Inhibition mechanism of \textit{Psidium guajava} leaf to dengue virus replication \textit{in vitro}

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Abstract. Dengue hemorrhagic fever is a disease caused by Dengue Virus (DENV) infection that carried by mosquito vector. The incidence of DHF in Indonesia (2013) is 41.25 cases per 100,000 population. However, until now there is no antiviral therapy for DHF. Last report explained the anti-Dengue activity in \textit{Psidium guajava} leaves extract that have potency to inhibits the DENV infection with IC\textsubscript{50} 7.2 \mu g/mL and CC\textsubscript{50} 153.18 \mu g/mL. But, the mechanism still unknown. This research measure the percentage of viral inhibition in the DENV surface protein inhibition and receptor inhibition of 2 times IC\textsubscript{50} extract using focus assay methods and followed by measure of cell viability using MTT assay. The results of focus assay for DENV surface protein inhibition is 58.24 \pm 17.40% and in DENV receptor inhibition is 8.56 \pm 6.29%. Then, the cell viability of attachment and receptor inhibition are 100.71 \pm 4.72% and 100.96 \pm 3.51% The bioactive compounds in the extract that acts as anti-Dengue are quercetin and hyperoside. But, the action of quercetin is not on DENV surface protein or inhibits the DENV receptors and hyperoside acts to inhibits DENV receptor. This novelty suggest maybe there are other compounds in \textit{Psidium guajava} leaves extract that acts as anti-Dengue.

1. Introduction

Dengue hemorrhagic fever (DHF) caused by Dengue virus (DENV) infection with mosquito as a vector [1]. The clinical manifestation of DHF are fever, muscle aches and or joint pain which is followed by leukopenia, rash, thrombocytopenia, lymphadenopathy and hemorrhagic [2]. According to the WHO, this disease is categorized as a danger infectious disease through the deadliest mosquitoes in the world [3]. Based on epidemiological studies, it is known that this disease occurs in the tropics and subtropics which more than 3.6 billion people [3]. Globally, there are more than 125 countries that are endemic to DENV [3,4]. Every year there are an estimated 30-100 million people with DENV infection and 500,000 people with DHF [1].

DENV is a flavivirus and has four serotypes that can be distinguished through molecular techniques and neutralization tests [5]. This is what causes the difficulty of making vaccines for
DEN. In addition, there is no specific antiviral therapy yet so that treatment is only fluid resuscitation [5].

Herbal medicines have been used as therapy of infectious disease [6]. In the development of antiviral drug to DENV, herbal medicines consider to have high potency to combat DENV [7]. One of the plants that has anti-DENV activity is *Psidium guajava* from family of Myrtaceae [8]. *P. guajava* leaf extract contain quercetin which capable to inhibit the formation of mRNA enzymes in viruses [9]. In addition, *P. guajava* leaf extract is also used to prevent bleeding in DHF cases due to increase platelet counts up to 100,000/mm³. Based on research conducted by Saptawati et al, *Psidium guajava* extract inhibit DENV infection with IC₅₀ and CC₅₀ values of 7.2 μg/mL 153.18 μg/mL [8]. However, the mechanism of DENV inhibition by *P. guajava* is still unknown. Therefore, in this study we evaluated by which mechanism *P. guajava* leaf extract Inhibit DENV replication in vitro.

2. Materials and methods

2.1 Preparation of *Psidium guajava* leaf extract

*P. Guajava* leaves were obtained from a previous study [10]. The ethanol extract of *P. Guajava* leaves was then dissolved in dimethyl sulphoxide (DMSO) with a concentration of 100 μg/mL. The extract was protected from light and stored at -20°C until use. For this study, we used extract at concentration of 2 x IC₅₀ value (14.4 ug/mL).

2.2 Propagation of cell line

Propagation of the Huh 7it-1 cells were done according to previous study [10]. We used cell at concentration of 5 x 10⁴ cell/mL, 200 μL/well in 48 well plate for evaluation of DENV inhibitory mechanism. To determine the toxicity, we used 96 well plate with 100 μl/well of the cells at concentration of 2.3 x 10⁴ cell/mL. All of the cells were incubated in 37°C with 5% CO₂ for 48 hours [12].

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 [10].

2.4 Determine of toxicity

The toxicity level of *P. guajava* leaf extract to the cell was determined by MTT assay for each mechanism. The MTT assay was performed according to previous study. The value of absorbance reading determined the value of cell viability as indicator of toxicity. The result of viability percentage of each mechanism was calculated as comparison between viability of treatment and control. In the 96 well plate with 100 ul/well of the cells at concentration of 2.3 x 10⁴ cell/mL was prepared 24 hours before used. For experiment of receptor inhibition, discard supernatant of the cell and add with medium contain extract at concentration of 2 time of IC₅₀ for 2 hours. After discard the medium, add with medium without extract and incubated for 3 days at 37°C with 5% CO₂. For pre infection inhibition, the cells were not exposed with extract, the viability of the cell was not measured. The MTT assay was performed according to previous study [10].

2.5 Attachment Inhibition

To determine attachment inhibitory mechanism, we used 2 different methods. The first method was blocking DENV protein surface to interrupt attachment process. The second method by blocking the receptor of DENV on the surface of the cells. To cover DENV surface protein, DENV at moi of 0.5 FFU/cell were added with extract to final concentration of 14.4 μg/mL. After 2 hours incubation, medium without extract was added and incubated for 48 hours. We used 0.01% DMSO as negative
control. To block DENV receptors, the medium of monolayer cells in 48 well plate was replaced with 50 μL/well medium containing P. guajava leaf extract with concentration of 2 time IC_{50} value (14.4 μg/mL). Then, incubated for 2 hours. Subsequently, the medium was removed and infected with DENV with moi of 0.5 FFU/cell. After incubation for 2 hours, we added with 100 uL/well of DMEM + 10% FBS and incubated for 48 hours at 37°C, 5% CO₂. The supernatant was harvested and evaluated the DENV titers by focus assay [11].

3. Results and discussions

3.1 Viability of the cell
Table 1 showed the percentage of cell viability after the intervention with P. guajava leaf extract in pre-infection such as inhibition of attachment and DENV receptors. The cell viability percentage for inhibiting DENV attachment was 100.71 ± 4.72%. The percentage of cell viability on DENV receptor intervention was 100.96 ± 3.51% (table 1). There was no significant different among all intervention (p value>0.05). The cell viability after treated with P. guajava leaf extract with a dose of 14.4 μg/mL (twice the IC_{50} value) does not have a toxic effect on the cell. Statistically, the cell viability values for the inhibition DENV protein surface and receptor interventions were not significantly different which proved that there was no difference between the two interventions on cell viability.

**Table 1.** Percentage of viability cell after various intervention. The experiment was done in triplicate

| Intervention          | 1  | 2  | 3  | Average ± SD |
|-----------------------|----|----|----|--------------|
| DENV protein surface  | 97 | 98 | 106| 100.71 ± 4.72|
| Receptor              | 95 | 101| 106| 100.96 ± 3.51|

3.2 Inhibition of DENV in each mechanism
Table 2 showed the percentage of inhibition DENV for attachment interventions, including inhibition of protein DENV surface and receptors which displayed in mean values and standard deviations. The percentage was comparison between treatment and control. The results of this study showed that the inhibition percentage of P. guajava leaf extract gives a value of 58.24 ± 17.40% in DENV adherence interventions or in inhibition protein surface. However, the percentage of DENV receptor inhibition was lower (8.56 ± 6.29%). Both mechanism was significantly different in the inhibition of DENV replication. Therefore, the administration of P. guajava leaf extract would be better for the DENV surface protein than the receptor inhibition.

**Table 2.** Percentage of Inhibition DENV replication with various intervention.

| Intervention          | Average of percentage inhibition ± SD |
|-----------------------|---------------------------------------|
| DENV protein surface  | 58.24 ± 17.40\(^a\)                   |
| Receptor              | 8.56 ± 6.29                           |

\(^a\)p value less than 0.01

From phytochemical analysis of ethanol leaf extract of P. guajava contains alkaloids, saponins, carbohydrates, tannins and flavonoids [12].The flavonoid of P. guajava has an antiviral activity to DENV. There are eight flavonoids in the extract, namely (2,6-dihydroxy-3-formaldehyde-5-methyl-4-O-(6″-O-galloyl-β-D-glucopyranosyl)-diphenylmethane; quercitrin; quercetin; kaempferol; reynoutrin; guajaverin; avicularin; isoquercitrin; hyperoside; 2,6-dihydroxy-3,5-dimethyl- 4-O- (6 ″-
O-galloyl-β-D-glucopyranosyl–benzophenone [9]. According to Saptawati’s research, et al which has an anti-dengue role in the extract of the P. guajava leaf is quercetin [8].

Zandi K, et al. [13] compared the antiviral to DENV activity of four flavonoids (quercetin, naringin, daidzein, hesperetin) in on Vero cells. The result indicated that quercetin was the only -nhibition of RNA through interactions with enzymes or proteins in viral replication complexes [16]. Then, the mechanism of anti-dengue activity of quercetin was viral replication through inhibition of RNA polymerase. The study stated that quercetin did not play a role in DENV adhesion and entry process [13]. In addition, hyperoside (including flavonoids) in water extract Houttuynia cordata is the main component of the extract and is able to protect cells from virus entry and inhibit viral activity after attachment (adsorption). Texeira R, et al also said that hyperoside might inhibit synthesis Intracellular RNA through interactions with enzymes or proteins in viral replication complexes [14]. The results of this study indicate the possibility of other types of flavonoids or other bioactive substances contained in the P. guajava leaf extract which might play a role in inhibiting the mechanism of DENV attachment due to inhibition of DENV surface protein.

4. Conclusion
The mechanism inhibition of psidium guajava leaf are on DENV surface protein with the inhibition is 58.24±17.40% and in DENV receptor inhibition is 8.56±6.29%.

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