

In vitro study of eight Indonesian plants extracts as anti Dengue virus

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Abstrak

Latar Belakang: Demam Berdarah Dengue (DBD) yang disebabkan oleh virus dengue masih merupakan masalah utama di negara tropis. Sampai saat ini belum tersedia vaksin maupun terapi yang efektif untuk DBD. Oleh karena itu, penelitian untuk menemukan antivirus spesifik untuk virus dengue sangat diperlukan. Indonesia kaya akan tumbuhan herbal yang mungkin berpotensi memiliki aktivitas antivirus, diantaranya adalah *Psidium guajava* (Jambu biji), *Euphorbia hirta* (Patikan kerbau), *Piper bettle* L. (Sirih), *Carica papaya* (Pepaya), *Curcuma longa* L. (Kunyit/turmeric), *Phyllanthus niruri* L. (Meniran), *Andrographis paniculata* (Sambiloto), dan *Cymbopogon citratus* (Serai). Penelitian sebelumnya menunjukkan bahwa beberapa tumbuhan herbal tersebut memiliki khasiat antibakteri, antivirus maupun keduanya. Namun, penelitian yang mengeksplorasi potensi beberapa herbal tersebut dalam melawan virus dengue masih terbatas.

Tujuan: Penelitian ini bertujuan untuk mengetahui apakah beberapa tanaman herbal tersebut berpotensi memiliki aktivitas antivirus terhadap virus dengue secara *in vitro*.

Metode: Ekstrak daun dari delapan tanaman herbal asli Indonesia diperoleh dari Solo, Jawa Tengah. Ekstrak kasar herbal tersebut diuji secara *in vitro* terhadap dengue virus serotype 2 (DENV-2) strain NGC menggunakan cell line Huh7it-1. Aktivitas antivirus beberapa ekstrak kasar tersebut diskriminasi dengan dosis 20 µg/mL. Ekstrak yang menunjukkan aktivitas penghambatan virus dengue, selanjutnya diuji dengan variasi dosis untuk menentukan CC_{50} and IC_{50} .

Hasil: Berdasarkan uji penapisan awal terhadap 8 ekstrak tanaman herbal dengan dosis 20 µg/mL, *Psidium guajava* (Jambu biji) dan *Carica papaya* (Pepaya) memiliki efek sitotoksik sebesar 11,3% dan 2,5% dan mampu menghambat replikasi virus dengue masing-masing hingga 92,6% dan 89,5%. Dose dependent assay pada *P. guajava* menunjukkan CC_{50} , IC_{50} dan indeks selektivitas berturut-turut sebesar 153,18 µg/mL, 7,2 µg/mL dan 21,28. Sedangkan *C. papaya* menunjukkan CC_{50} , IC_{50} dan indeks selektivitas berturut-turut sebesar 244,76 µg/mL, 6,57 µg/mL dan 37,25.

Kesimpulan: *Psidium guajava* dan *Carica papaya* memiliki potensi aktivitas antivirus melawan virus dengue *in vitro*. (Health Science Journal of Indonesia 2017;8(1):12-18)

Kata kunci: Virus Dengue, ekstrak herbal, aktivitas antivirus, *Psidium guajava*, *Carica papaya*.

Abstract

Background: Dengue hemorrhagic fever (DHF) caused by dengue viruses is still a major problem in tropical countries. Until nowadays, there is no vaccine or effective therapy is available as yet. Thus research on discovering specific antiviral against dengue is needed. Indonesia is rich in indigenous herbal plants, which may have potential antiviral activity, such as *Psidium guajava* (Jambu biji), *Euphorbia hirta* (Patikan kerbau), *Piper bettle* L. (Sirih), *Carica papaya* (Pepaya), *Curcuma longa* L. (Kunyit/turmeric), *Phyllanthus niruri* L. (Meniran), *Andrographis paniculata* (Sambiloto), and *Cymbopogon citratus* (Serai). Previous studies showed that these plants, some have antibacterial properties, antiviral properties or both. However, there is only limited study of these plants against dengue virus.

Objective: The aim of this study is to know whether these plants have potential activity against dengue virus *in vitro*.

Method: Leave extracts of eight indigenous herbal plants as mentioned before were originated from Solo, Central Java. The crude extracts were tested *in vitro* against dengue virus serotype 2 (DENV-2) strain NGC using Huh7it-1 cell line. Those crude extracts were screened for antiviral activity using doses of 20 mg/mL. Candidates that showed inhibition activity were further tested in various doses to determine CC_{50} and IC_{50} .

Result: From eight leave extracts tested with 20 µg/mL dose, *Psidium guajava* (Jambu biji) and *Carica papaya* (Pepaya) have cytotoxicity 11.3% and 2.5% respectively and inhibited virus replication up to 92.6% and 89.5% respectively. Dose dependent assay of *Psidium guajava* showed CC_{50} , IC_{50} and selectivity index 153.18 µg/mL, 7.2 µg/mL and 21.28 respectively. Whereas, *C. papaya* showed CC_{50} , IC_{50} and selectivity index 244.76 µg/mL, 6.57 µg/mL and 37.25 respectively.

Conclusion: *Psidium guajava* and *Carica papaya* have potential antiviral activity against dengue virus *in vitro*. (Health Science Journal of Indonesia 2017;8(1):12-18)

Keywords: Dengue virus, natural extract, antiviral activity, *Psidium guajava*, *Carica papaya*

Dengue hemorrhagic fever (DHF) is an infectious disease which is still a major problem in tropical countries, including Indonesia. It is caused by dengue viruses that belong to Flaviviridae. WHO showed that over 40% of world populations are at risk of DHF.¹ In 2012 number of cases were 57,204 with 1229 death in South East Asia.² Indonesian Ministry of Health data showed that the number of patients with DHF increased annually.³ In 2014 there were 71668 of DHF cases in 34 provinces with 641 death.⁴ In Central Java in 2013 incidence rate and fatality rate of DHF was 45.52 in 100.000 populations and 1.21% respectively, which was increasing from the previous year 19.29 in 100.000 population and 0,93%.⁵

Dengue appears in two forms, classic dengue fever and severe form. In severe form, Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) which may cause abdominal bleeding, hemorrhage and circulatory failure. If this severe form is not treated with prompt and proper management it will lead to the fatal cases.⁶

In addition, the morbidity and mortality from DHF is remaining high since there is no specific therapy for dengue. The management of cases is mainly supportive by fluid therapy. Thus, research on discovering specific antiviral against dengue is needed. Indonesia is rich in indigenous herbal plants, which may has potential antiviral activity, such as *Psidium guajava* (Jambu biji), *Euphorbia hirta* (Patikan kerbau), *Piper bettle* L. (Sirih), *Carica papaya* (Pepaya), *Curcuma longa* L. (Kunyit/turmeric), *Phyllanthus niruri* L. (meniran), *Andrographis paniculata* (Sambiloto), *Cymbopogon citratus* (Serai).

Psidium guajava (Jambu biji) contains a number of major active compounds which play a role for major biological activities, including anti-inflammatory, anti-plasmodial and antimicrobial properties.⁷ Other study by Gyuris *et al.* (2009) reported that *Euphorbia hirta* has antiviral activities. The aqueous and 50% MeOH extracts of this plant inhibit replication of SIV_{mac251} (simian immunodeficiency virus strain mac251), HIV-1 and HIV-2 viruses (human immunodeficiency virus types 1 and 2) in MT4 human T-Lymphocyte cells lines.⁸

Review on *Piper bettle* L. by Rekha *et al.* (2014) reported that the leaves of this plant have a tremendous potential biological activities such as antibacterial, antilarvasidal, immunomodulatory activities, etc.⁹ Sarala and Paknikar (2014) on their scientific literature review suggested that *Carica papaya* leaf extract have beneficial properties in dengue due to flavonoids

and other phenols compound. The *Carica papaya* leaf extract also rich several minerals which may balance the mineral deficiency caused by dengue virus and strengthen the immune cells against it.¹⁰ Moghadamtousi *et al.* (2014) reported that *Curcuma Longa* L. have antibacterial, antifungal and antiviral activities. This plant was found to be an inhibitor of HIV-1 and HIV-2 protease with IC₅₀ of 100 µM and 250 µM, respectively.¹¹ Aqueous and methanol extract of *Phyllanthus niruri* L. (Meniran) which contains lignan, tannin, polyphenol, alkaloid, flavonoid, terpenoid, steroid, coumarin and saponin inhibit DENV-2 activity.^{12,13} Tang *et al.* (2012) study on *Andrographis paniculata* (Sambiloto) and *Cymbopogon citratus* (Serai) based on cytophatic effect in vitro showed inhibition activity against DENV-1 75% and <50% respectively.¹⁴

Previous studies showed that these plants have antibacterial activity, antiviral activity or both.⁷⁻¹⁴ However, there is only limited study of these plants against dengue virus. This study aimed to know whether these plants have potential activity against dengue virus *in vitro*.

METHODS

Natural Extract Preparation

The crude extract of leave of eight indigenous herbal plants as mention before were originated from Solo, East Java were extracted in ethanol and dried, and dissolved in dimethyl sulphoxide (DMSO) in Laboratory of The Faculty of Science, University of Sebelas Maret, Surakarta, Central Java. The leaves were obtained from the plants grew in Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT) plantation in Tawangmangu, Karanganyar, Central Java. The extracts were protected from light and stored at -20°C. The stock solutions were then diluted in culture medium with the concentrations needed before conducting the assay.

Dengue Virus Propagation

DENV-2 strain NGC were obtained from National Institute of Health Research and Development, Ministry of Health Republic of Indoneisa with kind help of Dr. dr. Reni Herman, MBIomed and were propagated in Huh7it-1 cells in 2% FBS (Sigma, Aldrich, USA) DMEM (Gibco, Thermo Fisher Scientific, USA) medium at 37°C with 5% CO₂ for 5-7 days. Huh7it-1 cell propagation we used 10% FBS DMEM medium.

Dose-dependent assay

For dose dependent assay, we infected cells with DENV at a multiplicity of infection (MOI) of 0.5 FFU/cell. Firstly, the Huh7it-1 cells were prepared in 48 well-plate 5×10^4 cell/well. Then, the extract was prepared with twice its concentration in a 96-well round-bottomed plate. In a cold environment, afterwards, the virus was prepared with twice its MOI in the same 96-well plate and mixed with the prepared extract. Since there were no specific antiviral drugs to DENV as positive control, in this study we used medium and medium with DMSO (Sigma, Aldrich, USA) as positive and negative control. The mixture was then added to the Huh7it-1 cell after the medium was already removed. Next, incubation was done at 37°C with 5% CO₂ for 2 hours and agitated in every 30 minutes. Then, the mixture was disposed and the cell was rinsed two times with serum-free medium. After that, 3 wells of positive control and 3 well of negative control were prepared. Over-layer solution of natural extract was then added to the mixture. Afterwards, re-incubation was done for 48 hours. Lastly, the supernatant was harvested and stored in -80°C.

Cell Viability Assay

The cells were prepared in 96-well plate with 10% FBS DMEM medium and incubated for 24 hours. After the medium was removed, the extract solution was prepared with various concentrations. Controls DMSO at 0.3% and 0.1% as well as DMEM medium were put to the well plate with each 3 wells. Then, the prepared extract solution was added. The mixture was incubated for 48 hours. We added 10% MTT reagent (Sigma, Aldrich, USA) after removing the extract solution from each well. Re-incubation was done for 3 to 4 hours. Next, MTT reagent was discarded and DMSO 100% was added. Finally, the absorbance level was examined for further calculation of CC₅₀.

Focus Assay

The Huh7it-1 cell was prepared in complete medium with 2.3×10^4 cell/mL concentration and placed in a 96-well plate for 24 hours. Stored supernatant was thawed first and serially diluted in Medium with 2% FBS. The huh7it-1 cell was then taken out from the incubator and the medium was removed. Next, the diluted supernatant was added into the cells. They were then incubated for 2 hours at 37°C with 5% CO₂. The diluted supernatant was removed and

overlaid with methylcellulose 0.5%. We incubated again at 37°C with 5% CO₂ for 2 days. Two days later the infected cells in each well were fixed using 200 µL 3.7% formaldehyde per well and incubated for 15 minutes, and the formaldehyde was removed after 15 minutes. After the formaldehyde was removed, the cells were washed three times with 200 µL of PBS with period of 5 minutes in between. Immunostaining was then performed. After the PBS was removed, 100 µL of Tryton X 0.5% was added to each well. Afterwards, incubation was done for 30 minutes. The Tryton X was then removed from all wells. Next, the wells were rinsed with PBS 3 times. Then, 50 µL of 1/500 anti-dengue IgG from human serum was added. Incubation for 1 hour was then performed. After removing the antibody, each well was rinsed with PBS. After that, the second antibody, which was 50 µL of 1/500 human anti-IgG HRP (Sigma, Aldrich, USA), was added. It was incubated, removed, and rinsed again with the same method as before. Next, 100 µL of DAB (Thermo Scientific, USA) was added to each well. Last incubation was done for 5 to 15 minutes. Finally, the virus focus was observed under light microscope.

Statistical Analysis

The data was analyzed with unpaired t-test on GraphPad Prism 6. Normality tests of the data were performed using SPSS 21.

RESULTS

The effect of eight extracts to cell viability

The cytotoxic of eight natural extracts at 20µg/mL were showed at Table 1. A herbal extract is considered to have *in vitro* activity based on three criteria, i.e cell viability >50%, IC₅₀ ≤25 µg/mL and selectivity index >3.¹⁵⁻¹⁷ As shown in Table 1 the cell viability of all extracts were >50%, in other word all of extracts not toxic to the cells.

Screening of antiviral effect of eight extracts

As shown in Table 2, among the eight extracts tested *P. guajava* and *C. papaya* strongly inhibit virus replication (92.6% and 89.5% respectively) at 20 µg/mL dose.

Table 1. Cell viability of eight natural extracts at 20 µg/mL dose

Natural extract	Cell viability (%)
<i>Psidium guajava</i> (Jambu biji)	88.7
<i>Euphorbia hirta</i> (Patikan kerbau)	95.3
<i>Piper bettle</i> L (sirih)	96.2
<i>Carica papaya</i> (pepaya)	97.5
<i>Curcuma longa</i> L (Kunyit)	95.8
<i>Phyllanthus niruri</i> L (Meniran)	96.2
<i>Andrographis paniculata</i> (Sambiloto)	105.5
<i>Cymbopogon citrates</i> (Serai)	95.7

Table 2. Antiviral activity of eight natural extracts at 20 µg/mL dose

Natural extract	Inhibition (%)
<i>Psidium guajava</i> (Jambu biji)	92.6
<i>Euphorbia hirta</i> (Patikan kerbau)	34.7
<i>Piper bettle</i> L (sirih)	31.6
<i>Carica papaya</i> (pepaya)	89.5
<i>Curcuma longa</i> L (Kunyit)	46.4
<i>Phyllanthus niruri</i> L (Meniran)	36.1
<i>Andrographis paniculata</i> (Sambiloto)	46.2
<i>Cymbopogon citrates</i> (Serai)	-14.4

Cytotoxicity and antiviral activity of *Psidium guajava*

To ensure that extract was not toxic to the cell, the half cytotoxic concentration (CC₅₀) was measured by treating uninfected Huh7it-1 with various concentration of *P. guajava*, and cell viability was measured with MTT assay. The cell viability still showed a high level (almost 100%) after treated with *P. guajava* at concentration of 20 µg/mL. After treated with concentration more than 40 µg/mL, the viability decreased slightly. The CC₅₀ was analyzed from the linear regression equation of the percent viability. We found that the CC₅₀ of *P. guajava* was 153.18 µg/mL (Figure 1).

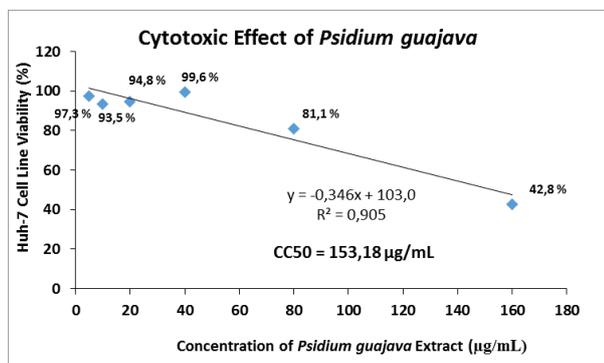


Figure 1. Linear regression curve of the cell viability after treatment with *P. guajava*

To determine antiviral effect of *P. guajava*, DENV was treated with various concentration of *P. guajava*. The infectivity decreased after treated with 40 µg/mL of *P. guajava*. In the concentration of 80 µg/mL and 160 µg/mL, we found no DENV infection. The IC₅₀ was analyzed from the linear regression equation of the percent infectivity. We found that IC₅₀ of *P. guajava* was 7.2 µg/mL (Figure 2). The selectivity index of *P. guajava* was 21.28.

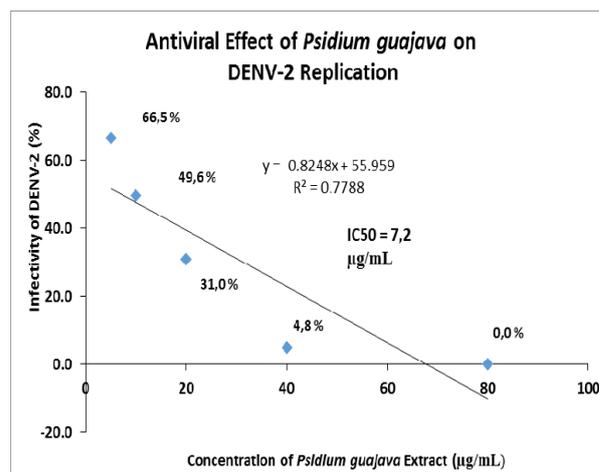


Figure 2. Linear Regression curve of the inhibition of DENV after treated with *P. guajava*.

Cytotoxicity and antiviral activity of *Carica papaya*.

From MTT assay we calculated the viability of cells and compared with viability after treated with DMSO. The viability showed that treatment with *C. papaya* at concentration up to 40 µg/mL was not toxic to the cells, while the cell viability decreased at treatment with 80 µg/mL. From the linear regression equation of the percent viability, we found that the CC₅₀ of *C. papaya* was 244.76 µg/mL (Figure 3).

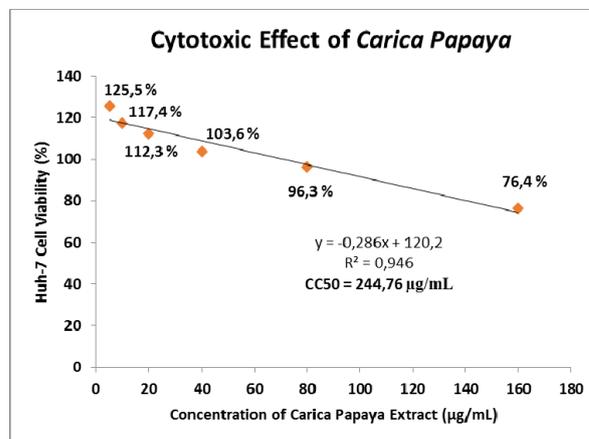


Figure 3. Linear regression curve of the cell viability after treatment with *C. papaya*

Similar with *P. guajava*, we also measured infectivity of DENV-2 after treated with *C. papaya*. Addition of *C. papaya* at the concentration of 40 µg/mL and more showed 100% inhibition to DENV replication. Lower doses such as 20 µg/mL, 10 and 5 µg/mL showed percentage of infectivity at the level of 3.7%, 25% and 48% respectively. From the linear regression equation of the percent infectivity, IC_{50} of *C. papaya* was 6.57 µg/mL (Figure 4). We found the selectivity index of *C. papaya* was 37.25.

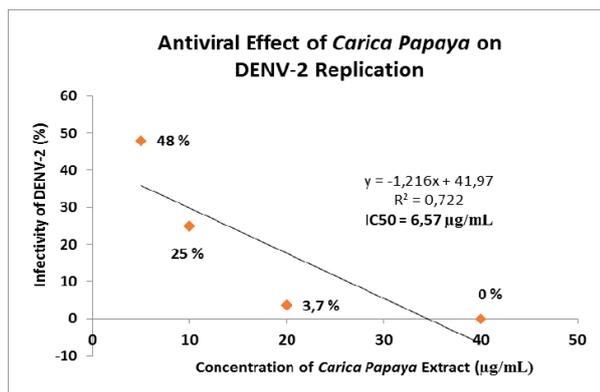


Figure 4. Linear Regression curve of the inhibition of DENV after treated with *C. papaya*

DISCUSSION

This research is aimed to know whether leaf extracts of *Psidium guajava* (Jambu biji), *Euphorbia hirta* (Patikan kerbau), *Piper bettle* L. (Sirih), *Carica papaya* (Pepaya), *Curcuma longa* L. (Kunyit/turmeric), *Phyllanthus niruri* L. (meniran), *Andrographis paniculata* (Sambiloto), and *Cymbopogon citratus* (Serai) had antiviral activity *in vitro* against DENV-2. As mentioned above, a herbal extract is considered to have *in vitro* activity based on three criteria, i.e cell viability >50%, $IC_{50} \leq 25$ µg/mL and selectivity index >3.¹⁵⁻¹⁷

Our screening results showed that *P. guajava* and *C. papaya* have inhibition activity 92.6% and 89.5% respectively, while the other extracts showed only inhibition activity less than 80%. All extract didn't show toxicity, i.e. cell viability >50%. These results suggest that *P. guajava* and *C. papaya* have good antiviral activity *in vitro*. This result is in agreement with study by Joseph et al. (2015), which also show that *C. papaya* leaf chloroform extract inhibit DENV-2 in LLC-MK2 cells with $CC_{50} > 1$ mg/mL and $EC_{50} > 1$ mg/mL.¹⁸

The extract of *Psidium guajava* was proven as an effective antiviral for dengue virus *in vitro*, might be caused this extract contained quercetin (derivate of flavonoid) that inhibit the activity of reverse transcriptase enzyme.¹⁹ *C. papaya* also contained flavonoid, beside other substance such as alkaloid, carbohydrate, saponin, phenol, glycosid, phytosterol, terpenoid and tannin.²⁰ Based on research of Zandi et al. (2011), quercetin can impede the replication of dengue virus serotype 2 (IC_{50} : 35.7 µg/mL) significantly. The exact antiviral mechanism is unknown, but it may similar to other flavonoid activity, i.e. by inhibition of RNA polymerase.²¹ Bioinformatic study suggest that quercetin from *C. papaya* leaf extract may inhibit NS2B-NS3 protease.²⁰

In screening test using 20 µg/mL extract, *Phyllanthus niruri* L. (Meniran), *Curcuma longa* L. (Kunyit/turmeric), inhibitory activity to DENV-2 replication in Huh7-it cells were 36.1% and 46.4% respectively, which meant no antiviral activity.

These results are different from previous report that suggests antiviral activity of these plant extracts against dengue virus. Aqueous and methanol extract of *P. niruri* L. inhibits viral absorption into cells and inhibit DENV-2 replication.¹³ However, study of *P. niruri* L. effect as antiviral is still limited. Methanol extract of *C. longa* was shown to interfere dengue virus replication by inhibiting NS2B/NS3 protease.²² The different results might be caused by different geographic source of the plants. Plants from different geographic conditions may adapt to environment and produce different secondary metabolites. Flavonoid, tannin, alkaloid and saponin content increase following increase of altitude of the region where it grows. This increase may be aimed to protect plants from stressor such as UV-B and low temperature.²³ Different results in this study may also caused by different dose used in screening test. We used 20 µg/mL, while Lee et al reports that the dose needed to inhibit 100% DENV2 replication is 250 µg/mL.¹³

Our results showed that DENV-2 inhibition of *Cymbopogon citratus* (Serai) and *Andrographis paniculata* (Sambiloto) leaf extracts were limited, i.e -14.4%, and 46.2% respectively, which were similar to previous study by Tang et al with flavonoid extract from *C. citratus* against DENV-1, but different from the same study which showed that methanol extract from *A. paniculata* inhibits DENV-1 replication in Vero E6 cells up to 75%.¹⁴

A. paniculata has flavonoid, lacton, tannin, saponin as reported by Widyawati (2007)²⁴; while *C. citratus* (Minyak serai or atsiri) composed of citronellal, citronellol, geraniol as reported by Wijesekara, (1973).²⁵ However, flavonoid in different plants may be various. Flavonoid of *A. paniculata* consists of *tetramethoxyflavanone* and *trimethoxyflavone*.²⁶

Despite the finding that *E. hirta* contains alkaloid, flavonoid, polyphenol and tannin by Okoli et al, which potentially has antiviral activity²⁷, our *in vitro* study only showed 34.7% inhibition against *DENV2*. This might be caused by the effect of side-chain of flavonoid or different class and molecular weight of tannin.

In conclusion, from screening antiviral activity of leave crude extract of eight indigenous herbal plants originated from Solo, East Java, *P. guajava* and *C. papaya* have potential antiviral activity against dengue virus *in vitro*. Further study is needed to confirm antiviral activity *in vivo*.

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