl gallate	−3.72	1.88	1

propyl gallate was relatively lower than other derivates of gallic acid which has more carbon chains, starting from butyl gallate until lauryl gallate [10].

Vero cells viability after propyl gallate administration

In general, propyl gallate is considered as a safe antioxidant in foods and cosmetic products with maximum concentration of 0.1% [11]. In this study, we found that Vero cells viability was 94.64 ± 0.4% and 95.31 ± 3.38% in post- and pre-post-group, respectively, with p value of 0.750. It showed that propyl gallate has relatively similar toxicity effects toward Vero cells in both groups. The previous study showed that propyl gallate had no toxic effects toward Vero cells in viral adhesion treatment, and increased cell viability with percentage of viability of 125 ± 1% [6]. Similar study also found that butyl gallate has percentage of viability of 108,69% in viral adhesion treatment [12]. The percentage of viability results over 100% in both studies shows that gallic acid derivates actually increase the viability of Vero cells [7], and support that propyl gallate was not toxic toward Vero cells. Similar result was found using Hep-2 cells [9]. Propyl gallate has toxic effects toward rat hepatocytes by causing mitochondrial dysfunction that leads to ATP deficiency [13]. Since mitochondrial dysfunction occurs intracellularly, it also explains how percentage of viability in replication group is lower than adhesion or adhesion-replication group [13]. The low cytotoxic effect is also due to the low number of carbon atoms on the gallic acid derivative compound. In general, gallic acid derivative compounds have low cytotoxic effects [9].

In silico analysis of binding energy between propyl gallate and DENV-2 proteins involved in replication

In silico analysis was done to support the *in vitro* results and to search for the mechanisms on how propyl gallate can inhibit DENV-2 replication. *In silico* analysis was done by docking method using AutoDock Tools 1.5.6 software. NS5, NS3 protease, and NS3 helicase proteins are prepared from RCSB Protein Data Bank with each code 5ZQK, 2FOM, and 2BHR, respectively. First of all, the water molecule of each protein was deleted by the command “delete water.” Then, the innate ligands binding to active sites of each molecule were separated. NS5 protein has an innate ligand which is SAM and NS3 protease has an innate ligand which is glycerol. NS5 and NS3 proteins were then given polar hydrogen atoms and interacted with propyl gallate; then, Kollman charges were given to macromolecule and Gasteiger charges were given to propyl gallate. The coordinates for docking are the coordinates of the active sites where the innate ligands had originally bound. The coordinates for NS5-propyl gallate docking were 51,158; −3,411; and 43,598 (x; y; z). The coordinates for NS3 protease-propyl gallate docking were 4,224; −4,002; 22,085 (x; y; z). Since NS3 helicase does not have any innate ligands, we used the center coordinates of the macromolecule, which was −3,479; 58,22; and 39,005 (x; y; z), assuming that coordinate was the active site of macromolecule-ligand interaction. Docking process was carried out by creating grid and dock files, then running the programs subsequently on AutoDock Tools, which was AutoGrid and AutoDock. The conformation in this study was expressed in the lowest binding energy (kcal/mol), the lowest inhibition constant (mM), and the sum of hydrogen bonds.

The docking results between propyl gallate and NS5 (Table 3) showed that the lowest binding energy was −3.49 kcal/mol with inhibition constant of 2.78 mM. It meant that the energy released during the interaction was −3.49 kcal/mol and the lowest concentration of propyl gallate needed to inhibit NS5 was 2.78 mM. Docking between NS5 and SAM shows that the lowest binding energy was −6.35 kcal/mol with inhibition constant of 22.21 mM. It shows that the bond between NS5 and SAM was more stable than NS5 and propyl gallate, which also means that NS5 was unable to compete with SAM for the active site. However, the potency of propyl gallate in inhibiting NS5 protein was higher than SAM, as the inhibition constant is lower.

Docking between propyl gallate and NS3 protease showed that the lowest binding energy from the interaction was −2.47 kcal/mol with inhibition constant of 15.6 mM. It meant that the energy released during the interaction was −2.47 kcal/mol and the lowest concentration of propyl gallate needed to inhibit NS3 protease was 15.6 mM. Since there was no binding energy of absolute value, comparation with the docking results of NS3 protease and glycerol was needed to validate the results. The docking results of NS3 protease and glycerol show that the lowest binding energy is −1.23 kcal/mol with inhibition constant of 124.75 mM. This could explain that the bond between propyl gallate and NS3 protease is more stable than the bond between glycerol and NS3 protease, and also, that propyl gallate is more potent in inhibiting NS3 protease than glycerol.

The docking results between propyl gallate and NS3 helicase show that the lowest binding energy is −3.72 kcal/mol with inhibition constant of 1.88 mM. It meant that the energy released during the interaction is −3.72 kcal/mol and the lowest concentration of propyl

gallate needed to inhibit NS3 helicase is 1.88 mM. Since there is no innate ligand on NS3 helicase, further docking comparations were not carried out.

A study by Kusuma *et al*. on 2020 found that the binding energy between propyl gallate and DENV protein involved in replication, which is protein E, is −3.21 kcal/mol with inhibition constant of 4.44 mM [6]. The results are higher compared to binding energy between propyl gallate with NS3 protease −2.47 kcal/mol), but lower compared to binding energy between propyl gallate with NS5 and NS3 helicase, which is −3.49 kcal/mol and −3.72 kcal/mol, respectively. The comparation of both analyses *in silico* shows that propyl gallate has strong binding stability for DENV-2 proteins involved in both viral adhesion and replication.

The *in vitro* results show that propyl gallate has a relatively low inhibition activity toward viral replication when compared toward viral adhesion and replication. From the *in silico* analysis, it is shown that although propyl gallate has good potency in inhibiting NS5, propyl gallate has a weaker binding stability toward NS5 when compared to the innate ligand SAM. However, in other docking results, propyl gallate is able to demonstrate good binding stability toward NS3 protease compared to the innate ligand glycerol, although with lower inhibition constant compared to NS5 and NS3 helicase. Other than that, propyl gallate also shows good binding stability and inhibition potency toward NS3 helicase.

The *in vitro* results which do not match the *in silico* results can be explained by some reasons. First of all, it is probable that propyl gallate may not enter the Vero cells effectively, leading to lack of demonstration regarding the inhibition activity toward viral replication. It may also be caused by the fact that binding stability between NS5 and SAM is better than NS5 and propyl gallate, which may lead to shift of binding by SAM. NS5 may have a more important role in DENV-2 replication compared to NS3. This is stated in a study by Oliveira, et al which explains that NS5 is the biggest protein (104 kDa) and the most conserved protein of DENV [14]. It means that NS5 does not undergo many mutations. A study by Klema *et al*. also supports the results of this study by explaining that the specific interactions between MTase and RdRp in NS5 protein is a strong determinant in DENV-2 replication [15]. Moreover, the comparation of *in vitro* data by Kusuma *et al*. in 2020 and this study supports that propyl gallate is more effective in inhibiting viral adhesion than viral replication [6].

This study has strengths and limitations. The strength of this study is the presence of *in silico* analysis that may explain further the findings from the *in vitro* analysis until the biomolecular level. The presence of *in silico* analysis also gives the plausible mechanism of action of propyl gallate in inhibiting DENV. The limitation of this study is that there is yet an analysis between NS3 helicase and an innate ligand, which is important to validate the docking results between propyl gallate and NS3 helicase, since the binding energy of a macromolecule-ligand interaction is not absolute. Another limitation of this study is that there is no method to ensure propyl gallate enters the Vero cells effectively; thus, the inhibition activity toward DENV-2 replication demonstrated in the *in vitro* results may be biased.

Conclusion

Propyl gallate compound has a relatively low inhibition activity toward DENV-2 replication *in vitro*. Propyl gallate shows no significant toxic effects toward Vero cells and is relatively safe to be used for future research. By *in silico* analysis, propyl gallate demonstrates good inhibition activity toward proteins involved in DENV-2 replication, such as NS5, NS3 protease, and NS3 helicase. From this study, it can be concluded that propyl gallate shows better promise to be a prophylactic drug than an antiviral drug for DENV-2. The author suggests for more studies to be conducted, especially using innate ligands of NS3 helicase to validate the results. Moreover, it is also recommended that *in vivo* studies from animals to humans are conducted to give more representative results to living creatures. For the next study, the author also recommends additional methods to ensure propyl gallate enters the cell, such as use of fluorescent labeling techniques and visualization with fluorescent microscope, so that future research may identify the actual activity of propyl gallate in inhibiting DENV-2 replication.

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